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The NET-effect of combining rituximab with belimumab in severe systemic lupus erythematosus

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ABSTRACT

Objective: In systemic lupus erythematosus (SLE) patients, excessive formation of neutrophil extracellular traps (NETs) is observed and their degradation is impaired. In vitro, immune complexes (ICx) trigger NET formation while NET-derived DNA is a postulated autoantigen for anti-nuclear autoantibodies (ANAs), found in SLE. Based on these self-perpetuating mechanisms in SLE, this study investigates whether interfering with ICx formation using a combination of rituximab (RTX) and belimumab (BLM) could decrease NET formation and ameliorate disease.

Methods: A phase 2A, open-label, single arm proof-of-concept study was performed wherein 16 SLE patients with severe, refractory disease were treated with a combination of CD20-mediated B-cell depletion with rituximab and sustained inhibition of B-cell activating factor BlyS with belimumab. Besides safety, the study's endpoints were chosen to address the concept of autoantibodies in relation to excessive NET formation.

Results: We demonstrated a surge of BlyS levels upon RTX-mediated B-cell depletion which was abrogated by subsequent BLM treatment. As such, therapeutic intervention with RTX + BLM led to specific reductions in ANAs and regression of excessive NET formation. RTX + BLM appeared to be safe and achieved clinically significant responses: low lupus disease activity state was achieved in 10 patients, renal responses in 11 patients and concomitant immunosuppressive medication was tapered in 14 out of the 16 patients.

Conclusions: This study provides novel insights into clinical beneficence of reducing excessive NET formation in SLE by therapeutic targeting ANA production with RTX + BLM. Altogether putting forward a new treatment concept that specifically ameliorates underlying SLE pathophysiology.

Trial registration: ClinicalTrials.gov NCT02284984.

1. Introduction

Systemic lupus erythematosus (SLE) is a systemic autoimmune disease characterized by the loss of tolerance to nucleic acids and their binding proteins. This results in the generation of anti-nuclear autoantibodies (ANAs), including anti-dsDNA, anti-chromatin and anti-histone autoantibodies. Neutrophil extracellular traps (NETs) have been demonstrated as prominent autoantigens leading to disease-relevant autoantibody production [1–8]. Excessive NET formation [9] together with the impaired degradation of NETs [10,11] has been associated with disease severity in SLE including the presence of lupus nephritis (LN), anti-dsDNA levels and complement usage [11]. The triggers of excessive NET formation in SLE

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have been associated with anti-RNP [4] and anti-LL37 [2] autoantibodies. Moreover, the impaired degradation of NETs is also associated with DNA-binding autoantibodies that block access for the DNAse-I complex which is functionally intact in SLE [12]. So, regression of excessive NET formation has been postulated as an important therapeutic goal in SLE [13].

Additional to NETs, which are proposed as important autoantigens for the development of ANAs, SLE patients display characteristics of B-cell hyperactivity [14] including the typical increase in circulating plasma cells [15,16]. Despite the eminent role of autoreactive B-cells in SLE, specific targeting of B-cells with rituximab (RTX) has been unsuccessful in randomized trials [17,18]. The well-described surge in circulating B-cell activating factor (BAFF) after B-cell depletion has been postulated to influence repopulation of autoreactive B-cells [19] and negatively impact the efficacy of rituximab [20,21]. The pathophysiological importance of BAFF is further supported by the increased serum BAFF levels in SLE patients compared to healthy subjects [22,23] and its association with disease activity [24], disease relapse [20] and increased numbers of circulating plasma cells [25]. The recently approved anti-BAFF monoclonal antibody, belimumab (BLM) [26], is able to specifically target these pathophysiological processes in SLE subsequent to rituximab treatment. Interestingly, a few case reports have reported the clinical use of combined anti-CD20 and anti-BAFF treatment in patients [27–30]: three of the cases had refractory lupus nephritis (LN) showing beneficial effects in response to combination therapy.

The present proof-of-concept study was designed to investigate the hypothesis that combination treatment of rituximab with belimumab (RTX + BLM) would have a synergetic reducing effect on autoantibody production and thereby diminishing NET formation in SLE patients. As a novel therapeutic approach in SLE, this proof-of-concept study was designed as a single-arm translational study aimed at determining the immunological effects while simultaneously evaluating safety and clinical responses in patients with severe, refractory disease eligible for rescue treatment with RTX + BLM.

2. Materials and methods

2.1. Clinical study

We conducted a phase 2, single-arm, proof-of-concept study in which SLE patients were included who had severe and refractory disease. 'Severe SLE' was defined as an SLE disease activity index (SLEDAI) score of 12 or more points or new, worse, or persistent SLE-related activity in major organs. Refractory disease was defined according to national Dutch guidelines [31]: 1) the failure of initial induction treatment for which a switch to another induction therapy regimen was already carried out; 2) intolerance or contraindication for cyclophosphamide and mycophenolate mofetil (MMF); 3) a second relapse within two years after the start of initial induction therapy; or 4) a relative contraindication for high-dose oral or intravenous prednisone. A renal biopsy was performed in 2 refractory LN patients prior to inclusion where the diagnosis active LN was uncertain. Patients were excluded if pregnant, had low peripheral B-cell counts ($<60 \times 10^6$ cells/liter), hypogammaglobulinemia (IgG<4.0 g/l), IgA deficiency (IgA<0.1 g/l), active infection or a history of primary immunodeficiency or active malignancy in the past 5 years.

Patients were treated with 1000 mg RTX at weeks 0 and 2 and with 10 mg/kg BLM at weeks 4, 6, 8 and then every 4 weeks. In accordance with international guidelines [32], any patient with active lupus nephritis or severe neurological involvement (e.g. transverse myelitis) received concomitant intravenous methylprednisolone pulse therapy (variable dose/regimens). High dose glucocorticoids were started at 1 mg/kg (maximum dose was 60 mg per day) and tapered towards a maintenance dose of 7.5 mg/day. The study was approved by the LUMC medical ethics committee and all patients provided written informed consent. The study was registered at ClinicalTrials.gov (NCT02284984).

2.2. Endpoints

Primary endpoints were decrease in autoantibodies and NET formation at 24 weeks. Secondary outcomes were seroconversion of anti-dsDNA autoantibodies, complement normalization, safety, feasibility, and clinical response. Autoantibodies were measured at screening, baseline and at 4, 12, and 24 weeks. Ex vivo NET induction was determined at screening, at week 12 and at week 24. Clinical response was investigated by determining the SLEDAI-2000 (SLEDAI-2K) [33] and the number of patients that achieved lupus low disease activity state (LLDAS) after 24 weeks. LLDAS was defined according to recent international recommendations [34]: 1) SLEDAI-2K ≤ 4, with no activity in major organ systems; 2) no new lupus disease activity; 3) physician global assessment ≤ 1; 4) prednisolone dose ≤ 7.5 mg per day; and 5) well-tolerated treatment with immunosuppressive drugs and/or biological agents [34]. In patients with lupus nephritis, renal responses were defined as follows: a complete renal response was achieved when proteinuria decreased to ≤ 0.7 g/24 h and normal serum albumin, stable kidney function and a normal urinary sediment were achieved. Partial renal response was achieved when proteinuria: >0.7–2.9 g/24 h with a decrease in proteinuria of $\geq 50\%$ from baseline, serum albumin >30 g/l and a stable kidney function as measured by serum creatinine. Urine sediment did not necessarily had to be normalized for achieving a partial renal response. All other patients were considered to be renal non-responders.

2.3. Preparation of neutrophils and ex vivo NET induction

Whole blood (20 ml) from healthy donors was collected into EDTA-coated tubes (BD, Franklin Lakes, NJ, USA). Neutrophils were isolated by density gradient centrifugation with a Ficoll-amidotrizoate gradient (LUMC, Leiden, The Netherlands) followed by erythrocyte lysis at 4 °C. Cells were counted using trypan blue, labelled with PKH26 (2 μM, Sigma-Aldrich, Saint-Louis, MO, USA), and 37,500 neutrophils per well were seeded into a 96-well culture plate (Falcon, Tewksbury, MA, USA) in phenol red-free RPMI 1640 medium (Life Technologies, The Netherlands) supplemented with 2% heat-inactivated fetal calf serum (FCS). To induce NETosis, neutrophils were stimulated for 3.75 h with one of the following: medium (negative control), 10% serum, 10% IgG-depleted serum, 250 or 25 μg/ml IgG, 25 nM phorbol 12-myristate 13-acetate (PMA) (Sigma-Aldrich, Saint-Louis, MO, USA) or IgG derived from intravenous immunoglobulin (IVIG; Sanquin, Amsterdam, the Netherlands), healthy controls and patients. When immobilized IgG was used, 10 μg/ml IgG was coated overnight at 4 °C in a 96-well Falcon plate, after which neutrophils were incubated in the wells. After stimulation, 1 μM of the impermeable DNA dye SYTOX green (Thermo Fisher, Waltham, MA, USA) was added for 15 min and then the neutrophils were fixed with 4% paraformaldehyde (PFA) (Added Pharma, Oss, the Netherlands).

2.4. NET visualization and quantification

NETs were visualized by confocal laser scanning microscopy (CLSM) using the automated BD Pathway 855 (BD Biosciences, San Jose, CA, USA), as described previously [35]. Briefly, 12 z-stacked images of 25 predefined high-power fields (HPFs) at 20×

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