Expression of Toll-like receptors and their detection of nuclear self-antigen leading to immune activation in JSLE

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Abstract

Objectives. Toll-like receptors (TLRs) essential in the functioning of the immune system have been implicated in the development of autoimmunity. TLR3, 7, 8 and 9 are capable of recognizing nucleic auto-antigens typical of SLE. Their expression correlates positively with disease activity in adult-onset SLE. This study aimed to determine the role of TLRs in JSLE and whether apoptotic neutrophils are a source of nuclear autoantigen being detected through TLR3, 7, 8 and 9, leading to an inflammatory response.

Methods. TLR3, 7, 8 and 9 mRNA and protein expression were measured in peripheral blood mononuclear cells (PBMCs) in JSLE patients compared with JIA and non-inflammatory controls. Activation of the TLRs by JSLE serum-induced apoptotic neutrophils was detected by measuring IFN-α mRNA and protein expression, and confirmed using myeloid differentiation factor 88 (MyD88) and TIR domain-containing adapter-inducing IFN-β (TRIF) inhibitors.

Results. JSLE patients have increased TLR3, 8 and 9 mRNA and protein expression compared with controls (P < 0.05). Incubation of PBMCs with apoptotic neutrophils demonstrated a dose–response relationship for IFN-α mRNA expression. Inhibition of TLR signalling by blocking MyD88 and TRIF signalling decreased IFN-α mRNA expression in PBMCs incubated with apoptotic neutrophils (P < 0.05).

Conclusions. This study demonstrated significantly increased TLR expression in JSLE compared with controls. Our data indicate that apoptotic neutrophils trigger TLR activation through their presentation of autoantigens. The role of TLRs in this inflammatory response was demonstrated by a dose–response relationship to apoptotic neutrophil concentration and confirmed by a decrease in IFN-α production after inhibition of TLR signalling.

Key words: Toll-like receptors, juvenile, SLE, neutrophil, apoptosis, nuclear self-antigen.

Introduction

Until recently, the induction of autoimmunity was thought to be primarily dependent on T- and B-cell dysfunction. However, it is now evident that the immune system is implicated in triggering autoimmunity [1]. Discovery of the Toll-like receptor (TLR) family significantly increased our understanding of how pathogen-associated molecular pattern (PAMP) recognition translates into innate and adaptive immune responses. Recent data have implicated TLRs in the pathogenesis of adult-onset SLE [2–4], RA [5] and diabetes [6].

SLE is the archetypal systemic autoimmune disease, characterized by a wide spectrum of clinical manifestations and autoantibody production. JSLE differs in important clinical aspects from adult-onset SLE, yet few studies explore its immunopathology. The most unifying feature among SLE patients is loss of tolerance to nucleic acid-binding nuclear antigens. The presence of autoantibodies raised against autoantigens, particularly antibodies directed against dsDNA, correlate closely with disease activity and are of diagnostic and prognostic value [7]. TLR3, 7, 8 and 9 can all recognize SLE-associated autoantigens [8]. Up-regulation of TLR mRNA occurs in peripheral blood mononuclear cells (PBMCs) from patients...
with adult-onset SLE [9]. Recently, TLR7 and 9 have been shown to have a key role in triggering activation of the type 1 IFN pathway in SLE, suggesting that inhibitors of TLR7 and 9 signalling could prove effective therapeutic options in SLE [10].

A potential source of these autoantigens is apoptotic cells [11]. Defects in these apoptotic cells are implicated in the pathogenesis of many diseases, including cancer, heart disease and several autoimmune diseases including SLE [12]. It is widely accepted [13], that apoptotic cells may be a potential source of autoantigens in SLE. There are very few studies demonstrating which apoptotic cells are important and the pathways activated; however, circulating levels of apoptotic neutrophils have been shown to correlate with disease activity and anti-dsDNA [14]. We have previously demonstrated an imbalance in both pro-apoptotic and anti-apoptotic factors in neutrophils and sera from patients with JSLE resulting in increased neutrophil apoptosis, augmented further by co-culture with the observed JSLE pro-apoptotic serum [15]. Furthermore, we have shown that dysregulated neutrophil apoptosis in JSLE relates to an imbalance in expression of factors within both the intrinsic and extrinsic apoptotic caspase pathways and their inhibitors [16].

We have demonstrated that during neutrophil apoptosis nuclear antigen (dsDNA) is exposed on the cell surface rather than within the cell as seen with viable neutrophils [17]. The increased neutrophil apoptosis induced by JSLE serum compared with control serum resulted in increased surface expression of nuclear antigens, which may provide an additional mechanism leading to the generation of autoantibodies in JSLE [17]. Here we investigate the expression of TLRs capable of recognizing SLE-associated autoantigens in JSLE, and whether apoptotic neutrophils are a source of such nuclear autoantigens, stimulating TLR signalling and resulting in an inflammatory response.

Patients and methods

Patients and controls

This study was approved by the Liverpool Paediatric Research Ethics Committee. Written informed patient or parental assent or consent was obtained from all subjects. JSLE patients fulfilled the revised ACR criteria for SLE [18] below the age of 17 years. Patients who fulfilled the modified ILAR criteria for JIA [14] were recruited as paediatric autoimmune disease controls. Paediatric non-inflammatory controls were defined as children with non-inflammatory musculoskeletal symptoms or those attending for elective surgery where no intercurrent infection was present. All patients were recruited from Alder Hey Children’s NHS Foundation Trust, Liverpool, UK. Standard data entry proformas were used. All samples were anonymized.

Cell preparation

Heparinized whole blood was collected and processed within 1 h. PBMCs were isolated by standard gradient centrifugation using Histopaque-1077 (Sigma-Aldrich). Neutrophils were isolated by one-step centrifugation through Polymorph Prep (Axis-Shield, Kimbolton, UK), following the manufacturer’s instructions. Contaminating erythrocytes were removed by hypotonic lysis. Neutrophils were routinely examined for purity and viability using trypan blue dye exclusion analysis immediately after isolation. Purity was confirmed using morphological analysis of cytopsin preparations. CD19+ B-cell population was separated from isolated PBMCs using an immunomagnetic cell selection procedure using EasySep™ mAb, magnetic nanoparticles and magnet following the manufacturer’s protocol (StemCell Technologies, Inc., Vancouver, BC, Canada).

Quantitative real-time PCR

Quantitative real-time PCR (qPCR) was used to assess TLR3, 7, 8 and 9 mRNA abundance in the PBMCs. RNA was extracted using the RNeasy miniprep kit (Qiagen) following the manufacturer’s instructions. The RNA concentration was determined at 260 nm, and purity was assessed by measuring the 260/280 nm ratio. First-strand cDNA synthesis was initiated from 0.5 μg total RNA using random hexamers (Promega, Madison, WI, USA) and avian myeloblastosis virus reverse transcriptase (Promega) using conditions as described by the manufacturer in a final volume of 25 μl. The primers used were as follows: TLR3 forward 5′-CCTGGTTGTTAATGGATTAA CGA-3′, reverse 5′-TGAGGTTAGTGTGCCAAAAGG-3′; TLR7 forward 5′-TTACCTGGATGAAAACAGGCTACT-3′, reverse 5′-TCAAGGCTGAGAAGCTGTAAAGCTA-3′; TLR8 forward 5′-CAGATAGACGGCGCTGACATCA-3′, reverse 5′-TGTCAGGGGATTGCCACTGA-3′; TLR9 forward 5′-TGAAGACTTCAACCCACTG-3′, reverse 5′-TGACCGTCACCAGGGTTG-3′; IFN-α forward 5′-GGAGTT TGATGGCAAACCAGT-3′, reverse 5′-CTCTCCTCGCAT CACACA-3′; and 18s forward 5′-TGCAGATACGCAGCAG ACTC-3′, reverse 5′-CGAGAATGTTAATCAAAGCAG-3′. All real-time PCR took place using the SYBR green fluorescence method with SYBR green qPCR master mix (Stratagene, La Jolla, CA, USA) as specified by the manufacturer. The real-time PCR reactions took place in triplicate on a MX3500 Multiplex Quantitative QPCR System (Stratagene) using standard default thermal cycling conditions. Non-template controls, loaded in triplicate, were prepared by replacing the cDNA fraction of the PCR with an equivalent volume of nuclelease-free water. Quantification of transcripts took place using the relative standard curve method. The 18s expression was monitored as an internal standard on the cDNA template; mRNA expression for each gene was normalized to this internal standard.

Protein analysis

To detect TLR3, 7, 8 and 9 protein expression using flow cytometry, the isolated cells were washed by centrifugation with buffer [PBS (pH 7.4), 0.5% BSA and 0.02% sodium azide] and fixed in BD Cytofix/Cytoperm (BD Biosciences) and incubated for 20 min at 4 °C. The cells
were washed twice in Perm/Wash buffer (BD Biosciences) and stained with anti-TLR8 antibody or FITC-labelled anti-TLR3, anti-TLR7 and anti-TLR9 or with isotype control mouse IgG (Imgenex) for 1 h at room temperature. Those cells incubated with the non-conjugated TLR8 primary antibody were incubated with a secondary antibody, FITC-labelled goat anti-mouse, for a further 30 min at room temperature. All cells were washed twice and analysed.

Induction of neutrophil apoptosis by JSLE serum

Neutrophils were isolated from non-inflammatory controls and resuspended at a concentration of $2 \times 10^9$ cells/ml in RPMI 1640 medium (Sigma-Aldrich, Poole, UK) with either 10% non-inflammatory control or JSLE sera and incubated at 37°C in an atmosphere consisting of 5% CO$_2$, as previously described [15]. Apoptosis (programmed cell death) and necrosis (uncontrolled cell death) [19] were measured by flow cytometry following 2 h of incubation with annexin V and propidium iodide (PI). Neutrophils were washed twice in RPMI 1640 media to remove any JSLE serum.

FITC-labelled annexin V and PI staining

Neutrophils were removed from culture and resuspended in Hanks’ balanced salt solution (HBSS) without phenol red (Invitrogen, Paisley, UK). FITC-labelled annexin V (Sigma-Aldrich) was added at a 1:100 dilution and cells were incubated at 4°C for 15 min, followed by incubation with 10 μg/ml of PI for 10 min at room temperature. Cells were pelleted at 400 g, resuspended in HBSS and then analysed by flow cytometry using an FC500 MPL flow cytometer (Beckman Coulter, High Wycombe, UK).

Stimulation of PBMCs with TLR ligands and apoptotic neutrophils

PBMCs ($2 \times 10^5$) were isolated and were either left unstimulated or incubated with 10 μg/ml poly I:C (TLR3 ligand), 1 μg/ml imiquimod (TLR7 ligand), 1 μg/ml ssRNA (TLR8 ligand), 1.5 μM ODN 2216 (TLR9 ligand; Invivogen, San Diego, CA, USA) or varying concentrations (0.5 × 10$^5$, 1 × 10$^5$ and 2 × 10$^5$) of JSLE serum-induced apoptotic neutrophils (PBMC concentration $1 \times 10^5$). All ligands were commercially bought and added at a dose within the manufacturers’ recommended reference range. Cells were incubated for 6 h at 37°C. Following incubation, the cell supernatants were stored at −20°C and the RNA was extracted from the cell pellets and stored at −80°C. Each experiment was repeated, using five independent donors.

IFN-α protein expression

IFN-α protein expression in cell supernatants from PBMCs incubated with either TLR ligands or apoptotic neutrophils was measured by ELISA purchased from eBioscience (San Diego, CA, USA) and was performed following the manufacturer’s instructions.

## TLR Inhibition assay

PBMCs ($2 \times 10^5$) isolated from non-inflammatory controls were either left alone or pre-treated with 10 μM myeloid differentiation factor 88 (MyD88) or TRIR domain-containing adapter-inducing IFN-β (TRIF) inhibitor (Invivogen) for 12 h at 37°C and 5% CO$_2$. Both sets of cells were then incubated with either 10 μg/ml poly I:C (TLR3 ligand) and 1.5 μM ODN2216 (TLR9 ligand) or 1 × 10$^5$ JSLE serum-induced apoptotic neutrophils for 6 h at 37°C and 5% CO$_2$. The level of apoptosis for each set of neutrophils was measured by flow cytometry using annexin V staining. Following incubation, the cell supernatants were stored at −20°C and the RNA was extracted from the cell pellets and stored at −80°C. Each experiment was repeated using five independent donors.

## Measurements

Statistical analysis used SPSS 15.0. The Friedman non-parametric test was used to analyse multiple-related groups. Following a significant value ($P < 0.05$), further statistical analysis was carried out on the data. Comparisons between JSLE and control patients were made using the Mann–Whitney test. Spearman’s rank correlation coefficient assessed any correlations.

## Results

### Clinical characteristics of patients

Twenty-one JSLE patients were studied, diagnosed at a mean (range) age of 10.6 (2.6–16.1) years; 8 (38%) were male, 13 were white British, 1 Pakistani, 3 Indians, 2 Bangladeshis and 2 other mixed race. The 17 non-inflammatory controls had a mean (range) age of

<table>
<thead>
<tr>
<th>Biomarker/activity parameter, mean (range)</th>
<th>Current medication, n</th>
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<tbody>
<tr>
<td>Age at sampling, years 11.2 (3.0–17.4)</td>
<td>HCQ 12</td>
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<tr>
<td>ESR mm/h (2–8)$^a$ 17 (1–87)</td>
<td>DMARD$^b$ 10</td>
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<tr>
<td>Total white cell count, 10$^9$/l 8.56 (2.45–26.3)</td>
<td>Prednisolone 12</td>
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<tr>
<td>Neutrophil count, 10$^9$/l 4.78 (1.31–15.57)</td>
<td>Dose, mean (range), mg 17 (5–45)</td>
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<td>Lymphocyte count, 10$^9$/l 2.74 (0.09–13.64)</td>
<td>Previous CYC 5</td>
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<tr>
<td>Complement C3, g/l (1.10–1.98)$^b$ 1.14 (3.4–27.29)</td>
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<tr>
<td>Complement C4 g/l (0.19–0.56)$^b$ 0.2 (0.12–0.38)</td>
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<tr>
<td>DsDNA titre, IU/ml (&lt;7)$^a$ 22 (0–280)</td>
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<td>IgG, g/l (7.4–13.9)$^a$ 12.5 (3.4–27.3)</td>
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<td>Physician’s global score VAS (0–100) 21.25 (0–95)</td>
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<td>BILAG 2004 score 2.68 (0–15)</td>
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$^a$Normal laboratory value range. $^b$Either MTX, AZA, CYC or MMF. VAS: visual analogue score.
12.2 (6.1–16.4) years; 8 (47%) were male, 13 were white British, 2 Chinese and 1 black Caribbean. Table 1 presents biomarker, physician’s global activity scores, BILAG disease activity index score and medication for the JSLE patients.

The 16 JIA control patients studied had a mean age of 11.5 (3.1–17.6) years; seven (44%) were male and all were white British. Seven had systemic onset, four had polyarticular, four had oligoarticular and one had psoriatic-associated JIA. The mean number of swollen joints across all JIA subtypes was 2.5 (range 0–18) with a range of disease activity from mild to severe.

**TLR mRNA expression in PBMCs**

PBMCs were isolated from whole blood from JSLE (n = 19) and JIA (n = 7) patients and non-inflammatory controls (n = 17). TLR mRNA expression was measured by qPCR, normalized to 18s mRNA expression (Fig. 1A). There was a statistically significant increase in TLR3 (P = 0.001), TLR8 (P = 0.001) and TLR9 (P = 0.002) mRNA expression in JSLE compared with JIA and non-inflammatory controls. TLR7 mRNA expression was higher in JSLE than both JIA and non-inflammatory controls, but was not statistically significant.

**TLR protein expression in PBMCs**

PBMCs were isolated from whole blood from JSLE (n = 13) and JIA (n = 5) patients and non-inflammatory controls (n = 13). TLR protein expression was determined by FITC-labelled TLR-specific antibody detection via flow cytometry (Fig. 1B). It was found that TLR3 protein expression was significantly increased in JSLE compared with non-inflammatory (P = 0.009) and JIA (P = 0.016) controls, as was TLR8 (control P = 0.009; JIA P = 0.009) and TLR7 (P = 0.009).

**Fig. 1** Increased TLR expression in JSLE patients compared with controls. PBMCs were isolated from JSLE (n = 19) and JIA (n = 7) patients and non-inflammatory controls (n = 17). TLR3, 8 and 9 mRNA expression was significantly higher in JSLE compared with controls (A; P < 0.05). TLR7 mRNA expression was higher in JSLE than controls, but was not statistically significant. TLR3, 8 and 9 protein expression was significantly increased in JSLE compared with controls (B; P < 0.05). B cells were also isolated from JSLE (n = 7) and JIA (n = 7) and non-inflammatory controls (n = 6). TLR7 and 9 mRNA expression was significantly higher in JSLE compared with controls (C; P < 0.05). TLR3 and 8 mRNA expression was higher in JSLE compared with controls, but this difference was not statistically significant (P = 0.097 and P = 0.073, respectively).
TLR9 (control \( P = 0.009 \); JIA \( P = 0.009 \)) protein expression. Of note, TLR3 (\( P = 0.009 \)) and TLR8 (\( P = 0.028 \)) protein expression was significantly increased in JIA as compared with non-inflammatory controls. A significant positive correlation was observed between TLR9 mRNA and protein expression and global BILAG DAS (\( r^2 = 0.92, \ P < 0.001 \) and \( r^2 = 0.71, \ P < 0.001 \), respectively). There was no other significant correlation between the expression of TLRs and other biomarkers of disease activity.

**TLR mRNA expression in B cells**

B cells were isolated from JSLE (\( n = 7 \)) and JIA (\( n = 7 \)) and non-inflammatory controls (\( n = 6 \)). TLR mRNA expression was measured by qPCR and normalized to 18s mRNA expression (Fig. 1C). TLR7 (\( P = 0.038 \)) and TLR9 (\( P = 0.030 \)) mRNA expression was significantly higher in JSLE compared with non-inflammatory controls. TLR3 (\( P = 0.097 \)) and TLR8 (\( P = 0.073 \)) mRNA expression was higher in JSE compared with non-inflammatory controls, but this difference was not statistically significant. TLR7 (\( P = 0.078 \)) and TLR9 (\( P = 0.038 \)) mRNA expression was also higher in JSLE compared with JIA controls.

**TLR ligand-induced IFN-\( \gamma \) mRNA and protein expression**

Control PBMCs were stimulated with specific TLR3, 7, 8 and 9 ligands. After 6 h, the cell supernatant was collected and RNA extracted from the cell pellet. TLR mRNA expression was measured and normalized to 18s. TLR3 (\( \times 1.4 \)-fold), TLR8 (\( \times 2.4 \)) and TLR9 (\( \times 3.78 \)) mRNA expression was increased compared with unstimulated cells. IFN-\( \gamma \) protein expression was measured by ELISA. IFN-\( \gamma \) protein expression under TLR3 (\( \times 1.2 \)-fold), TLR8 (\( \times 1.2 \)) and TLR9 (\( \times 1.9 \)) stimulation was increased compared with unstimulated cells.

**Neutrophils that undergo apoptosis in JSLE serum induce a greater IFN-\( \gamma \) response from PBMCs than neutrophils incubated in control serum**

PBMCs and neutrophils were isolated from non-inflammatory controls (\( n = 5 \)). Neutrophils were incubated with either control or JSLE serum. Incubation with control serum induced 5% and 20% apoptosis, respectively. After 2 h, neutrophils incubated with either control or JSLE serum were incubated with PBMCs. After 6 h, PBMCs were harvested and IFN-\( \gamma \) mRNA expression was measured, normalized to 18s. Neutrophils that had undergone apoptosis in JSLE serum induced a greater IFN-\( \gamma \) mRNA expression in PBMCs (\( \times 4.8 \)-fold) than those incubated in control serum (\( P < 0.05 \); Fig. 2A).

**Dose-response relationship**

PBMCs and neutrophils were isolated from non-inflammatory controls (\( n = 3 \)). PBMCs were incubated with varying concentrations of neutrophils that had undergone apoptosis in JSLE serum. PBMCs and neutrophils were incubated at a concentration of \( 1 \times 10^5 \) PBMCs : \( 5 \times 10^4 \) neutrophils (2 : 1), \( 1 \times 10^5 \) PBMCs : \( 1 \times 10^5 \) neutrophils (1 : 1) and \( 1 \times 10^5 \) PBMCs : \( 2 \times 10^5 \) neutrophils (1 : 2). The \( 2 \times 10^5 \) of unstimulated PBMCs acted as the experimental control. After 6 h of incubation, RNA was extracted.

![Increased IFN-\( \gamma \) expression from PBMCs following incubation with JSLE serum-induced apoptotic neutrophils. PBMCs and neutrophils were isolated from non-inflammatory controls (\( n = 5 \)). Neutrophils were incubated with JSLE serum were incubated with PBMCs. After 6 h, PBMCs were harvested and IFN-\( \gamma \) mRNA expression was measured (A). JSLE serum-induced apoptotic neutrophils induced a greater IFN-\( \gamma \) mRNA expression in PBMCs (\( \times 4.8 \)-fold) than those incubated in control serum (A; \( P < 0.05 \)). Control PBMCs (\( n = 3 \)) were incubated with varying concentrations of neutrophils that had undergone apoptosis in JSLE serum (B; PBMC ratio to neutrophils 2 : 1, 1 : 1 and 1 : 2). After 6 h, PBMCs were isolated and IFN-\( \gamma \) mRNA expression was measured by qPCR. PBMC IFN-\( \gamma \) mRNA expression occurred in a dose-response manner to apoptotic neutrophil concentration (B).](https://www.rheumatology.oxfordjournals.org/issue/828)
from the cell pellet. IFN-α mRNA expression was measured by qPCR. All groups expressed a significantly higher IFN-α mRNA expression as compared with unstimulated PBMCs (2:1, P = 0.02; 1:1, P = 0.02; 1:2, P = 0.02). IFN-α mRNA expression occurred in a dose-response manner to the concentration of apoptotic neutrophils. IFN-α mRNA expression was significantly higher in the 1:2 group as compared with the 2:1 group (P = 0.035). Neutrophils incubated in control or JSLE serum alone expressed very little IFN-α mRNA (Fig. 2B).

Apoptosis correlates with TLR mRNA expression

PBMCs and neutrophils were isolated from non-inflammatory controls (n = 4). Neutrophils were incubated with 10% JSLE serum for 2 h and the percentage of apoptosis was measured by flow cytometry. PBMCs were incubated with neutrophils at different percentages of apoptosis. After 6 h, cell RNA was extracted and TLR3, 7, 8 and 9 mRNA expression was measured by qPCR. An increasing percentage of apoptosis positively correlated with TLR3 (r² = 0.591; P = 0.004), TLR7 (r² = 0.551; P = 0.006), TLR8 (r² = 0.470; P = 0.14) and TLR9 (r² = 0.608; P = 0.003) expression.

Inhibition of TLR signalling decreases TLR-induced IFN-α production in PBMCs

PBMCs and neutrophils were isolated from non-inflammatory controls (n = 5). TRIF inhibitory peptide (Invivogen) or MyD88 inhibitory peptide was used to inhibit TLR3 or TLR7-9 signalling, respectively. Apoptotic neutrophils, TLR3 or TLR9 ligand and apoptotic neutrophil as compared with those not incubated with MyD88 or TRIF inhibitor (P < 0.05).

**Discussion**

This study aimed to determine the expression of TLRs capable of recognizing nuclear autoantigens associated with SLE and to investigate whether apoptotic neutrophils in JSLE are a source of nuclear autoantigen(s) detected through the innate immune system by the TLR pathway leading to the induction of an inflammatory response. First, it demonstrated significantly increased TLR3, 8 and 9 expression (mRNA and protein) in JSLE PBMCs compared with both autoimmune inflammatory (JIA) and non-inflammatory controls. It found that JSLE B cells also displayed significantly increased mRNA expression of TLR7 and 9 compared with controls. These data are in
agreement with previous work demonstrating significantly increased expression of these same TLRs in adult-onset SLE [9, 20].

TLR activation was determined by measuring downstream IFN-α expression from PBMCS following exposure to TLR ligands or co-culture with apoptotic neutrophils (induced by co-culture with JSLE serum). Both resulted in increased IFN-α production in a dose–response manner, with IFN-α production increasing as the relative concentration of apoptotic neutrophils increased. These data support the hypothesis that apoptotic neutrophils are triggering TLR activation through their presentation of autoantigens. Signalling through TLRs can be broadly categorized into two pathways, namely, the MyD88-dependent and the TRIF-dependent pathways. All TLRs, except TLR3, activate the MyD88 pathway, whereas TLR3 and 4 activate the TRIF pathway [21]. The specific role of TLR activation by apoptotic neutrophils in this study was thus demonstrated by a significant decrease in IFN-α production after inhibition of TLR signalling through the blocking of MyD88 and TRIF.

Marked synergy occurs in the production of pro-inflammatory mediators following stimulation of multiple TLRs together. Simultaneous activation of different TLR pathways may therefore create an environment in which the breakthrough of tolerance is likely to occur [5]. Decreased IFN-α expression in this study following inhibition of both MyD88 and TRIF signalling indicates that apoptotic neutrophils stimulate more than one TLR at a time, and therefore induce an increased pro-inflammatory environment, as described in RA [5].

The hypothesis that TLR activation leads to downstream effects important in the generation of the disease phenotype of lupus is supported by a case report describing the remission of long-standing SLE after development of an acquired deficiency of the TLR7 and 9 signalling pathway in peripheral B cells [22]. The same acquired immunodeficiency has been documented in 18 SLE patients, with clinical improvement occurring in 12 of these [23]. In our study, a significant positive correlation was observed between TLR9 expression and DAS (measured by BILAG), suggesting that TLR expression may be an important factor influencing disease severity in JSLE. Previously, studies have shown increased TLR expression in adult-onset SLE to correlate with parameters of disease activity, including anti-dsDNA titres, with stimulation of TLR9 being shown to directly lead to anti-dsDNA production [3, 9]. Methylation of human DNA is one of the mechanisms TLRs use to discriminate between self and non-self, and hypomethylated DNA, as seen in SLE [24], may bind to and stimulate TLR9, leading to autoantibody production.

Many investigators point to apoptosis as a mechanism for autoantigen exposure in SLE. However, the direct evidence behind this in terms of mechanistic pathway is scarce. TLR activation leads to the production of IFN-α, a key player in the pathogenesis of SLE. Increased serum levels of IFN-α and the IFN-α gene signature exhibited by JSLE patients are thought to significantly contribute to the development and maintenance of autoimmunity in SLE through the chronic activation of autoreactive T and B cells [25]. Data demonstrating the direct link between apoptosis, TLR activation and IFN-α have been few until now. Immune complexes of SLE IgG and nucleic acids derived from necrotic and late apoptotic cells have been shown to induce IFN-α production through the TLR pathway [26]. Apoptosis induced through UV light exposure demonstrated the ability of apoptotic material to stimulate an autoimmune reaction. Here, we demonstrate for the first time apoptotic neutrophils as a potential source of nuclear autoantigens are detected by TLRs, leading to activation and a downstream increase in IFN-α expression in JSLE. Neutropenia is a frequent occurrence in SLE, and increased rates of apoptosis are thought to contribute to this [27]. Neutrophils undergo rapid constitutive apoptosis and, because of their vast number, represent an enormous potential apoptotic cell burden. Studies have shown increased levels of circulating apoptotic neutrophils in adult-onset SLE and JSLE, which positively correlate with markers of disease activity [14, 15, 28].

IFN-α production in response to apoptotic neutrophils through TLR up-regulation and stimulation described in this study was significantly associated with, and therefore a likely contributor to, this inflammatory response, even if not solely responsible in vivo. As the experiment was carried out under controlled conditions, the only influential variable on TLR expression/activation was the presence of apoptotic neutrophils.

A dual inhibitor of both TLR7 and 9 has recently been developed, which potently inhibits IFN-α produced by plasmacytoid dendritic cells (pDCs) in response to DNA and RNA containing pathogens and immune complexes proven to promote serum IFN-α levels in SLE [26]. Chronic stimulation of pDCs through TLR7 and 9 by self-nucleic acid-containing autoantibodies resulted in induction of IFN-α and reduced therapeutic activity of glucocorticoids. The investigators concluded that the use of inhibitors of TLR signalling could be effective CS-sparing drugs. This is particularly relevant to JSLE patients who suffer more severe illness than adult-onset SLE [29] and therefore require proportionally higher doses of steroids over a longer disease duration, consequently suffering the more severe treatment-related morbidity. This study provides further evidence that TLR inhibition does have an anti-inflammatory effect in JSLE, highlighting a practical application for this therapy in JSLE.

Conclusion

This study has shown for the first time that apoptotic neutrophils activate TLR signalling pathways resulting in increased IFN-α production. All of these factors have been shown to be positively correlated with disease activity in SLE [2, 9, 15]. In a time with exciting new prospects for potential new therapeutics in lupus, this study provides further evidence supporting the role of TLRs and the benefit of using TLR inhibition therapy in JSLE.
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**Disclosure statement:** The authors have declared no conflicts of interest.

## References

Clinical vignette

Unilateral leg swelling in an adolescent girl

A 9-year-old girl presented with a 10-day history of swelling of the left leg that occurred spontaneously and gradually increased in size by 33%. She reported discomfort and pain in the calf and upper thigh. The skin of the left lower leg was pink and hot, and pitting oedema was noted. The rest of the history and examination were unremarkable. MRI (Fig. 1) was suggestive of a lymphatic abnormality and a diagnosis of lymphoedema praecox was confirmed with lymphoscintigraphy.

Lymphoedema praecox (also known as Meige’s disease) is the most common form of primary lymphoedema. It results from a congenital abnormality in the lymphatic vessels. The delay between birth and clinical manifestation may be due to an exacerbation of the underlying lymphatic defect by an event such as trauma or infection [1]. Lymphoedema praecox affects approximately 1 per 100 000 females and 1 per 400 000 males [2], with the peak incidence occurring during puberty [2]. Management is conservative and is focused on avoiding tight clothing or trauma to the area. Lymphoedema praecox is an important differential diagnosis in teenagers, particularly females, presenting with acute swelling of the leg.

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References


Fig. 1 Magnetic resonance scan suggestive of lymphoedema praecox.