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ORIGINAL RESEARCH

Distinct Autoantibody Profiles in Systemic Lupus Erythematosus Patients are Selectively Associated with TLR7 and TLR9 Upregulation

Sudhir Kumar Chauhan · Vikas Vikram Singh · Richa Rai · Madhukar Rai · Geeta Rai

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Abstract
Purpose Systemic lupus erythematosus (SLE) patients have a wide array of autoantibodies against nuclear antigens. The two predominant classes of these autoantibodies are directed either against dsDNA or RNA-associated antigens (extractable nuclear antigens; ENA). Nucleic-acid sensing Toll-like receptors (TLRs) that recognize dsDNA and RNA, have been well implicated in some murine models of SLE. We took up this study to identify if unique TLR expression patterns are associated with distinct autoantibody profiles in SLE.
Methods We segregated the patients into three subsets distinguished on the basis of autoantibody response either against dsDNA or ENA or both. We determined the mRNA expression of TLR3, 7, 8, and 9 by real-time reverse-transcription PCR in peripheral blood leucocytes (PBLs) of the SLE patients of all three subsets. TLR7 and 9 protein expression was determined by western blotting in PBLs and by flow cytometry on B-cells and monocytes. The serum interferon-alpha (IFN-α) and anti-dsDNA/-ENA autoantibodies were detected using enzyme-linked immunosorbant assay.
Results We report differential and unique TLR expression patterns associated with different autoantibody profiles. The presence of anti-ENA and anti-dsDNA autoantibodies in SLE patients was associated with elevated levels of TLR7 and TLR9 respectively. The TLR9 mRNA expression was further augmented in SLE patients with Glomerulonephritis. Interestingly, anti-dsDNA$^+$ ENA$^+$ patients displayed higher serum IFN-α and interferon regulatory factor 7 mRNA expression than patients with either anti-dsDNA or anti-ENA autoantibodies alone.
Conclusion Characteristic TLRs expression profile associated with distinct autoantibody repertoire is suggestive of differential immuno-regulatory pathways operative in different subsets of SLE patients.

Keywords Toll-like receptor · systemic lupus erythematosus · autoantibody · dsDNA · extractable nuclear antigen · glomerulonephritis

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Introduction

Systemic lupus erythematosus (SLE) is an autoimmune disease characterized by a diverse range of autoantibodies against nuclear and cytoplasmic components. The SLE patients display great heterogeneity in both their autoantibody repertoire and clinical manifestations [1, 2]. The two major groups of autoantibody populations in SLE are targeted either against dsDNA or against RNA-associated proteins like Sm, RNP, SS-A, SS-B, etc. (also known as extractable nuclear antigens; ENA). Anti-dsDNA autoantibodies are considered to be highly pathogenic and have been implicated in many SLE-associated disease manifestations, especially glomerulonephritis (GN) [3, 4]. Although anti-dsDNA autoantibodies are regarded as the hallmark of SLE, there are large numbers of SLE patients who predominately have autoantibodies against ENA [5]. The etiology of SLE being multifactorial, there is no general unifying theory that leads to the basis for this distinctive autoantibody profile in SLE.

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However, recent reports suggesting the influence of Toll-like receptors (TLRs) on specific autoantibody generation in a murine model [6, 7] led us to explore whether TLR expression was part of an underlying mechanism for autoantibody distinction in SLE patients. TLRs are pattern recognition receptors recognizing specific molecular patterns of invading pathogens [8] and so far 10 functional TLRs have been identified in humans [9]. They are essential for regulating the immune response and their role in various autoimmune diseases including SLE has been widely documented [10]. Recent reports have indicated the role of TLR7 and TLR9 in generation of autoantibodies against ENA and dsDNA respectively in a murine model of lupus [7, 11, 12]. Apart from murine models, in vitro studies using SLE patient's blood cells have revealed upregulation of TLR7 and TLR9 [13–15]. However, the association of TLR7 and TLR9 with anti-ENA and anti-dsDNA autoantibodies respectively has not been established in humans.

In light of the above findings, we hypothesized that SLE patients characterized into different subsets based on autoantibody profile (anti-dsDNA or anti-ENA) could be further distinguished by novel associations and expression patterns of TLRs. The fact that nucleic-acid sensing TLRs exhibit selective specificity for their ligands (TLR3/7/8 recognize RNA whereas TLR9 senses dsDNA) and our earlier report that there are differences in the gene expression of two groups of genetically defined non-inbred rabbits with distinct autoantibody profiles (anti-ENA vs. -dsDNA autoantibodies) [16, 17] provided a rational basis to conduct this investigation.

In this study we determined the expression of nucleic-acid sensing TLRs (TLR3, TLR7, TLR8, and TLR9) in different subsets of SLE patients distinguished on the basis of autoantibodies against either dsDNA or ENA. We observed preferential upregulation of TLR7 and TLR9 expression in patients with anti-ENA and anti-dsDNA autoantibodies respectively. The TLR9 mRNA expression was further increased in SLE patients with GN and anti-dsDNA positivity. In addition, we have demonstrated higher serum interferon-alpha (IFN-α) and interferon regulatory factor 7 (IRF7; an IFN-inducible gene) mRNA expression in patients with autoantibodies against both dsDNA and ENA compared to patients with anti-dsDNA or anti-ENA only. Our finding that TLRs influence autoantibody patterns in SLE patients may offer a new approach for studying molecular and cellular events in the development and progression of SLE.

Methods

Subjects

Forty-seven patients, diagnosed with SLE, according to American College of Rheumatology 1997 revised criteria [18], attending the outpatient department from January 2009-February 2013 at Sir Sunder Lal Hospital, Banaras Hindu University, Varanasi were enrolled for the study. After prior approval from our institutional ethics committee, informed consent was obtained from all participants and the study was performed in accordance with the 1964 declaration of Helsinki and its later amendments. All patients were female (median age: 28 years, range: 15–56 years) and most of them were on medications (Table I) generally including prednisolone, hydroxychloroquine, and non-steroidal anti-inflammatory drugs. The disease activity of SLE patients was scored using the Systemic Lupus Erythematosus Disease Activity Index 2000 (SLEDAI-2000) [19]. The descriptive clinical features and SLEDAI-2000 scores of SLE patients are shown in Table I. SLE patients were divided into three subsets based on their serum autoantibody profile as determined by indirect ELISA. Subset 1 included 18 patients (S01-S18) positive for autoantibodies against dsDNA only (anti-dsDNA$^+$); subset 2 comprised of 18 patients (S30-S47) positive for anti-ENA autoantibodies only (anti-ENA$^+$) and subset 3 included 11 patients (S19-S29) possessing autoantibodies against both dsDNA and ENA (anti-dsDNA$^+$ ENA$^+$). Twenty-five age- and sex-matched healthy individuals (median age: 27 years, range: 20–58 years, and all females) served as controls. Peripheral blood (5 ml) was collected in sterilized heparin-coated tubes and some blood was coagulated to obtain serum.

Separation of Peripheral Blood Leukocytes (PBLs) and RNA Isolation

Whole blood (1 ml) was mixed with 4 ml of RBC lysis buffer (155 mM NH_4Cl, 12 mM $NaHCO_3$, 0.1 mM EDTA) and incubated at room temperature for 10 min to lyse the RBCs. The PBLs were recovered by centrifugation at 300 g and washed with 5 ml of phosphate buffer saline. Total RNA was extracted from the PBLs using 1 ml of TRI Reagent™ (Sigma-Aldrich, St Louis, MO) following manufacturer's instructions. Briefly, the cell lysate was mixed with 200 μl of chloroform, centrifuged at 12,000 g for 15 min at 4 °C. The aqueous phase was aspirated, mixed with equal volume of isopropanol and centrifuged at 12,000 g for 10 min at 4 °C. The RNA pellet was washed with 1 ml of 75 % ethanol, air-dried and dissolved in nuclease-free water. The quality and quantity of RNA was assessed using a NanoDrop® ND1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE) and RNA integrity was determined by running the RNA samples on 1 % denaturating formaldehyde-agarose gel.

cDNA Synthesis

The RNA was reverse transcribed using a high capacity cDNA reverse transcription kit (Applied Biosystems,

Table I Clinical details of SLE patients

Patient I.D.	Age (years)	Anti-dsDNA	Anti-ENA	Clinical manifestations	SLEDAI-2000 (score)	Medications
S01	20	+	–	*Glomerulonephritis*, arthritis, cutaneous, pericarditis	15	Prednisolone
S02	24	+	–	Cutaneous	5	Prednisolone
S03	35	+	–	Cutaneous	5	Eltroxin
S04	56	+	–	Arthritis, cutaneous	8	Prednisolone, HCQ
S05	35	+	–	*Glomerulonephritis*, pericarditis, hepatomegaly	9	Carvedilol, Lasilactone
S06	50	+	–	Arthritis, cutaneous, oral ulcers	11	Esomeprazole
S07	30	+	–	Arthritis, cutaneous	11	Cernevit
S08	28	+	–	Arthritis, cutaneous	8	NSAIDs
S09	32	+	–	Pleuritis, arthritis, cutaneous, oral ulcers	31	Prednisolone, HCQ
S10	37	+	–	Arthritis, cutaneous	8	Prednisolone
S11	28	+	–	*Glomerulonephritis*, leucopoenia, anaemia	15	Prednisolone
S12	22	+	–	Myositis	6	Prednisolone, HCQ
S13	45	+	–	Arthritis, cutaneous	11	NSAIDs
S14	17	+	–	Arthritis, oral ulcers, cutaneous	14	Prednisolone
S15	40	+	–	*Glomerulonephritis*,arthritis	10	Prednisolone, HCQ
S16	16	+	–	Oral ulcers, cutaneous	8	Prednisolone
S17	36	+	–	Arthritis, oral ulcers, cutaneous	13	Phentermine
S18	38	+	–	*Glomerulonephritis*, arthritis, cutaneous	13	Prednisolone, HCQ
S19	25	+	+	Arthritis, cutaneous, oral ulcers	12	Prednisolone, NSAIDs
S20	18	+	+	*Glomerulonephritis*, cutaneous	8	Eltroxin, Mysolone-N
S21	25	+	+	*Glomerulonephritis*, arthritis, oral ulcers	12	NSAIDs
S22	16	+	+	Cutaneous	6	N.A.
S23	24	+	+	*Glomerulonephritis*, cutaneous	6	Promethazine
S24	36	+	+	Arthritis	8	Prednisolone, HCQ
S25	28	+	+	*Glomerulonephritis*, anaemia	6	Prednisolone
S26	22	+	+	*Glomerulonephritis*, arthritis, cutaneous	15	N.A.
S27	46	+	+	Arthritis, cutaneous	9	HCQ, NSAIDs
S28	32	+	+	Arthritis, cutaneous	8	Alfacalcidol
S29	27	+	+	Oral ulcers, cutaneous	16	Prednisolone, HCQ
S30	40	–	+	*Glomerulonephritis*, cutaneous	6	Fexofenadine
S31	24	–	+	Arthritis, cutaneous, oral ulcers	8	Prednisolone
S32	32	–	+	Arthritis, cutaneous, oral ulcers	12	Prednisolone, HCQ
S33	28	–	+	Oral ulcers, cutaneous, leucopoenia	5	Prednisolone, HCQ
S34	36	–	+	Arthritis, oral ulcers, cutaneous, anaemia	9	Prednisolone, NSAIDs
S35	42	–	+	Arthritis, cutaneous, oral ulcers	9	Prednisolone, HCQ
S36	34	–	+	Neurological, arthritis, cutaneous, oral ulcers	33	Prednisolone, HCQ
S37	36	–	+	Arthritis, cutaneous	6	Prednisolone
S38	26	–	+	Pericarditis, arthritis	14	Prednisolone
S39	32	–	+	*Glomerulonephritis*, arthritis	30	Prednisolone, HCQ
S40	32	–	+	Myositis, arthritis	8	Prednisolone, HCQ
S41	18	–	+	Neurological, cutaneous, hepatomegaly, splenomegaly	20	Prednisolone, Epsolin
S42	26	–	+	Pericarditis, arthritis	6	N.A.
S43	15	–	+	*Glomerulonephritis*, arthritis	17	Prednisolone
S44	19	–	+	Arthritis	8	Prednisolone, HCQ
S45	27	–	+	*Glomerulonephritis,* arthritis, cutaneous	20	Prednisolone, HCQ
S46	22	–	+	Arthritis, myositis, cutaneous	9	Indometacin
S47	30	–	+	Arthritis, myositis, cutaneous	10	Prednisolone

All patients were female; Glomerulonephritis is italicized as it represented the major end-organ disease in our cohort of patients

HCQ Hydroxychloroquine, *NSAIDs* Non-steroidal anti-inflammatory drugs, *N.A.* Medications history not available

Foster City, CA) as per manufacturer's directions. Briefly, RNA was treated with RNase-free DNase (New England Biolabs, Ipswich, MA) to remove genomic DNA. After DNase treatment, RNA (2 μg) was added to 10 μl of RT mix containing 2 μl of RT random primer (10X), 0.8 μl of dNTPs mix (100 mM), 2 μl of RT buffer (10X), 1 μl of MultiScribe™ Reverse Transcriptase (50 U/μl) and 1 μl of RNase inhibitor (10 U/μl). The reaction mix (20 μl) was incubated at 25 ^{0}C for 10 min followed by incubation at 37 ^{0}C for 2 h. The reverse transcriptase was then inactivated by heating the reaction mixture at 85 ^{0}C for 5 min followed by chilling at 4 ^{0}C.

Real-time PCR

Real-time PCR was performed for TLRs (3, 7, 8 and 9) and IRF7. All mRNA sequences were retrieved from NCBI database (http://www.ncbi.nlm.nih.gov). The gene-specific primers were designed either using Autoprime software or taken from published reports [20, 21] and synthesized at Integrated DNA Technologies (Coralville, IA). Primer sequences and corresponding product size are given in Table II. The PCR reactions were carried out using quantifast syber green PCR mix (Qiagen, Germantown, MD) in an Applied Biosystems 7,500 real-time PCR system at 95 °C for 10 min, followed by 40 cycles at 95 °C for 15 sec and at primer-specific annealing temperature (Table II) for 1 min. The threshold cycle (Ct) was defined as the fractional cycle number at which the fluorescence exceeds the fixed threshold. The β-actin gene was selected as an internal control to normalize the gene expression data. The relative expression of each gene was determined using $2^{-\Delta\Delta Ct}$ method, where $DDC_t = (DC_{t\ SLE} - DC_{t\ Controls})$.

Detection of Autoantibodies

Anti-ENA and anti-dsDNA autoantibodies were detected in sera of SLE patients using indirect ELISA kits according to manufacturer's instructions (Aesku diagnostics, Wendelsheim, Germany). The anti-ENA ELISA kit had wells coated with a mixture of 6 ENA (Sm, RNP, SS-A, SS-B, Jo-1 and Scl-60). Briefly, 100 μl of standards and diluted sera (1:101) samples were added to the wells coated with dsDNA or ENA, and incubated for 30 min at room temperature. The fluid was aspirated and wells were washed three times with wash buffer. The anti-human-IgG conjugated to HRP (100 μl) was added to the each well and incubated for 15 min at room temperature. The fluid was again aspirated and wells were washed three times with wash buffer. Substrate (100 μl) was added and incubated at room temperature for 15 min. On appearance of the blue color, reaction was stopped adding 100 μl of stop solution (1 M HCl) per well and absorbance was measured at 450 nm wavelength using an ELISA reader (Bio-Rad Laboratories, Hercules, CA).

IFN-α ELISA

Sera IFN-α levels of SLE patients and controls were determined using sandwich IFN-α ELISA kit (Beckman coulter,

Table II Gene-specific primers for real-time PCR

Gene	Primers (Sequences)	Annealing temperature	Product size (bp)	NCBI Reference Sequence	Reference
TLR3	F- 5′ GGCTAGCAGTCATCCAACAGAA 3′	60 °C	167	NM_003265.2	AutoPrime software[a]
	R- 5′ TCATCGGGTACCTGAGTCAACT 3′				
TLR7	F- 5′ TTTACCTGGATGGAAACCAGCTA 3′	60 °C	73	NM_016562.3	[20]
	R- 5′ TCAAGGCTGAGAAGCTGTAAGCTA 3′				
TLR8	F- 5′ TTATGTGTTCCAGGAACTCAGAGAA 3′	58 °C	82	NM_138636.4	AutoPrime software[a]
	R-5′TAATACCCAAGTTGATAGTCGATAAGTTTG 3′				
TLR9	F- 5′ CCACCCTGGAAGAGCTAAACC 3′	60 °C	161	NM_017442.3	[21]
	R- 5′ GCCGTCCATGAATAGGAAGC 3′				
IRF7	F- 5′ CCTGGTGAAGCTGGAACC 3′	58 °C	80	NM_001572.3	AutoPrime software[a]
	R- 5′ TGCTATCCAGGGAAGACACAC 3′			NM_004029.2	
				NM_004031.2	
β-actin	F- 5′ CTTCCTGGGCATGGAGTC 3′	58 °C	87	NM_001101.3	AutoPrime software[a]
	R- 5′ TACAGGTCTTTGCGGATGTC 3′				

[a] Web address: www.autoprime.de

Marseille, France) according to manufactures instructions. Briefly, standards or sera samples (50 μl) and diluent (50 μl) were added to the wells coated with antibody against human IFN-α and incubated for 2 h at room temperature. The fluid was aspirated and wells were washed three times with wash buffer. Biotinylated monoclonal antibody against IFN-α (50 μl) and streptavidin-HRP conjugate (100 μl) was added to the each well and incubated for 30 min at room temperature. The wells were washed three times and substrate (100 μl) was added. Following incubation at room temperature for 15 min, reaction was stopped adding 50 μl of stop solution (2 N H_2SO_4) and absorbance was measured at 450 nm wavelength.

Western Blotting

TLR7 and 9 protein expression was determined by western blotting using a standard protocol. Briefly, PBLs from SLE patients and controls (2×10^6) were lysed in a cell lysis buffer (50 mM Tris–HCl, 0.1 mM EDTA, 1 mM dithiothreitol, and 1 mM phenylmethylsulfonylfluoride) on ice for 20 min with occasional vortexing. The cell lysate was centrifuged at 12,000 g for 10 min at 4 °C to remove sheared genomic DNA and other cellular debris. The supernatant containing protein fraction was collected and quantified using Bradford method [22]. Protein (100 μg) was separated on a 10 % sodium dodecyl sulphate-polyacrylamide gel and blotted onto PVDF membrane. The membrane was blocked with 5 % bovine serum albumin in Tris buffered saline-Tween-20 (TBST) for 2 h at room temperature, washed, and incubated with mouse anti-human TLR7 or mouse anti-human TLR9 antibody (1:100 dilution, Imgenex, San Diego, CA) overnight at 4 °C. The membrane was then washed three times with TBST and incubated with HRP-conjugated rabbit anti-mouse antibody (1:1,000 dilution) for 2 h at room temperature. Detection of TLR7 and 9 proteins was performed by enhanced chemiluminescence detection method using immobilon™ reagent (Millipore Corporation, Billerica, MA). The rabbit anti-human GAPDH antibody (Imgenex, San Diego, CA) was used to determine the expression of GAPDH housekeeping control for normalizing the differences in protein quantity and quality across the samples. The TLR7 and TLR9 protein levels were expressed as a ratio of TLR7 or 9 band intensity normalized against GAPDH.

Flow Cytometry

Expression of TLR7 and 9 on $CD19^+$ B-lymphocytes and $CD14^+$ monocytes of SLE patients and controls was determined by flow cytometry. PBLs were stained for cell surface antigens with APC-conjugated mouse anti-human CD19 and CD14 antibodies or corresponding isotype controls (BD biosciences, San Diego, CA). Cells were then stained with PE-labelled mouse anti-human TLR7 and FITC-labelled mouse anti-human TLR9 antibodies or corresponding isotype controls (Imgenex, San Diego, CA). The data was acquired and processed using BD caliber flow cytometer (BD biosciences, San Diego, CA). Briefly, for cell surface antigen staining, APC-conjugated anti-CD14/CD19 monoclonal antibodies at recommended dilutions were added to 100 μl of cell suspension and incubated at room temperature for 15 min. Following staining, cell suspension was centrifuged and washed in wash buffer (DPBS containing 1 % FBS and 0.09 % sodium azide) and resuspended in Cytofix/Cytoperm™ Reagent (BD biosciences, San Diego, CA) for cell fixation and permeabilisation and incubated for 20 min at room temperature. The cell pellet was washed once with perm/wash buffer followed by addition of anti-human TLR7-PE or anti-human TLR9-FITC antibody and incubated for 30 min at room temperature. The cell pellet was washed again with perm/wash buffer, and then resuspended in 0.5 ml of 2 % paraformaldehyde in PBS. The stained cells were stored at 4 °C until acquisition.

Statistical Analysis

The statistical significance between two groups was determined using the non-parametric Mann–Whitney *U* test. For correlation studies spearman rank correlation was used. A p-value less than 0·05 was considered as statistically significant. All statistical calculations were done using GraphPad prism software v.5.0 (GraphPad Software, San Diego, CA).

Results

Preferential Increase in Expression of TLR7 in Anti-ENA^+ Patients and TLR9 in Anti-$dsDNA^+$ Patients

The mRNA expression of nucleic acid sensing TLRs 3, 7, 8, and 9 was studied. The grouping of the SLE patients into different subsets based on different autoantibody populations revealed varying patterns of expression of TLRs. The TLR7 and TLR9 mRNA levels were found to be significantly higher in SLE patients of all three subsets (anti-$dsDNA^+$, anti ENA^+, and anti-$dsDNA^+$ ENA^+) than controls (Fig. 1a, b). Upon inter-subset analysis, it was noted that TLR7 expression was highest in the anti-ENA^+ subset followed by the anti-$dsDNA^+$ ENA^+ subset. The expression of TLR7 in the anti-$dsDNA^+$ subset was significantly lower than in the other two subsets ($p<0.05$) (Fig. 1a). In contrast, TLR9 expression was higher in the anti-$dsDNA^+$ and anti-$dsDNA^+$ ENA^+ subsets than in the anti-ENA^+ subset ($p<0.05$) (Fig. 1b). It is noteworthy that the SLEDAI-2000 scores among the patients with either autoantibody specificity were comparable (Table I). There was

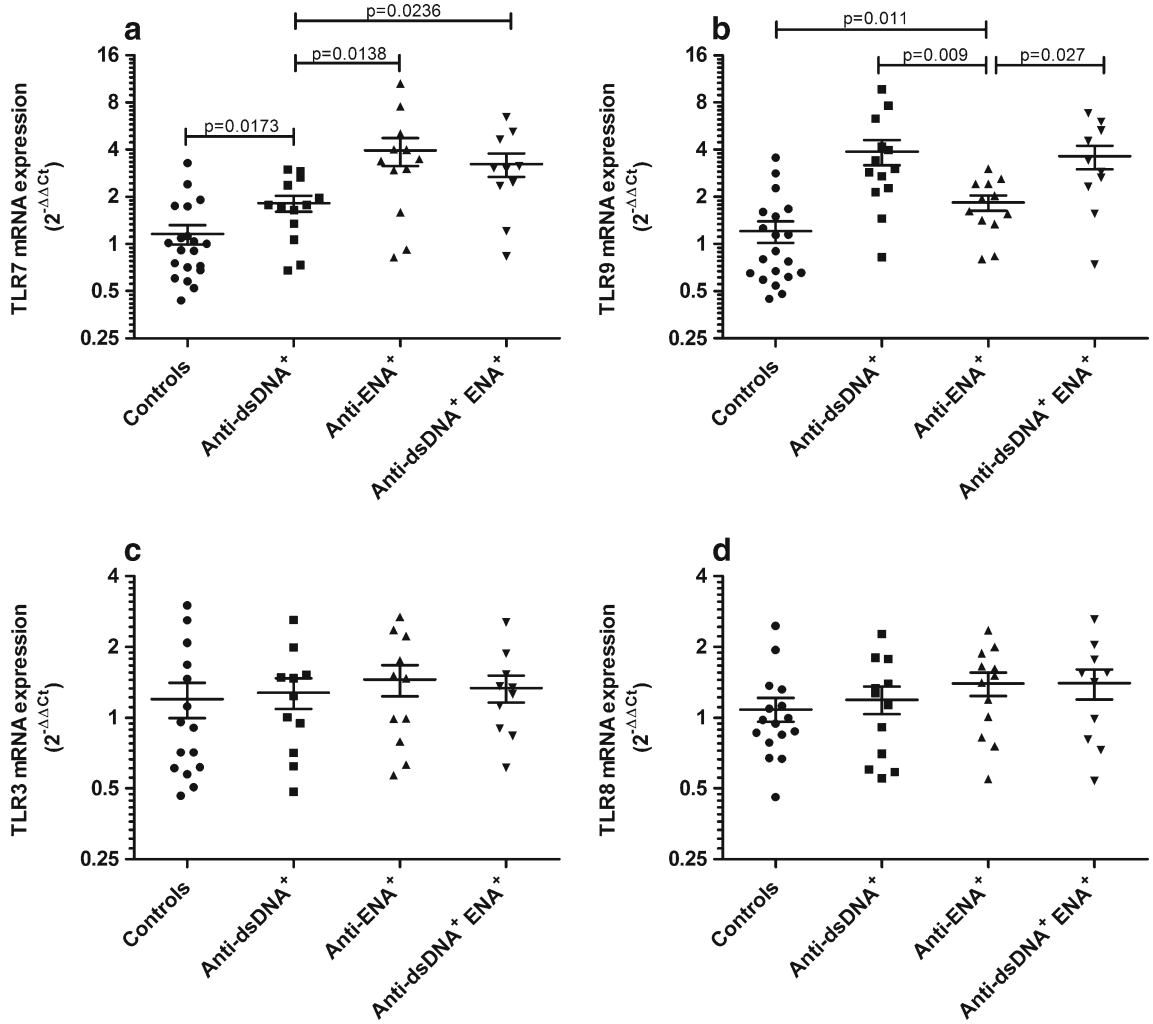

Fig. 1 mRNA expression levels of TLRs (3, 7, 8, and 9) as determined by real-time PCR in different subsets of SLE patients (anti-dsDNA$^+$, anti-ENA$^+$, and anti-dsDNA$^+$ ENA$^+$). The X-axis represents the different SLE subsets and Y-axis represents the mRNA expression of TLRs in terms of $2^{-\Delta\Delta Ct}$. **a** TLR7 mRNA expression was higher in anti-ENA$^+$ and anti-dsDNA$^+$ ENA$^+$ patients compared to patients with anti-dsDNA only. **b** TLR9 mRNA expression was higher in anti-dsDNA$^+$ and anti-dsDNA$^+$ ENA$^+$ patients compared to patients with anti-ENA alone. **c** TLR3 mRNA expression and **d** TLR8 mRNA expression was not significantly different among the different autoantibody subsets of SLE patients

no significant difference in the expression levels of TLR3 and TLR8 between SLE patients and controls (Fig. 1c, d). We further analyzed protein expression of TLR7 and 9 in PBLs of SLE patients (n=14) and healthy controls (n=5). We observed that TLR7 protein expression was higher in anti-ENA$^+$ patients compared to anti-dsDNA$^+$ patients whereas TLR9 protein levels were higher in anti-dsDNA$^+$ patients (p<0.05) (Fig. 2).

Selective Expression of TLR7 and 9 on B-cells of SLE Subsets

After observing the preferential upregulation of TLRs in different SLE subsets using PBLs, we wanted to identify the contribution of specific cell types. We therefore compared the TLR7 and 9 expressions on CD19$^+$ B-cells and CD14$^+$ monocytes of SLE patients (n=9) and controls (n=5). We observed that B-cells of anti-ENA$^+$ patients had higher TLR7 levels than anti-dsDNA$^+$ patients (p<0.05) (Fig. 3a, c). On the other hand anti-dsDNA$^+$ patients showed higher TLR9 levels on B-cells as compared to anti-ENA$^+$ patients (p<0.05) (Fig. 3b, d). We also compared the numbers of CD19$^+$ B-cells between anti-ENA$^+$ and anti-dsDNA$^+$ patients, and found no difference in the frequency of these cells (data not shown). In contrast to B-cells, there was no difference in TLR7 and 9 protein expressions on monocytes of the two SLE subsets (data not shown).

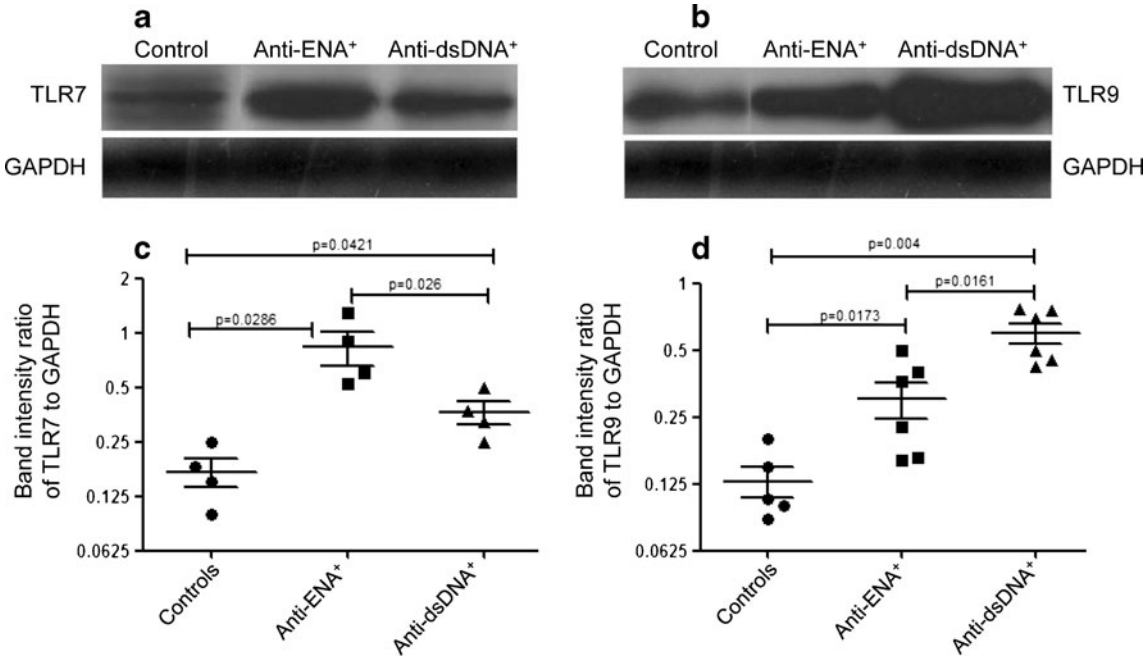

Fig. 2 TLR7 and 9 protein expression in PBLs of SLE patients and controls. **a** Representative figure showing TLR7 and **b** TLR9 protein expression in PBLs of anti-ENA$^+$ and anti-dsDNA$^+$ SLE patients and controls. GAPDH was used as a housekeeping control. **c** Band intensity ratio of TLR7 protein to GAPDH was higher in anti-ENA$^+$ patients compared to anti-dsDNA$^+$ patients and controls. **d** Band intensity ratio of TLR9 protein to GAPDH was higher in anti-dsDNA$^+$ patients compared to anti-ENA$^+$ patients and controls

Presence of Anti-dsDNA Autoantibodies, TLR9 Upregulation, and Glomerulonephritis

As GN patients represented the major end-organ disease subset in our SLE patients and anti-dsDNA autoantibodies have been linked with GN, we compared the TLR7 and TLR9 mRNA expression between SLE patients with or without GN. Of the 10 GN patients studied, 3 were anti-dsDNA$^+$, 5 were anti-dsDNA$^+$ ENA$^+$, and 2 were anti-ENA$^+$. We observed that TLR9 mRNA expression was significantly upregulated in patients with GN compared to patients without GN ($p<0.05$) (Fig. 4a). The association of TLR9 upregulation already being established with anti dsDNA autoantibodies, we further wanted to check whether presence of GN had any additional effect on TLR9 expression. We divided anti-dsDNA$^+$ patients in two groups based on presence (anti-dsDNA$^+$/GN$^+$) and absence of GN (anti-dsDNA$^+$/GN$^-$). We observed a significant increase in TLR9 expression in the dsDNA$^+$/GN$^+$ group as opposed to the anti-dsDNA$^+$/GN$^-$ group ($p<0.05$) (Fig. 4b). We further analyzed for correlation between titers of anti-dsDNA autoantibodies and TLR9 mRNA levels in GN patients and found they did not correlate with each other (data not shown). No significant change was observed in TLR7 mRNA expression in SLE patients with or without GN (data not shown).

Serum IFN-α Levels and IRF7 mRNA Expression were the Highest in Anti-dsDNA$^+$ ENA$^+$ Subset of Patients

Patients of all three SLE subsets (anti-dsDNA$^+$, anti ENA$^+$, and anti-dsDNA$^+$ ENA$^+$) had higher serum IFN-α and IRF7 mRNA expression compared to controls ($p<0.05$) (Fig. 5). Among the different SLE subsets, we observed that serum IFN-α levels were significantly higher ($p<0.05$) in dsDNA$^+$ ENA$^+$ subset than the other two subsets which did not differ significantly among themselves. A similar pattern of expression was observed for IRF7 gene (Fig. 5).

Discussion

The present investigation focused on identifying variations in expression of nucleic acid sensing TLRs in SLE patients with different autoantibody profiles. Our finding that the expression of TLR7 and TLR9 is associated with anti-ENA and anti-dsDNA autoantibodies respectively extends the earlier observations made using a murine model [6, 7, 12] to human SLE patients. The findings point towards differential immuno-modulating mechanisms operative in the patients with different autoantibody specificities. Interestingly, absence of such association patterns with other RNA sensing TLRs (TLR3 and TLR8) suggests that there are unique regulatory processes related to TLR7 and TLR9

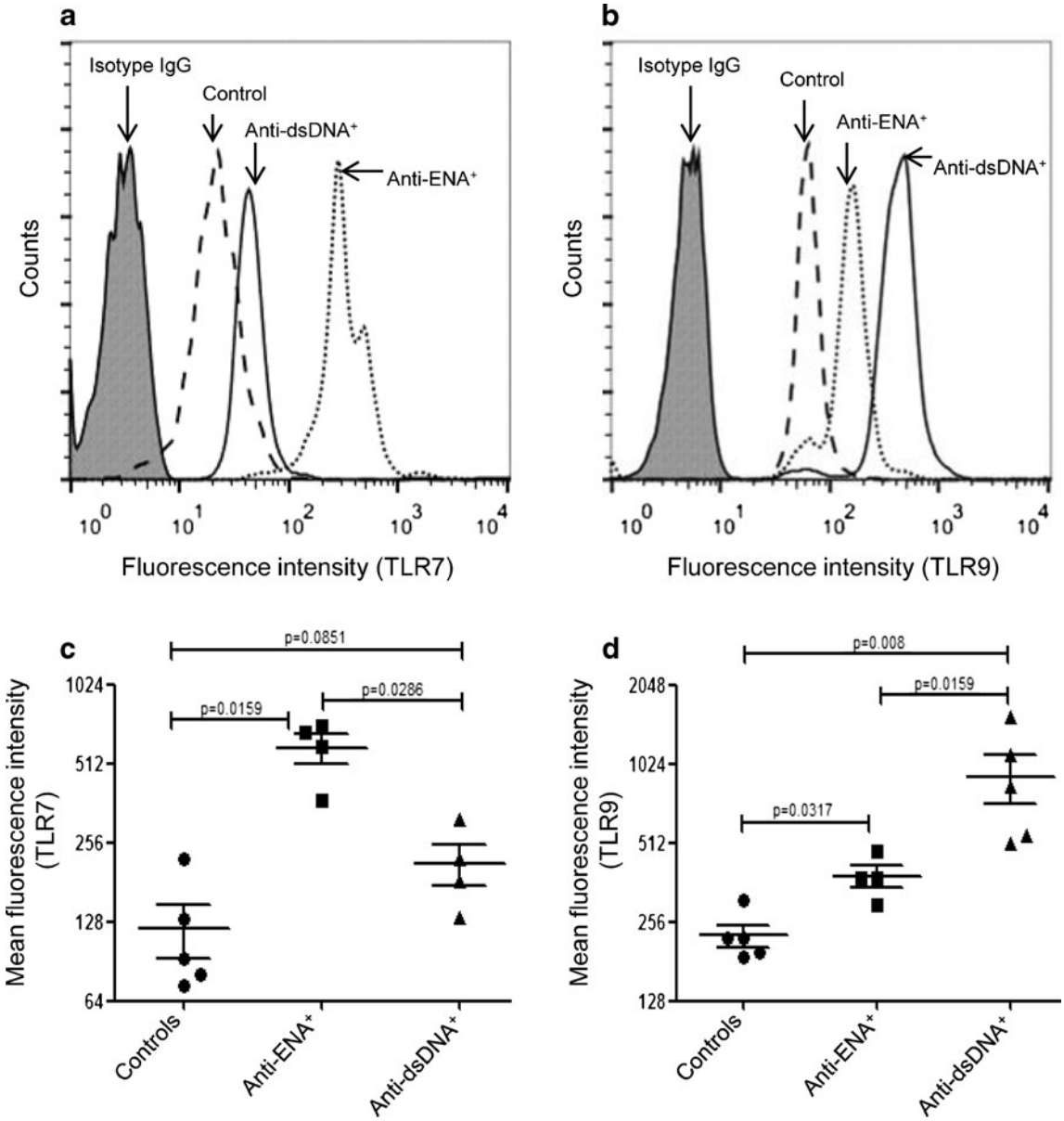

Fig. 3 TLR7 and 9 expression on B-cells of SLE patients and controls. **a** Representative staining pattern of TLR7 and **b** TLR9 on B-cells of anti-ENA$^+$ (dotted line), anti-dsDNA$^+$ (solid line) SLE patients and controls (dashed line). Isotype IgG is represented by filled histogram. **c** Mean fluorescence intensity of TLR7 was higher in anti-ENA$^+$ patients compared to anti-dsDNA$^+$ patients and controls. **d** Mean fluorescence intensity of TLR9 was higher in anti-dsDNA$^+$ patients compared to anti-ENA$^+$ patients and controls

signaling. A report using a murine lupus model suggested that the increase in autoantibodies reflected increased TLR gene dosage [23]. Future investigations may reveal whether the elevated expression of these TLRs is induced via some stimuli that lead to the induction of autoantibodies or alternatively, development of anti-ENA or -dsDNA autoantibodies precedes the upregulation of TLR7 or TLR9. Preferential upregulation of TLR7 and 9 on B-cells of anti-ENA$^+$ and anti-dsDNA$^+$ SLE patients was an interesting observation. Since B-cells are the antibody producing cells with central role in SLE pathogenesis [24], it is possible that selective upregulation of specific TLRs could be a part of mechanism leading to generation of autoantibodies from specific B-cell clones. DNA-containing immune complexes can activate autoreactive B-cells to produce autoantibodies by co-engagement of B-cell receptor (BCR) and TLR9 in a sequential fashion [25]. Further BCR-TLR dual engagement model for autoantibody production was subsequently extended by showing that RNA-associated autoantigens could also stimulate autoantibody production in a TLR7 dependent process [26].

Functional abnormalities in the cells of myeloid origin such as monocytes, neutrophils and dendritic cells have also

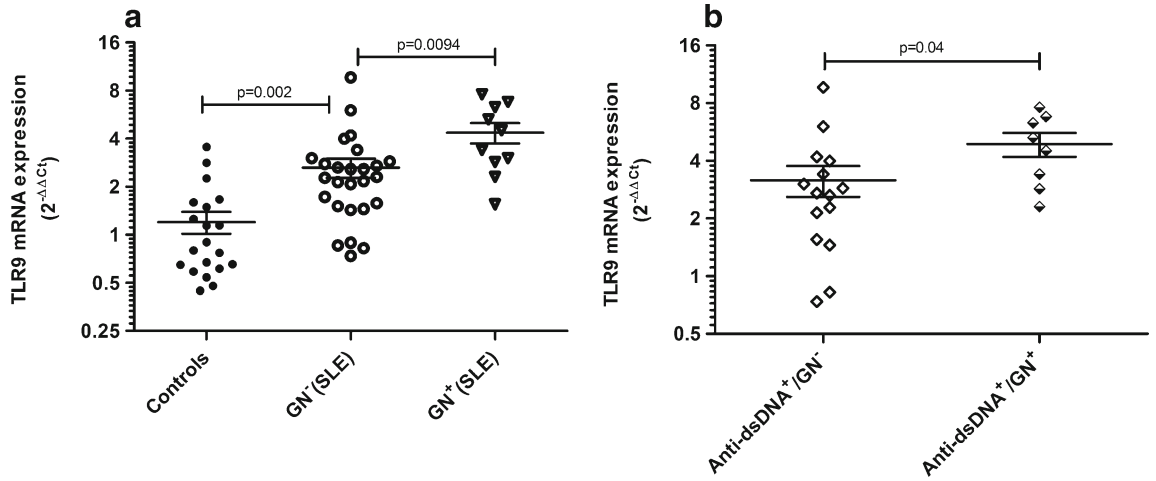

Fig. 4 TLR9 mRNA expression levels in SLE patients with or without GN as determined by real-time PCR. **a** TLR9 mRNA expression was higher in GN^+ as compared to GN^- SLE patients. **b** Anti-$dsDNA^+/GN^+$ patients had higher TLR9 mRNA expression compared to anti-$dsDNA^+/GN^-$ patients

been widely documented in SLE [27–29]. We observed no difference in TLR7 and 9 expressions on monocytes of SLE patients and controls, and in another study TLR9 expression levels of plasmacytoid dendritic cells have been shown to be similar between SLE patients and healthy subjects [30]. It would however be interesting to examine the variation in TLRs expression in different monocyte subpopulations as well as other cell types of myeloid origin like neutrophils and myeloid dendritic cells, etc., in SLE subsets with different autoantibody specificity. Our observation of elevated TLR7 and 9 in different SLE subsets may also be extended further to evaluate the activation status of these TLRs by studying downstream signaling molecules like MyD88, TRAF, IRAK, etc. Some studies have documented enhanced nuclear translocation of NF-kB [31] and activation of mitogen activated kinases [32] in SLE patients. Ongoing in-depth functional studies in our lab will elucidate the status of various signaling pathway proteins involved in TLR activity. Furthermore, proteomic studies in progress will serve to enhance our understanding on various other signaling pathways affected in SLE.

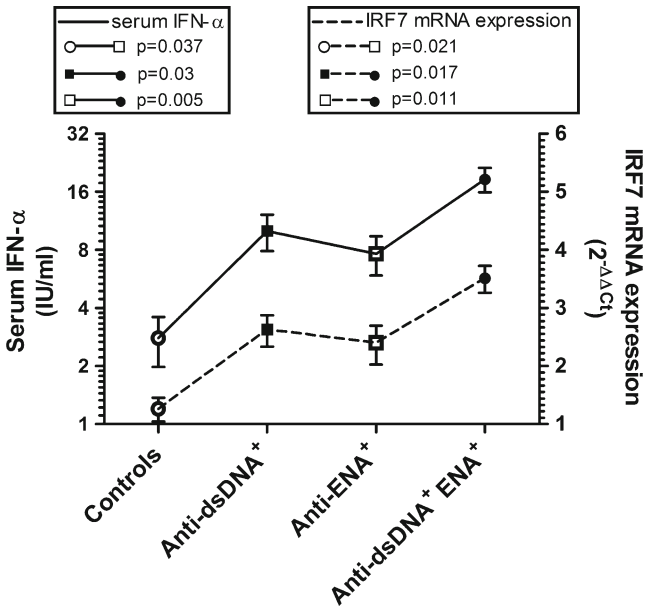

Fig. 5 Serum IFN-α (—) and IRF7 (….) mRNA expression levels in SLE patients with different autoantibody specificities. Patients positive for both anti-dsDNA and anti-ENA autoantibodies had higher serum IFN-α and IRF7 mRNA expression levels compared to patients with either anti-dsDNA or anti-ENA autoantibodies only. Serum IFN-α levels are expressed in IU/ml and represented at left Y-axis. IRF7 mRNA levels are expressed in terms of $2^{-\Delta\Delta Ct}$ and represented at right Y-axis. The X-axis represents the different SLE subsets. The boxes at top left (serum IFN-α) and at top right (IRF7 mRNA expression) represent the results of statistical analysis between different SLE subsets and controls

As anti-dsDNA autoantibodies have been implicated in GN, our result showing high TLR9 expression in the GN^+ patients was anticipated. What interested us more, was the observed higher upregulation of TLR9 in anti-$dsDNA^+/GN^+$ patients as compared to the anti-$dsDNA^+/GN^-$ patients. This indicates that the presence of anti-dsDNA autoantibodies may not be the sole factor correlating with TLR9 up regulation, presence of GN appears to augment TLR9 expression further. The combined effect of these two clinical entities on the observed expression levels of TLR9 may reflect anti-dsDNA induced extensive damage in renal tissues, exposing a multitude of dsDNA fragments that otherwise remain sequestered in the cells. Although pathogen-derived dsDNA fragments are natural ligands for TLR9, the overt release of dsDNA fragments from the inflamed kidney may stall TLR9's ability to discriminate between self and non-self leading to enhanced induction of this receptor. Although interplay between anti-dsDNA autoantibodies and TLR9 towards the development of GN is obvious, more in-depth

studies are needed to elucidate the direction of this link. Ongoing proteomic studies and microarray based investigations in progress may help address this question.

IRF7 is the key transcriptional regulator involved in TLR signaling leading to IFN-α induction [33]. High serum IFN-α is noted in SLE [34, 35] and an interferon-inducible gene signature is known to be present in some SLE patients' peripheral blood mononuclear cells [36, 37]. Recently, Niewold et al. [38] observed higher serum IFN-α activity in SLE patients positive for autoantibodies against both dsDNA and RNA-associated antigens compared to patients with only one of the autoantibody types. When we analyzed serum IFN-α and IRF7 mRNA levels in patient subsets with varying autoantibody specificities, we confirmed and extended the above report by showing that IRF7 mRNA levels too, along with serum IFN-α levels are higher in anti-dsDNA$^+$ ENA$^+$ SLE patients. As has been suggested by in vitro models, nucleic-acid immune complexes formed by these autoantibodies could activate endosomal TLRs resulting in IFN-α production [39]. An additive effect of both TLR7 and TLR9 upregulation in the anti-dDNA$^+$ENA$^+$ subset could account for this observation.

Conclusion

Differential TLRs expression profile identified in our study utilizing a new sub-grouping approach suggest differential mechanisms are operative in patients with different autoantibody repertoire. This observation could be of importance if TLRs are to be exploited for therapeutic interventions in SLE. Further our data indicate that other SLE related pathological and molecular events may also be evaluated in the light of differences in the autoantibody repertoire.

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Conflict of interest The authors declare that they have no conflict of interest.

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