Pharmacological inhibition of TLR9 activation blocks autoantibody production in human B cells from SLE patients

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Abstract

Objectives. Toll-like receptor 9 (TLR9), which recognizes hypomethylated DNA [cytosine-phosphate-guanine (CpG)], plays a role in the maintenance of serological memory and has been recently implicated in the pathogenesis of SLE. We previously reported that in vitro TLR9 triggers memory B-cell differentiation into antibody-producing cells, and that the MyD88-inhibitor ST2825 blocks TLR9-induced plasma cell (PC) generation. Here, we investigated whether memory B cells produce autoantibodies in SLE patients with active disease or in clinical remission, and whether ST2825 could inhibit PC generation in SLE patients.

Methods. Peripheral blood mononuclear cells from 10 SLE patients in clinical remission and 2 with active SLE were cultured in the presence of CpG with or without ST2825. Phenotypical analysis of CpG-stimulated cells was performed by flow cytometry. Supernatants were collected to measure antibody production by ELISA and to detect autoantibodies by IF.

Results. CpG-induced TLR9 stimulation caused autoantibody secretion in patients with active disease and in the majority of patients in clinical remission. Inhibition of MyD88 completely blocked the de novo generation of PCs and the secretion of autoantibodies.

Conclusions. Autoreactive B cells persist in SLE patients during disease remission in the circulating B-cell memory pool. TLR9-dependent activation of memory B cells by pathogens could be one of the mechanisms triggering relapses in SLE. Compounds targeting the TLR/MyD88 pathway may be used as novel therapeutic tools to treat acute disease and to prevent relapses in SLE patients.

Key words: SLE, Memory B cells, Toll-like receptor 9, ST2825, MyD88, Autoantibodies, Cell signalling, Cytosine-phosphate-guanine.

Introduction

The production of autoantibodies is a hallmark of disease and plays a pathogenic role in SLE, causing tissue and organ damage [1, 2]. Autoantibodies may be detectable in the serum several years before the onset of clinical symptoms, and their concentration correlates with the development of clinical disease [3].

To date, very little is known about the role of each B-cell subset in the onset and progression of SLE, and on the molecular mechanisms that lead to generation and activation of autoreactive B cells in humans. Owing to a defect in early self-tolerance checkpoints, SLE patients...
show high frequency of circulating self-reactive and polyreactive mature-naive B cells [4, 5]. In contrast, comparable frequencies of self and polyreactive immunoglobulin (IgG) memory B cells were observed in SLE patients and healthy donors [6, 7]. Therefore, in SLE patients autoreactive IgG antibodies may arise from either polyreactive or non-reactive precursors by somatic hypermutation, just as they occur in healthy individuals [7]. Surprisingly, self-reactive antibodies have never been found in IgM memory of healthy donors [8].

In recent years, accumulating evidence suggests a role for Toll-like receptors (TLRs) in autoimmunity [9]. Results obtained in animal models of SLE suggest that TLR7 (a receptor for single-stranded RNA) and TLR9 (a receptor for hypomethylated DNA) play a central role in the pathogenesis of autoimmunity in mice [10]. These receptors exclusively rely on MyD88 adaptor protein to transduce their signals and to elicit biological responses. It has been recently shown that severe kidney disease of aged MRL/lpr mice is attenuated in the absence of both TLR7 and TLR9 or MyD88 [11]. TLR9 and MyD88 are also required to generate class-switched anti-DNA antibodies in autoimmune mice [12]. Despite a clear involvement of TLRs in the generation of autoantibodies in mice, there is still paucity of information regarding their pathogenic role in humans.

MyD88-deficient patients do not display autoreactive antibodies or develop autoimmune disease [13] and patients with acute SLE secrete anti-dsDNA antibodies upon TLR9 activation seems to be a mechanism that thwarts autoimmunity in humans, with the TLR/MyD88 pathway representing a potential molecular target for novel drugs.

We recently demonstrated that memory B cells isolated from the peripheral blood of healthy donors express high levels of TLR9 compared with mature B cells. Cytosine-phosphate-guanine (CpG) acted as a polyclonal activator by promoting memory B-cell proliferation and differentiation into CD27*CD38* plasma cells (PCs), secreting IgM, IgA and IgG antibodies [15]. Transitional B cells, a low-frequency circulating population in the adult [16], also responded to CpG by differentiating into IgM memory-like B cells that mostly produced IgM antibodies. Mature B cells exposed to CpG survived better but did not divide nor produced antibodies. Therefore, IgG antibodies produced by cultured peripheral blood mononuclear cells (PBMCs) upon TLR9 engagement mostly derive from the memory B-cell compartment in the adult [15].

We have previously reported that specific MyD88 inhibitors blocked signalling downstream of TLRs by interfering with MyD88 self-association, hence preventing the recruitment of the IRAK1/IRAK4 complex [17]. One of such MyD88 inhibitors, namely, ST2825, was able to block the TLR9-induced activation of memory B cells and impaired their differentiation into PCs, thereby preventing antibody secretion [18]. In this study, we used TLR9 stimulation as a tool to detect the presence of autoreactive memory B cells in the peripheral blood of SLE patients in clinical remission and in acute phase. Our results indicate that ST2825 completely abolished production of autoantibodies in CpG-stimulated PBMCs from SLE patients by blocking PC differentiation. The inhibition of B-cell activation and autoantibody production through ST2825 indicates that targeting the TLR/MyD88 pathway may represent a good strategy to develop drugs interfering with the molecular mechanisms of disease pathogenesis in SLE.

Materials and methods

Synthesis of ST2825

The peptidomimetic compound ST2825 was synthesized in the chemistry laboratory at Sigma-tau Industrie Farmaceutiche Riunite S.p.A, as previously described [18].

Subjects

Samples of peripheral blood were obtained from 12 SLE patients, 2 of whom had active disease and 10 were in remission. All patients were treated at the Ospedale Pediatrico Bambino Gesù (OPBG), Unit of Rheumatology (Rome, Italy) and their recruitment to this study conformed to the ethical guidelines of the 1975 Declaration of Helsinki. The study was approved by the OPBG ethical committee. Eight age-matched healthy blood donors were used as controls. Additional demographic, clinical and serological parameters, as well as the therapeutic regimen, were performed at the time of analysis and are shown in Table 1.

Cell preparation and flow cytometric analysis

PBMCs were isolated by Ficoll-Paque Plus (Amersham Pharmacia Biotech, Little Chalfont, UK) density-gradient centrifugation. The lymphocyte-enriched cells were washed in PBS (1×), stained with the appropriate combination of FITC, phycoerythrin (PE), allophycocyanin (APC), cyochrome or biotinlabelled antibodies, followed by streptavidin-Red670, streptavidin–APC (Caltag, San Francisco, CA, USA), Monoclonal clone HIB19 (anti-CD19), clone ML5 (anti-CD2), clone M-T271 (anti-CD27) and clone HIT2 (anti-CD38) were obtained from BD Biosciences (San Diego, CA, USA), while anti-IgM Fc5 fragment specific was obtained from Jackson ImmunoResearch Laboratories (West Grove, PA). Dead cells were excluded from analysis by sideforward scatter gating. All analyses were performed on a FACSCanto (Becton and Dickinson, Sunnyvale, CA) interfaced to a PC FACSDiva software (BD Biosciences, San Diego, CA, USA). Fifty thousand gated events on living cells were analysed, whenever possible, for each sample.

Proliferation assay

Before stimulation, peripheral blood lymphocytes were labelled with 5-chloromethylfluorescein diacetate (CMFDA) at a final concentration of 0.1 μg/ml (CellTracker CMFDA; Molecular Probes, Eugene, OR) [18]. The cells were cultured at 5 × 10^5 cells per well in 96-well plates (Becton Dickinson, San Jose, CA) in complete RPMI 1640 (InvivoGen, San Diego, CA, USA), supplemented with 10% FBS (Hyclone Laboratories, Logan, UT). ST2825 was
added to the medium at 1, 3, 10, 30 or 60 μM in the presence or absence of 2.5 μg/ml CpG (ODN 2006; Hycult Biotechnology, Uden, The Netherlands). For the inhibition of antibody production induced by CpG, we used ST2825 at the optimal concentration of 10 μM. Cell proliferation was measured on Day 7 by flow cytometry.

IF analysis
The detection of ANA autoantibodies was performed using the diagnostic automation ANA/Hep2 test system, a standardized kit to measure ANA in human serum (AnaFluor Kit; Diagnostic, Stillwater, MN, USA). Screening of autoantibodies was also carried out by measuring IgG reactivity in an indirect immunofluorescent assay using rat liver/kidney/stomach tissue slides according to the manufacturer procedure (Astra Prodotti Diagnostici, Milan, Italy). Slides were analysed in a confocal microscope (Olympus FV1000; Olympus Life Science Research Europa GmbH, Munich, Germany) and all images acquired at ×20 magnification.

ELISA immunoassay
Secreted Igs were detected on Day 7 by ELISA. Briefly, 96-well plates (Corning Inc., Corning, NY) were coated overnight with purified goat anti-human IgA plus IgG, plus IgM (Jackson ImmunoResearch Laboratories). After washing with PBS containing 0.05% Tween and blocking with PBS containing 1% gelatin (1 h, room temperature), plates were incubated for 1 h at 37°C with the supernatants of the cultured cells. After washing, plates were incubated for 1 h with peroxidase-conjugated fragment goat anti-human IgA or IgG or IgM antibodies (Jackson ImmunoResearch Laboratories). The assay was developed with O-phenylenediamine tablets (Sigma-Aldrich, St. Louis, MO, USA) as a chromogenic substrate. Absorbance at 405 nm was measured, and Igs concentrations were calculated by interpolation from the standard curve.

Statistical analysis
Data were analysed using the StatView statistical MacIntosh program (StatView Software, San Diego, CA) and significance of differences between experimental variables determined with the paired Student’s t-test. A level of P < 0.05 was considered statistically significant.

Results
ST2825 blocks TLR9-induced PC differentiation in healthy donors
ST2825 was first tested on CpG-stimulated PBMCs from healthy donors at varying concentrations to ascertain toxicity. Cultured cells were first stained with the viable cell tracker CMFDA to analyse proliferation of activated cells. Next, after in vitro stimulation in the presence or absence of ST2825 for 7 days, the cells were collected, stained for CD19, CD27 and analysed by flow cytometry. The number of B cells (CD19pos) recovered from the culture conditions using tissue culture medium, 1, 3, 10 and 30 μM ST2825 was not significantly different. In contrast, the highest concentration of ST2825 (i.e. 60 μM) was rather toxic and significantly affected B-cell number (Fig. 1A). For each experimental condition depicted in Fig. 1A, we calculated the percentage of PCs (CMFDA low, CD19low and CD27bright) in the B-cell gate [15, 18]. ST2825, used at 10 μM, completely inhibited CpG-induced PC generation (Fig. 1B). Culture supernatants were then collected to perform ELISA. Concordant with the absence of PCs observed in the presence of 10 or 30 μM ST2825, we found a complete inhibition of antibody secretion for all isotypes (Fig. 1C). Based on these results, subsequent experiments were conducted using 10 μM ST2825. Interestingly, we did not observe the low percentage of spontaneous PCs generated in unstimulated cultures.

Table 1 Profile of SLE patients

<table>
<thead>
<tr>
<th>Name</th>
<th>Age, years</th>
<th>Sex</th>
<th>Disease activity</th>
<th>ANA</th>
<th>Anti-dsDNA</th>
<th>Organ involvement</th>
<th>Steroid therapy</th>
<th>Other immunosuppressive therapies</th>
<th>SLEDAI</th>
</tr>
</thead>
<tbody>
<tr>
<td>SLE1</td>
<td>19</td>
<td>F</td>
<td>CR</td>
<td>4+</td>
<td>1:640</td>
<td>Cutaneous, arthritis</td>
<td>0.36 mg/kg/e.o.d.</td>
<td>MMF, CYA</td>
<td>17</td>
</tr>
<tr>
<td>SLE2</td>
<td>20</td>
<td>F</td>
<td>Relapse</td>
<td>3+</td>
<td>1:1280</td>
<td></td>
<td>0.32 mg/kg/e.o.d.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SLE3</td>
<td>17</td>
<td>F</td>
<td>CR</td>
<td>3+</td>
<td>1:320</td>
<td></td>
<td>0.29 mg/kg/e.o.d.</td>
<td></td>
<td>2</td>
</tr>
<tr>
<td>SLE4</td>
<td>20</td>
<td>M</td>
<td>CR</td>
<td>1+/-</td>
<td>0</td>
<td></td>
<td>0.25 mg/kg/e.o.d.</td>
<td></td>
<td>0</td>
</tr>
<tr>
<td>SLE5</td>
<td>20</td>
<td>M</td>
<td>Flare</td>
<td>3+</td>
<td>0</td>
<td>Cutaneous</td>
<td>0.6 mg/kg/d</td>
<td>MMF</td>
<td>4</td>
</tr>
<tr>
<td>SLE6</td>
<td>22</td>
<td>F</td>
<td>CR</td>
<td>1+</td>
<td>0</td>
<td></td>
<td>0.42 mg/kg/e.o.d.</td>
<td></td>
<td>0</td>
</tr>
<tr>
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<td>22</td>
<td>F</td>
<td>CR</td>
<td>2+</td>
<td>0</td>
<td></td>
<td>0.35 mg/kg/e.o.d.</td>
<td></td>
<td>0</td>
</tr>
<tr>
<td>SLE8</td>
<td>20</td>
<td>F</td>
<td>CR</td>
<td>1+</td>
<td>1:160</td>
<td></td>
<td>0.34 mg/kg/e.o.d.</td>
<td></td>
<td>0</td>
</tr>
<tr>
<td>SLE9</td>
<td>27</td>
<td>F</td>
<td>CR</td>
<td>3+</td>
<td>0</td>
<td></td>
<td>0.32 mg/kg/e.o.d.</td>
<td></td>
<td>0</td>
</tr>
<tr>
<td>SLE10</td>
<td>12</td>
<td>F</td>
<td>CR</td>
<td>3+</td>
<td>1:40</td>
<td>Cutaneous</td>
<td>0.6 mg/kg/d</td>
<td>AZA</td>
<td>2</td>
</tr>
<tr>
<td>SLE11</td>
<td>28</td>
<td>F</td>
<td>CR</td>
<td>1+/-</td>
<td>0</td>
<td></td>
<td>0.19 mg/kg/e.o.d.</td>
<td></td>
<td>0</td>
</tr>
<tr>
<td>SLE12</td>
<td>17</td>
<td>F</td>
<td>CR</td>
<td>2+</td>
<td>1:320</td>
<td></td>
<td>AZA, HCQ</td>
<td></td>
<td>2</td>
</tr>
</tbody>
</table>

Patients in complete clinical remission are indicated as complete remission (CR). Patients 1–11 were receiving oral administration of prednisolone as steroid therapy. Other immunosuppressive therapy: AZA, ciclosporin (CYA), CYC, MMF, HCQ. e.o.d.: every other day; F: female; M: male.
when ST2825 was used. Since this low percentage was calculated from B cells that had proliferated (CMFDA$^{\text{low}}$), it follows that ST2825 also blocked spontaneous PCs differentiation from ex vivo-activated memory B cells.

ST2825 prevents TLR9-dependent in vitro PC generation in SLE patients

Memory B cells respond to CpG by differentiating into PCs secreting IgM, IgA or IgG [15, 19]. Transitional B cells can be activated through TLR9 and generate IgM-secreting cells [15]. Given the low frequency of transitional B cells in healthy control (HC) peripheral blood, their contribution to in vitro IgM secretion is virtually negligible. Consequently, the lack of immunoglobulins observed when ST2825 was added to CpG-stimulated cultures is likely due to inhibition of memory B-cell differentiation into antibody-secreting cells. In order to test this hypothesis, PBMCs from 8 healthy donors and 12 SLE patients were stained with CMFDA and cultured for 7 days in the presence of CpG with or without 10 μM ST2825. Concurrent phenotype analysis before cell culture was performed to identify the relative frequency of memory cells (CD19$^{\text{pos}}$CD27$^{\text{pos}}$), PCs (CD19$^{\text{low}}$CD27$^{\text{bright}}$CD38$^{\text{bright}}$) and transitional B cells (CD24$^{\text{bright}}$CD38$^{\text{bright}}$) in individual donors and patients. No significant differences were observed in the frequencies of memory B cells in HCs compared with SLE patients [mean 23.23 (12.36) vs 28.62 (17.34)% of total B cells] and in the PC subset [mean 0.30 (0.25) vs 0.6 (0.7%)]. The frequency of transitional B cells in SLE patients was slightly increased, even though the difference was not statistically significant [mean 5.01 (1.9) vs 8.87 (8.53%); P > 1]. At the end of culture, samples were stained with suitable combinations of antibodies against CD19 and CD27 (15) to identify PCs (Fig. 2A and B). Notably, no PCs were found when cells were stimulated in the presence of ST2825 (Fig. 2A). Thus, TLR9 stimulation with CpG promotes PC differentiation and antibody secretion in both HC and SLE patients and inhibition of the MyD88 pathway by ST2825 completely abrogates these mechanisms.
**Fig. 2** ST2825 inhibits TLR9-dependent PC differentiation and antibody secretion in SLE patients. (A) Phenotypic analysis of CD19\textsuperscript{pos} B cells from one representative HC and SLE patient cultured with CpG in the absence or presence of ST2825 inhibitor (10 \textmu M). Memory B cells CD27\textsuperscript{pos/bright} CMFDA\textsuperscript{pos} (A, mem) and PCs CD27\textsuperscript{bright}CMFDAlow (A, PC) are indicated in rectangular boxes with their relative percentages within the CD19\textsuperscript{pos} B-cell pool. (B) Histograms show the percentage of PCs obtained from experiments performed with 8 different HCs and 12 SLE patients. Each bar represents a single individual. (C) ELISA immunoassay was performed on culture supernatants of PBMCs from healthy controls (n = 8) and SLE patients (n = 8). Each bar shows IgM, IgA and IgG concentrations in CpG and in ST2825+CpG-stimulated cultures from single subjects. Unt: untreated.
ST2825 inhibits secretion of autoantibodies in SLE patients

As TLR9 stimulation promotes antibody secretion in SLE B cells, we next investigated whether autoantibodies were present in the supernatants of CpG-stimulated PBMCs from SLE patients in comparison with HC individuals. To this end, we incubated supernatants from CpG-stimulated HC and SLE cells with Hep-2 cells and assessed the presence of bound IgG by confocal microscopy. TLR9 stimulation induced ANA IgG secretion in SLE PBMCs (Fig. 3). No autoantibodies were detected in the supernatants from HC stimulated with CpG.

To confirm these results, supernatants from cultured cells obtained from eight HC and nine SLE patients were assayed for the presence of autoantibodies on rat tissue slides, a method usually employed for clinical diagnosis of autoimmune diseases [20, 21]. In the supernatant of CpG-stimulated PBMCs from HCs we never detected autoantibodies. In contrast, immunoglobulins reactive against rat stomach, kidney and liver were produced by stimulated B cells from SLE patients (Fig. 4 and supplementary figures 5 and 6, available as supplementary data at Rheumatology Online). ST2825 was able to block autoantibody production in CpG-stimulated cells. Table 2 summarizes the results obtained by the semi-quantitative analysis of each sample. In particular, we observed that immunoglobulins reactive against rat liver (six out of nine), kidney (six out of nine) and stomach (seven out of nine) were produced by CpG-stimulated B cells of SLE patients. The patients analysed were mostly in remission and only Patients 4 and 7 were in the active phase of disease (Table 1).

Thus, our results indicate that B cells capable of producing autoantibodies persist in the blood of SLE patients during disease remission. Autoantibodies were present in the majority of SLE patients (seven out of nine), and this production was completely abolished by the presence of ST2825 in the culture (Table 2), indicating that it occurs through an MyD88-dependent pathway.

Discussion

B cells producing autoreactive IgGs directed against intracellular antigens play a central role in the pathogenesis of SLE. High-affinity autoantibodies contribute to immunopathology by forming immune complexes that accumulate in vital organs causing inflammation and tissue damage. Blocking the generation of autoreactive PCs represents a challenging task for the development of new therapeutic drugs for SLE.

The number of B-cell-directed therapies in the pipeline for the treatment of autoimmune diseases is increasing steadily [22]. Rituximab (anti-CD20) has been proposed as a therapeutical tool in SLE, consequent to serious adverse effects of conventional immunosuppressive agents [23]. However, rituximab cannot deplete PCs, as these do not express CD20 on their surface. The effectiveness of
this drug is still under debate since the Exploratory Phase II/III SLE Evaluation of Rituximab (EXPLORER) trial with patients with active non-renal SLE produced entirely negative results and the Lupus Nephritis Assessment with Rituximab (LUNAR) trial in patients with active lupus nephritis was announced to be negative [24].

In vivo evidence from TLR7- and TLR9-deficient lupus-prone mice highlighted these receptors as potential targets for therapy. These studies revealed a clear involvement of TLR9 in the generation of anti-DNA antibodies and for TLR7 in the generation of anti-RNA antibodies [25]. Lupus-prone mice deficient for MyD88 or both TLR7 and TLR9 showed ameliorated kidney disease compared with TLR9-deficient mice [11], suggesting that pharmacological inhibition which specifically targets both TLR7 and TLR9 or the common MyD88 protein adaptor should be the ideal candidate for clinical application [26].

ST2825 was modelled on the structure of the consensus heptapeptide RDVLPGT within the BB-loop of MyD88 TIR domain [17, 18]. The inhibitory effect of ST2825 was due to specific interference with TIR/TIR homotypic interaction of its putative target MyD88 that prevents the formation of the IRAK1/IRAK4 complex [18]. A similar in vitro inhibitory activity was also demonstrated in human B cells triggered by TLR9/CpG stimulation [18]. Moreover, additional in vivo studies showed that oral administration of ST2825 in mice inhibited IL-1β-induced production of IL-6 in a dose-dependent way [18], and significantly protected against left ventricular enlargement in a permanent ligation model of acute myocardial infarction [27], highlighting

**Fig. 4** ST2825 inhibits CpG-induced secretion of autoantibodies against rat stomach. PBMCs from SLE patients and HCs were cultured as described in ‘Materials and Methods’ section. Culture supernatants from PBMCs of representative ANA+ SLE patient (A) and HC (B) analysed by IF. Culture supernatants were incubated with slides containing sections of rat stomach. Auto-antibodies were detected with anti-Igs FITC (green). Evans Blue (red). Magnification: ×20. Unt: untreated.
The absence of autoantibodies is indicated as __/C0__. The autoantibody production is expressed by an arbitrary scale with a maximum value of +++ and a minimum value of +.

Performed with PBMCs untreated (Unt) or treated with the indicated stimuli, namely, CpG or CpG+ST2825. The intensity of tissue sections. Culture supernatants from eight HCs and nine SLE (SLE 1–9 of Table 1) patients were analysed. Cultures were semi-quantitative analysis of autoantibody fluorescence intensity against rat liver, kidney, and stomach detected by IF on rat tissue sections.

Our findings indicate that ST2825 interferes with TLR9-dependent biological responses that may drive and sustain autoimmunity in humans. ST2825 inhibits PC differentiation and antibody secretion in SLE B cells from SLE patients in acute phase and clinical remission, thus representing a valuable therapeutic adjunct not only to counteract disease progression but also to prevent disease relapse.

The present results, showing that interference with the TLR9/MyD88 pathway translates in inhibitory effects on CpG-induced _in vitro_ activation and differentiation of human B cells, demonstrate that targeting this pathway is a promising approach to develop novel therapeutic tools [29, 30] and prompt further _in vivo_ investigations to assess the efficacy and safety of our experimental approach.

<table>
<thead>
<tr>
<th>No.</th>
<th>Liver</th>
<th>Kidney</th>
<th>Stomach</th>
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<tbody>
<tr>
<td></td>
<td>Unt</td>
<td>CpG</td>
<td>ST2825</td>
</tr>
<tr>
<td>HC (1–8)</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>SLE1</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>SLE2</td>
<td>–</td>
<td>+++</td>
<td>–</td>
</tr>
<tr>
<td>SLE3</td>
<td>–</td>
<td>++</td>
<td>–</td>
</tr>
<tr>
<td>SLE4</td>
<td>–</td>
<td>+++</td>
<td>–</td>
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<td>SLE5</td>
<td>–</td>
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<td>–</td>
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<td>SLE6</td>
<td>–</td>
<td>+++</td>
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<td>SLE8</td>
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<td>–</td>
<td>–</td>
</tr>
<tr>
<td>SLE9</td>
<td>–</td>
<td>++</td>
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Semi-quantitative analysis of autoantibody fluorescence intensity against rat liver, kidney, and stomach detected by IF on rat tissue sections. Culture supernatants from eight HCs and nine SLE (SLE 1–9 of Table 1) patients were analysed. Cultures were performed with PBMCs untreated (Unt) or treated with the indicated stimuli, namely, CpG or CpG+ST2825. The intensity of the autoantibody production is expressed by an arbitrary scale with a maximum value of +++ and a minimum value of +. The absence of autoantibodies is indicated as __/C0__.

We have previously shown that CpG-induced TLR9 stimulation is sufficient to promote proliferation and differentiation of memory B cells into PCs secreting antibodies of all isotypes [15]. In the present study, we demonstrate that ST2825 inhibits the secretion of autoantibodies from SLE patients by blocking the de novo TLR9-induced generation of autoreactive PCs from memory B cells. As TLR9 stimulates the memory compartment [15], antibody secretion represents the entire memory B-cell repertoire of the circulating specificity. Transitional B cells do not significantly contribute to the CpG-induced antibody production due to their low frequency. In addition, transitional B cells differentiate into IgM memory-like B cells, a subset in which autospecificities have not yet been described [8, 15].

We observed that TLR9 stimulation-induced PC differentiation and antibody production in both HC and SLE patients without significant differences in the frequency of PCs and levels of Ig concentrations of HCs compared with SLE patients (Figs 3 and 4). These results suggest that the TLR9-induced responses in SLE B cells are normal and comparable with the HC in terms of differentiation and Ig levels.

Among the tested SLE patients, seven were in clinical remission and 71.4% of them (five out of seven) secreted autoantibodies upon TLR9 engagement, hence indicating that autoreactive memory B cells persist in the blood of SLE patients even during clinical remission. Therefore, the correlation between infections and relapses in SLE might be explained by the TLR-dependent activation of a pre-existing repertoire of memory B-cell containing autospecificity.

The present results, showing that interference with the TLR9/MyD88 pathway translates in inhibitory effects on CpG-induced _in vitro_ activation and differentiation of human B cells, demonstrate that targeting this pathway is a promising approach to develop novel therapeutic tools [29, 30] and prompt further _in vivo_ investigations to assess the efficacy and safety of our experimental approach.

**Rheumatology key messages**

- Memory B cells from SLE patients in acute phase and clinical remission produce autoantibodies upon TLR9 stimulation.
- An MyD88 synthetic inhibitor completely abolishes the TLR9-induced response and blocks autoantibody production.
- Selective interference with the TLR9/MyD88 pathway may lead to development of novel therapeutics for SLE patients.

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Supplementary data

Supplementary data are available at Rheumatology Online.

References