Concise report

Up-regulation of the endoplasmic reticulum transmembrane protein UNC93B in the B cells of patients with active systemic lupus erythematosus

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Abstract

Objectives. The transmembrane endoplasmic reticulum (ER) protein UNC93B plays an essential role in the normal response to signalling through intracellular Toll-like receptor (TLR)3, TLR7, TLR8 and TLR9. In the current study, we examined the level of UNC93B expression on peripheral B cells from patients with active SLE, and investigated any correlation with SLE pathogenesis.

Methods. Peripheral blood mononuclear cells (PBMCs) and B cells from 43 active SLE patients were analysed by quantitative RT–PCR to determine the precise levels of UNC93B mRNA. We also analysed UNC93B protein expression on B cells from SLE patients using immunoblotting.

Results. The expression of UNC93B mRNA on PBMCs from active SLE patients was significantly higher than that of controls (P < 0.05). The intracellular expression level of UNC93B protein on CD20⁺ B cells from active SLE patients was also higher than in the controls. Moreover, the expression of UNC93B on B cells from lupus patients correlated significantly with high titres of anti-dsDNA antibody (P < 0.05).

Conclusions. Up-regulation of the ER membrane protein UNC93B on human lupus B cells suggests that TLR9 and UNC93B play a partial role in the pathogenesis of SLE by inducing defective peripheral B-cell tolerance.

Key words: Systemic lupus erythematosus, Innate immunity, Toll-like receptor, Toll-like receptor 7, Toll-like receptor 9, B cell, UNC93B1, Anti-dsDNA antibody, Autoimmune disease, Myeloid differentiation factor 88.

Introduction

SLE is a systemic autoimmune disease characterized by the generation of autoantibodies directed against nuclear DNA and nuclear proteins [1, 2]. Although it is generally considered that autoimmunity is related to adaptive immunity, a recent study has demonstrated that abnormalities of the innate immune system may also be related to the pathogenesis of autoimmune disease [3]. Toll-like receptor (TLR) activation initiates the innate immune response by inducing the expression of antimicrobial genes and inflammatory cytokines. Activation of TLR also enhances adaptive immunity via the activation of dendritic cells (DCs). Several mechanisms have been proposed to explain the production of autoantibodies in diseased B cells, including impaired survival or apoptosis signalling that may prevent negative selection, dysfunctional complement or inhibitory Fc receptors, and the activation of TLR in response to the accumulation of apoptotic bodies.

We recently reported that higher expression levels of TLR9 on peripheral blood B cells in active SLE patients correlated with CH50 and SLEDAI, and induced the production of anti-dsDNA antibody and IL-10 synthesis via TLR9–CpG ligation [4]. The response following TLR9 triggering was also found to be dependent on intracellular trafficking of the receptors themselves between the transmembrane endoplasmic reticulum (ER) protein and endosomes [5, 6]. Tabeta et al. [7] identified triple D (3d)
mice that showed defects in TLR3, TLR7 and TLR9 signalling, as well as Classes I and II MHC-restricted antigen presentation. The mutation was subsequently identified as a single histidine-to-arginine substitution (H412R) in the polytopic transmembrane protein Unc93b.

Unc93b is known to be essential for TLR3, TLR7 and TLR9 signalling in both humans and mice, and physically directs interactions with these TLRs to reach endosomes, where they bind ligands and initiate signalling [6–9]. The Unc93b gene is expressed in human DCs and B cells, but not T cells [10], and in the absence of Unc93b cells are unable to respond to TLR stimulation [9]. Moreover, with the exception of TLR3, triggering of all TLRs induces their recruitment to the intracytoplasmic domain of complexes formed with the adaptor protein myeloid differentiation factor 88 (MyD88) and IL-1 receptor-associated kinase-4 (IRAk-4), which mediate signalling of these receptors [10]. Interestingly, patients deficient in IRAk-4, MyD88 and Unc93b demonstrate an accumulation of autoreactive B cells expressing ANA, but do not display autoreactive antibodies in their serum or develop autoimmune disease [10].

In the current study, we examined the levels of IRAk-4, MyD88 and Unc93b expression on peripheral blood B cells isolated from patients with active SLE, and investigated any correlations with clinical parameters.

Patients and methods

Patients and clinical data assessments

We obtained peripheral blood from 43 active SLE patients, 20 inactive SLE patients (Table 1) and 40 healthy controls [age: 27.8 (3.5) mean (s.d.); male/female: 18/22]. All SLE patients fulfilled the 1997 revised criteria of the ACR [11]. We assessed clinical data including SLEDAI, serum levels of complement C3, C4, CH50 and anti-dsDNA antibody production using a RIA for each SLE patient. This study was approved by the ethics committee of Juntendo University and was undertaken in accordance with the principles outlined in the Declaration of Helsinki. Signed informed consent was obtained from all patients prior to the onset of the experiment.

Cell preparation and stimulation

Peripheral blood mononuclear cells (PBMCs) were isolated using Lymphoprep (Nycomed Pharma AS, Oslo, Norway) and collected at a concentration of 1 x 10^6 cells/ml. For B-cell isolation, B cells were labelled with anti-human CD20 antibody coupled with colloidal paramagnetic microbeads (Miltenyi Biotech, Bergisch Gladbach, Germany) and isolated using AutoMACS (Miltenyi Biotech). Flow cytometric analysis revealed that B cells were isolated at a purity of >93%.

Isolated SLE B cells differentiated for 3 days were re-plated in 96-well round bottom plates at 1 x 10^5 cells/well. Cells were then stimulated with medium, oligodeoxynucleotides (ODNs) 2006 (5'–TGTCGTTTTTGCTTTTTGCT GTT-3'), ODN2216 (5'–GGGGACGATGTCGGGG-3') and ODN-TTAGGG (5'–TTTAGGTTAGGTTAGGTTA GGG-3') (Invivogen, San Diego, CA, USA), as described previously [4].

Unc93b mRNA analysis

Total RNA was isolated from 1 x 10^6 PBMCs, B cells and 1 x 10^5 stimulated B cells using the RNeasy Mini kit (Qiagen, Valencia, CA, USA). RT reactions were undertaken using the High Capacity cDNA Reverse Transcription Kit (Applied BioSystems, Foster City, CA, USA) according to the manufacturer's protocol. Quantitative RT-PCR (Q-PCR) was performed in a 25-μl reaction volume containing 12.5 μl of Power SYBR Green Master Mix (Applied BioSystems), 10 nM forward and reverse primers for Unc93b (5'–TGATCCTGCACATCGACGAG and 3'–GGAG GGAACATCATCCACTT), IRAK-4 (5'–GCAGGAATAGAAG and 3'–GCTTCAAATCTCCTTATGTGAAACT) and ODN-TTAGGG (5'–TTTAGGTTAGGTTAGGTTA GGG-3') (Invivogen, San Diego, CA, USA), as described previously [4].

Table 1 Characteristics of patients

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Active SLE patients (n = 43)</th>
<th>Inactive SLE patients (n = 20)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, mean (s.d.), years</td>
<td>34.7 (9.5)</td>
<td>34.9 (6.6)</td>
</tr>
<tr>
<td>Sex, males/females, n</td>
<td>19/24</td>
<td>9/11</td>
</tr>
<tr>
<td>Duration of SLE, mean (s.d.) years</td>
<td>5.15 (4.84)</td>
<td>5.14 (4.35)</td>
</tr>
<tr>
<td>SLEDAI, mean (s.d.) (range)</td>
<td>8.33 (4.75) (4–22)</td>
<td>2.85 (1.93) (0–3)</td>
</tr>
<tr>
<td>C3, mean (s.d.) (range) mg/dl</td>
<td>59.1 (26.4) (12–103)</td>
<td>87.8 (22.8) (53–144)</td>
</tr>
<tr>
<td>C4, mean (s.d.) (range) mg/dl</td>
<td>11.9 (8.86) (0–32)</td>
<td>16.9 (7.11) (4–30)</td>
</tr>
<tr>
<td>CH50, mean (s.d.) (range) U/ml</td>
<td>24.1 (14.1) (7–46)</td>
<td>35.2 (10.1) (14–48)</td>
</tr>
<tr>
<td>Anti-DNA antibody, mean (s.d.) (range) IU/ml</td>
<td>72.4 (75.2) (2.7–300)</td>
<td>7.93 (7.21) (0–30)</td>
</tr>
<tr>
<td>Prednisolone, n (%), mean dosage (mg/day)</td>
<td>25 (62.5), 11.47</td>
<td>13 (65) 7.92</td>
</tr>
<tr>
<td>Immunosuppressive therapy</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>Renal manifestation, n (%)</td>
<td>14 (35)</td>
<td>3 (15)</td>
</tr>
<tr>
<td>Arthritis, n (%)</td>
<td>17 (42.5)</td>
<td>4 (20)</td>
</tr>
<tr>
<td>Cytopenia, n (%)</td>
<td>10 (25)</td>
<td>1 (0.5)</td>
</tr>
<tr>
<td>CNS, n (%)</td>
<td>4 (10)</td>
<td>1 (0.5)</td>
</tr>
<tr>
<td>Cutaneous manifestation, n (%)</td>
<td>18 (45)</td>
<td>9 (45)</td>
</tr>
<tr>
<td>Serositis, n (%)</td>
<td>2 (5)</td>
<td>2 (1)</td>
</tr>
</tbody>
</table>
CCCTCGGATTGGTACAG) or β-actin (5'-GGACTTGCAGAAGAGATG and 3'-AGCAGTTGGCGTACA) and 1 μg of cDNA. The cycling conditions were 95°C for 10 min, followed by 40 cycles at 95°C for 15 s and 60°C for 1 min for denaturing and annealing-extension, respectively. UNC93B levels were measured on an ABI PRISM 7500 Sequence Detection System (Applied Biosystems). PCR products were separated on a 2% agarose gel and stained with ethidium bromide. UNC93B mRNA levels were normalized to β-actin for each sample.

Western blot analysis

B-cell whole-cell lysate (10 μl) was separated on 10% SDS–polyacrylamide gels and transferred to polyvinylidene difluoride membranes (Millipore, Billerica, MA, USA). Membranes were then blocked with blocking buffer containing phosphate-buffered saline-0.5% Tween-20 (PBS-T) and 3% skim milk for 90 min, and washed three times in 0.5% PBS-T. UNC93B protein was detected using rabbit anti-human UNC93B antibody (Imgenex, San Diego, CA, USA) diluted in blocking buffer (final concentration was 0.25 μg/ml) and incubated for 2 h at room temperature. The membrane was then incubated with a 1:5000 dilution of rabbit secondary alkaline phosphatase antibody (Santa Cruz Biotechnology, San Diego, CA, USA) for 90 min. After three washes in 0.5% PBS-T, the membranes were stained with 5-Bromo-4-Chloro-3'-Indolylphosphatase p-Toluidine salt Table; Inactive SLE patients-Prednisolone/nitro-blue tetrazolium chloride (BCIP/NBT)/NBT solution (KPL, Gaithersburg, MD, USA).

Statistical analysis

Statistical analysis was performed using the Mann–Whitney U-test and Spearman’s correlation coefficient. The results of the ODN-stimulated assay were analysed using the Kruskal–Wallis test and Sheffe’s method. Statistical significance was defined as P < 0.05.

Results

Expression of UNC93B on SLE B cells

We initially examined the expression of UNC93B mRNA on PBMCs from SLE patients and healthy controls.

**Fig. 1** Expression of UNC93B on B cells isolated from active SLE patients and healthy controls.
As shown in Fig. 1A, UNC93B mRNA on PBMCs from SLE patients was expressed at significantly higher levels than in the healthy controls ($P = 0.0021$).

We previously reported that high expression levels of TLR9 were present on the peripheral blood B cells derived from active SLE patients. Therefore, to assess whether the mechanisms underlying the production of autoreactive B cells involves TLR9, we next examined UNC93B mRNA and protein expression on B cells from lupus patients and healthy controls. The expression of UNC93B mRNA on CD20+ B cells from active SLE patients was significantly higher than the healthy controls ($P = 0.0042$) and inactive patients ($P = 0.045$) (Fig. 1B and C). We also found that the intracellular expression of UNC93B protein in CD20+ B cells from active SLE patients was higher than that in the healthy controls and inactive SLE (Fig. 1D). In contrast, the expression levels of IRAK-4 and MyD88 mRNA on B cells did not significantly differ between active SLE patients and healthy controls (supplementary figure 1, available as supplementary data at Rheumatology Online).

We next investigated whether TLR9–CpG interaction induced activation of UNC93B. We have previously shown that B cells isolated from active SLE patients express high levels of TLR9. Given these results, we stimulated active B cells from SLE patients with CpG ODN. As shown in supplementary figure 2 (available as supplementary data at Rheumatology Online), B cells stimulated with ODN2006 ($P = 0.0049$) and ODN2216 ($P = 0.045$) demonstrated enhanced expression of UNC93B mRNA.

**Correlation with clinical data**

We next investigated the relationship between UNC93B mRNA expression on B cells isolated from active SLE
patients and laboratory data (CH50, C3, C4 and titre of anti-dsDNA antibody). We found a significant correlation between UNC93B mRNA levels on B cells and anti-dsDNA antibody production (Fig. 1E, \( P = 0.00002, R = 0.688 \)) or C3 (Fig. 1F, \( P = 0.0002, R = -0.558 \)) or SLEDAI (Fig. 1G, \( P = 0.00003, R = 0.651 \)) in active lupus patients.

**Discussion**

In the current study, we demonstrate that UNC93B mRNA expression levels in PBMCs and B cells isolated from active SLE patients were significantly higher than those in healthy controls and inactive SLE patients when assessed using Q-PCR and western blotting. We also show that the expression levels of IRAK-4 and MyD88 mRNA on B cells from SLE patients was not significantly higher than those in the healthy controls. Furthermore, the expression levels of UNC93B mRNA correlated significantly with the production of anti-dsDNA antibody or the amount of C3, or the point of SLEDAI in active SLE patients.

It is well established that the levels of intracellular TLRs including TLR7, TLR8 and TLR9 correlate with the pathogenesis of murine SLE, and that TLR9 in particular plays an essential role in the pathogenesis of murine lupus models [12]. In SLE patients, we have recently demonstrated that TLR9 present on the peripheral blood B cells is also associated with the pathogenesis of SLE [4]. More recently, some reports have suggested that TLR7 and TLR9 expression levels, and TLR9 levels on CD19+ B cells from SLE patients, are significantly higher than those in healthy controls [13, 15]. In contrast, TLR3 expression does not appear to be up-regulated in human lupus [14, 15]. Thus, it is suggested that TLR7 and TLR9 may play a specific role in the pathogenesis of SLE.

Previous reports have demonstrated that UNC93B specifically controls trafficking of nucleotide-sensing TLRs, such as TLR7 and TLR9 [6]. Isnardi et al. [10] reported that signalling through IRAK-4 and MyD88 complexes plays a major role in the establishment of central B-cell tolerance in the bone marrow by counter-selecting developing autoreactive B cells including ANA-expressing cells. Moreover, they were able to show that UNC93B-deficient patients displayed an increased frequency of polyreactive new emigrant B cells [10]. As TLR3, TLR7, TLR8 and TLR9 are the only receptors that require functional UNC93B, and given that TLR3 signalling does not require IRAK-4 and MyD88 formation, it appears likely that TLR7, TLR8 and TLR9 are responsible for the removal of autoreactive B cells in the periphery [10]. Little is known about the cells and molecules that regulate human peripheral tolerance, and about how TLR7, TLR8 and TLR9 may be involved in these regulatory processes. Our recent data revealed that up-regulation of B cell activating factor of the TNF family (BAFF), which is known to be a B-cell survival factor present on SLE B cells, may prevent the removal of autoreactive B cells by increasing their survival in the periphery of SLE patients [16]. In addition, our current data showed that the expression levels of IRAK-4 and MyD88 mRNA on B cells from active SLE patients were not significantly greater than those in the healthy controls. We considered the possibilities that the UNC93B interacts directly with TLR9 and MyD88/IRAK4 signalling pathway triggered by not only TLR7, TLR8, TLR9 but also via TIR domain-containing adaptor protein (TIRAP) from TLR4, and another one is that it is more powerful with LPS-TLR4 signalling than with CpGdDNA-TLR9 signalling.

In conclusion, we report up-regulation of the ER membrane protein UNC93B on B cells in patients with active SLE and a significant correlation with the clinical data and disease activity. These data suggest that TLR9 signalling through UNC93B plays a partial role in the pathogenesis of SLE by preventing peripheral B-cell tolerance.

**Rheumatology key messages**

- UNC93B is present in intracellular B cells in active SLE patients.
- UNC93B mRNA correlated significantly with the levels of anti-dsDNA antibody present in SLE patients.
- Intracellular TLR and UNC93B play an essential role in SLE pathogenesis.

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