Impaired degradation and aberrant phagocytosis of necrotic cell debris in the peripheral blood of patients with primary Sjögren's syndrome

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ABSTRACT

Aberrant removal of necrotic debris is considered a feature with inflammatory consequences in SLE. Herein, primary Sjögren’s syndrome (SS) patients were investigated for the first time for the capacity of their sera to degrade secondary necrotic cell remnants (SNEC) and DNA (endonuclease DNase1 activity), as well as for uptake of SNEC by blood-borne phagocytes. For comparison, specimens from unselected SLE and RA patients and from healthy blood donors (HBD) were also studied. Compared to HBD, the sera from SS and SLE patients studied (but not RA) were found to exhibit significantly impaired capacity for degradation of SNEC (both for \( p = 0.007 \) and deficient DNase1 activity (both for \( p < 0.0001 \)). The deficient DNase1 activity in SS and SLE sera did not owe to decreased DNase1 protein levels. It correlated inversely with increased serum levels of circulating nucleosomes and cell-free DNA (\( p < 0.0001 \)), as well as with the disease activity indices of SS (\( r = -0.445, p = 0.0001 \)) and SLE (\( r = -0.500, p = 0.013 \)). In vivo whole blood analyses, SS and SLE patients (but not RA) also manifested significantly increased SNEC-phagocytosis by monocytes and granulocytes (all for \( p < 0.0001 \)) that also correlated with disease severity indices of SS (\( p = 0.001 \)) and SLE (\( p = 0.01 \)). In various cross-admixture experiments, such aberration was found to reside in the hyperfunctional activity of phagocytes, the impaired degrading activity of serum DNase1 and the SNEC-binding capacity of serum IgG of SS and SLE patients. The sera of SS and SLE patients (but not of RA) induced significantly SNEC-phagocytosis by healthy monocytes that correlated inversely with the DNase1 activity (\( r = -0.634, p < 0.0001 \)). In line with this, the inhibition of DNase1 in HBD sera by G-actin was found to lead to significantly diminished SNEC degradation and increased SNEC uptake by healthy phagocytes (\( p = 0.0009 \)), supporting the important physiologic role of serum DNase1 in the prevention of SNEC-phagocytosis. Purified serum IgG preparations from SS and SLE patients manifested increased binding to SNEC and were able to enhance significantly the engulfment of SNEC by healthy phagocytes both directly (under serum-free conditions, \( p < 0.0001 \)) and via the prevention of physiologic degradation of SNEC by serum, most likely due to their “shielding” against endonuclease digestion (\( p = 0.0005 \)). These data indicate that upon cell necrosis, the immune system of SS and SLE patients may be overly exposed to the necrotic debris, a fact that probably holds a key role in the pathogenesis of inflammatory and autoimmune reactions observed in these disorders.

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Abbreviations: SNEC, secondary necrotic material (necrotic cell remnants); SRED, single radial enzyme diffusion assay; PI, propidium iodide; SS, primary Sjögren’s syndrome; SLE, systemic lupus erythematosus; RA, rheumatoid arthritis; HBD, healthy blood donors.

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1. Introduction

Dead cells and cellular debris may elicit inflammatory reactions, however, under healthy conditions this is effectively prevented by their rapid elimination from tissues [1]. On the other hand, experimental evidence from patients and animal models indicates that systemic lupus erythematosus (SLE) is characterized by defective clearance of apoptotic cells, whereby chronic autoimmune reactions and organ damage are thought to ensue [2,3]. Normally, dying cells are rapidly cleared at early stages of apoptosis via mechanisms that involve several types of membrane receptors and serum bridging molecules, including components of the complement system [4–6]. However, in the absence of efficient uptake, the apoptotic cells proceed to the state of “late apoptosis” or “secondary necrosis”, where cells lose their cell membrane integrity and spill out their content, including modified autoantigens and “danger signals” such as alarmins [7–9]. Although such secondary necrotic material (termed SNEC) may be rendered accessible to the cells of the immune system, recent studies indicate that SNEC is not engulfed by phagocytes from healthy individuals, whereas it is readily ingested by monocytes and granulocytes from SLE patients, thus resulting in the secretion of several proinflammatory cytokines [10,11]. In fact, SNEC normally undergoes efficient degradation by serum DNase1 with the assistance of serum cofactors [12,13], a fail-safe mechanism that is reportedly defective in SLE [14,15]. In lupus patients, serum autoantibodies to nuclear antigens, such as anti-dsDNA, have been also reported to promote the uptake of SNEC by blood-borne phagocytes [10,11,16]. Taken together, the impaired phagocytosis of apoptotic cells in conjunction with increased uptake of necrotic material by peripheral blood phagocytes of SLE patients appears to contribute to the pathogenesis of the disease [17].

Primary Sjögren’s syndrome (SS) is a chronic autoimmune disorder with a broad spectrum of clinical symptoms that extends from disease confined to the exocrine glands (organ-specific exocrinopathy) to various extraglandular manifestations (systemic disease) and the development of B-cell lymphoma [18]. SS patients present polyclonal B-cell hyperactivity, illustrated by profound hypergammaglobulinemia, multiple autoantibodies, circulating immune complexes and complement consumption [18,19]. SLE shares with SS the above features of B-cell hyperactivity, as well as the frequent occurrence of defective uptake of apoptotic cells by phagocytes (Manoussakis et al., submitted). In this context, we presently sought to comparatively investigate the handling and removal of necrotic cell debris by the peripheral blood components of SS, SLE and RA patients, including the degradation of SNEC and DNA by serum and the uptake of SNEC by phagocytes.

2. Patients and methods

2.1. Patients

Blood specimens were obtained after informed consent from 70 consecutive unselected patients with primary SS, 32 with SLE and 15 with rheumatoid arthritis (RA) using established criteria [20–22] (Table 1), as well as from 50 healthy blood donors (HBD) matched for age and sex to the SS (HBD-1; n = 25) and SLE groups (HBD-2; n = 25). The study was approved by the Medical Council of “Laikon” University Hospital. At the time of investigation, none of the patients or controls studied displayed evidence of infection, including past or current infection by hepatitis viruses or human immunodeficiency virus. Patients’ medical records were retrospectively analyzed for demographic variables, clinical and laboratory features. At the time of the study, patients studied were assessed for disease activity by calculation of ESSDAI (for SS) [23], SLEDAI (for SLE), DAS28 (for RA) and for disease severity by SSD1 (for SS) [24], SLICC-ACR (for SLE) [25] and by global disease severity index (for RA). Patients with SS were also analyzed for the presence of extraglandular manifestations (arthritis, Raynaud’s phenomenon, purpura, peripheral neuropathy, vasculitis, and hepatic, pulmonary, or renal involvement), as well as for the presence of manifestations of type-I disease, as previously [26]. In RA patients, the occurrence of extra-articular disease (subcutaneous nodules, secondary Sjögren’s syndrome, episceritis/scleritis, serositis, interstitial lung disease, and vasculitis) was recorded. The global severity of RA (assigning patient’s global disease condition and not just the inflammatory status of the joints) was rated independently and blindly to the other results by two of the authors (MMN and HMM), on a graded scale ranging from 0 (mildest disease) to 10 (most severe disease) (Table 1). All blood specimens were processed immediately. Sera were stored in aliquots at −20 °C until tested.

<table>
<thead>
<tr>
<th>Features</th>
<th>SS</th>
<th>SLE</th>
<th>RA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, years, median (range)</td>
<td>57.5 (33–78)</td>
<td>39.5 (19–42)</td>
<td>62.0 (42–80)</td>
</tr>
<tr>
<td>Sex, women: men</td>
<td>66:4</td>
<td>30:2</td>
<td>11:4</td>
</tr>
<tr>
<td>Disease duration, years, median (range)</td>
<td>10 (1–22)</td>
<td>10 (1–20)</td>
<td>9 (2–15)</td>
</tr>
<tr>
<td>Sicca manifestations, no. positive (%)</td>
<td>70 (100)</td>
<td>0 (0.0)</td>
<td>1 (6.7)</td>
</tr>
<tr>
<td>Disease activity, median (range)</td>
<td>8.4 (0–30)</td>
<td>4 (0–16)</td>
<td>5.4 (3–9)</td>
</tr>
<tr>
<td>Disease severity, median (range)</td>
<td>7 (2–7)</td>
<td>2 (0–7)</td>
<td>2.7 (1–5.2)</td>
</tr>
<tr>
<td>Type-I SS disease, no. positive (%)</td>
<td>51 (72.8)</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>ANA, titer †, median (range)</td>
<td>320 (0–2560)</td>
<td>640 (0–2560)</td>
<td>0 (0–2560)</td>
</tr>
<tr>
<td>Anti-Ro/SSA, no. positive (%)</td>
<td>48 (68.6)</td>
<td>13 (40.0)</td>
<td>0 (0.0)</td>
</tr>
<tr>
<td>Anti-La/SSB, no. positive (%)</td>
<td>22 (31.4)</td>
<td>0 (0.0)</td>
<td>0 (0.0)</td>
</tr>
<tr>
<td>Anti-dsDNA, no. positive (%)</td>
<td>0 (0.0)</td>
<td>14 (43.7)</td>
<td>0 (0.0)</td>
</tr>
<tr>
<td>Anti-chromatin, no. positive (%)</td>
<td>0/27 (0.0)</td>
<td>5/18 (22.2)</td>
<td>0 (0.0)</td>
</tr>
<tr>
<td>Anti-histone, no. positive (%)</td>
<td>0/27 (0.0)</td>
<td>7/18 (38.9)</td>
<td>0 (0.0)</td>
</tr>
<tr>
<td>Anti-C1q, no. positive (%)</td>
<td>5/27 (18.5)</td>
<td>9/18 (50.0)</td>
<td>0 (0.0)</td>
</tr>
<tr>
<td>Low serum C3 and/or C4, no. positive (%)</td>
<td>42 (60.0)</td>
<td>21 (65.6)</td>
<td>3 (20.0)</td>
</tr>
<tr>
<td>Low serum C1q, no. positive (%)</td>
<td>6/22 (27.3)</td>
<td>5/14 (35.7)</td>
<td>ND</td>
</tr>
<tr>
<td>RF, no. positive (%)</td>
<td>22 (66.7)</td>
<td>NA</td>
<td>11 (73.3)</td>
</tr>
<tr>
<td>Cryoglobulinemia, no. positive (%)</td>
<td>20 (28.6)</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

NA: non-applicable; ND: not determined.

Median serum levels of C3 (range) were in SS: 104 mg/dL (60–168), in SLE: 91 mg/dL (39–154) and in RA: 107 mg/dL (82–163). Median serum levels of C4 (range) were in SS: 19 mg/dL (2–46), in SLE: 14 mg/dL (4–33) and in RA: 23 mg/dL (16–37). Median serum levels of C1q (range) were in SS: 17.6 mg/dL (3.5–43.6) and in SLE: 18.5 mg/dL (12.0–25.0).

† Defined as described in Patients and Methods.

a Low serum C3 < 90 mg/dL and C4 < 20 mg/dL.

b Low serum C1q < 15 mg/dL.

c Low serum C1q < 15 mg/dL.
DNase1 activity was expressed as DNase1 equivalent units/mL, by comparison to a standard curve generated using human recombinant DNase1 (Qiagen), whereas in ELISA assay, the results were expressed as DNase1 activity index (range: 1–100%, corresponding to 0.3–5000 pg/mL DNase or 1 μkU/mL to 16 μkU/mL [DNase enzyme in Kunitz units]). In the SRED assay, arbitrary cut-off value of DNase1 activity was set at two standard deviations below the corresponding mean of HBD, and accordingly, donors with higher values were termed as “degraders”, whereas the remainder were designated as “non-degraders”. Serum protein levels of DNase1 were measured by a commercially available ELISA assay (Life Sciences Advanced Tech, Inc, assay sensitivity: 1 ng/mL), according to the instructions of the manufacturer. DNA in serum specimens (500 μL) was extracted using the Qiaamp DSP Virus Kit (Qiagen, Hilden, Germany) and the levels of cell-free DNA were determined by real-time quantitative polymerase chain reaction to amplify a 87-bp fragment of the human β-globin gene using the LightCycler Control Kit DNA (Roche Diagnostics), as previously [28].

2.3. SNEC preparation and SNEC degradation assays

Secondary necrotic cell derived material (SNEC) was produced as described previously [11]. In brief, cell death was induced in peripheral blood mononuclear cells from an HBD by treatment at 56°C for 30 min. SNEC was obtained by consecutive incubation at 37°C in RPMI medium supplemented with 5% human AB serum for 24–36 h. SNEC was labeled with 10 μg/mL propidium iodide (PI: the fluorescent DNA dye Sigma) and stored at −20°C until use. Microscopically, SNEC corresponded to amorphous granular debris of disrupted cells and nuclei with occasional small aggregate formation, and were not found to possess any features of neutrophil extracellular traps as determined by PI-staining. The capacity of sera for degradation of SNEC was assayed by flow cytometry, as previously [11] with some modifications. In brief, PI-stained SNEC were either mock-treated or treated with serum specimens at 37°C in different concentrations (0–82% in PBS) and at different time points (0–120 min), followed by addition of EDTA (5 mM) to inhibit nuclease activity. Prior to analyses, specimens of untreated PI-stained SNEC and sera alone were run to define a gate (FL2-log vs. SSC-log plot) that distinguished SNEC from particles present in the sera. Gated events of serum-treated PI-stained SNEC were analyzed by flow cytometry and expressed as percentage of mock-treated SNEC. The SNEC degradation capacity of DNase1 was appraised as above in mock-treated SNEC or SNEC treated with human recombinant DNase1 (Qiagen, 1 U/mL in DNase1 buffer or PBS, for 0–60 min at 37°C). To inhibit serum DNase1, scaled concentrations of an HBD serum (10–50% v/v) were treated with EDTA (5–20 mM), heat (56°C, for 30-min) or the DNase1-specific inhibitor G-actin (rabbit skeletal; Sigma Aldrich, 400 μg/mL). To determine the possible protective effect of IgG from patients and controls on SNEC degradation by serum and DNase1, SNEC were mock-treated or pretreated with purified serum IgG preparations (1 mg/mL for 1-hr at 37°C) and subsequently were incubated with an HBD serum (10%) or recombinant DNase1 (20 U/mL) 1-hr at 37°C. The protection of SNEC from degradation was comparatively determined (IgG-pretreated SNEC over mock-treated SNEC) by flow cytometry as above (calculation of the ratio of gated events detected), as well as by direct evaluation of the morphology and intensity of PI staining by immunofluorescence microscopy.

2.4. Purification of serum IgG immunoglobulins and assessment of their binding to SNEC

Total immunoglobulin IgG was isolated from serum samples of autoimmune patients and HBD using the Melon Gel purification kit (Pierce), according to the manufacturer’s protocol, aliquoted and stored at −20°C. Protein yields were quantified spectrophotometrically and purification was routinely found to be more than 95% by SDS-PAGE electrophoresis. The binding of serum IgG to SNEC was assessed by flow cytometry. In brief, SNEC derived from 2×10^6 cells were resuspended in 1% BSA/PBS and incubated for 30-min at 4°C with or without purified IgG (50 μg/mL) obtained from sera of autoimmune patients or HBD. After washing, SNEC were incubated with 4 μL of FITC-labeled rabbit anti-human IgG for 30-min at 4°C in the dark and analyzed by flow cytometry.

2.5. Ex-vivo assessment of phagocytosis of necrotic cell derived material (SNEC-phagocytosis) in whole blood

SNEC-phagocytosis was quantitatively assessed by flow cytometry in peripheral blood specimens of 21 SS, 29 SLE, 10 RA and 28 HBD, as previously described [11]. In brief, parallel specimens (100 μL) of heparinized freshly drawn whole blood were each mixed with 10 μL of SNEC and were incubated at either 37°C (evaluation of phagocytosis) or on ice (to prevent phagocytosis). The incubation time was optimized at 4 h, as previously [11]. Subsequently, red blood cells were lysed with a lysing buffer (BD, Pharmingen) and the samples were analyzed by flow cytometry (Facscaibur, BD). Granulocytes and monocytes were separately gated, based on their forward- and side-scatter properties, and the ingestion of propidium iodide-labeled SNEC was estimated as SNEC-phagocytosis index (SNEC-PhI) which was calculated as the product of the percentage of gated fluorescence-positive cells by their mean fluorescence intensity.

2.6. Cross-admixture experiments for the assessment of the influence of serum factors and of phagocytes' function in SNEC-phagocytosis

Total peripheral blood cells were isolated from heparinized whole blood derived from HBD and autoimmune patients (100 μL) by centrifugation (1200 rpm, 10-min) and plasma aspiration. Following hemolysis and washing, healthy peripheral blood cells were subsequently admixed with 40 μL of culture medium (DMEM) or with serum derived from other HBD or autoimmune patients and SNEC-phagocytosis assays were performed as described above. Such cross-admixture SNEC-phagocytosis experiments were also used to appraise the physiologic role of serum DNase1 in the degradation of necrotic cell debris and the prevention of SNEC uptake by blood-borne phagocytes using healthy human sera that were either mock-pretreated or pretreated with the DNase1-inhibitor G-actin (Sigma, 5–800 μg/mL, for 10-min at room temperature). To address the net effect of IgG antibodies in the uptake of SNEC, similar cross-admixture SNEC-phagocytosis assays were also carried out, using whole blood monocytes from HBD and PI-labeled SNEC pretreated with purified IgG immunoglobulins (1 mg/mL) obtained from sera of autoimmune patients and HBD. Finally, cross-admixture SNEC-phagocytosis experiments were also performed employing peripheral blood phagocytes derived from SS and SLE patients and SNEC treated with serum or purified IgG from autoimmune patients or HBD.

2.7. Statistical analyses

Correlations were calculated using the Spearman’s rank correlation coefficient. Group comparisons were performed by Mann–Whitney rank sum test. For paired comparisons, Wilcoxon signed-rank test and one-way analysis of variance were used when appropriate. Analyses were conducted using SPSS 15.0 and Graph Pad 5.0 softwares. The results were expressed as median and
Fig. 1. The sera from SS and SLE patients manifest significantly impaired capacity to degrade SNEC and DNA. A. Time-course studies by flow cytometry demonstrating the reduced capacity of sera from SS patients to degrade SNEC, as compared to sera from HBD. Similar results were obtained using sera from SLE patients (not shown). B–D. Comparative evaluation of serum specimens derived from HBD and patients with RA, SS and SLE for SNEC degradation (B) and DNase1 activity by the SRED assay (C) and by ELISA (D). The horizontal lines indicate the median levels in each group, whereas the numbers in boxes indicate the percentages of individuals with significantly decreased SNEC degradation or DNase1 activity, as defined by values found two standard deviations below the corresponding mean of HBD studied. E–F. Linear correlation of serum DNase1 activity values obtained in the various samples tested between SRED and ELISA assays (E), as well as between DNase1 activity values obtained by SRED assay and the SNEC degradation assay (F). G–H. Inverse correlation between serum DNase1 activity values (by SRED assay) and the indices of disease activity of patients with SS (G, ESSDAI) and SLE (H, SLEDAI) studied. SRED; single radial enzyme diffusion assay, ESSDAI: EULAR Sjogren’s Syndrome Disease Activity Index, SLEDAI: Systemic Lupus Erythematosus Disease Activity Index.

range, and correlations with two-tailed p-values less than 0.05 were considered statistically significant.

3. Results

3.1. SNEC are significantly degraded by sera of healthy individuals, owing to the activity of serum DNase1

In preliminary experiments, SNEC preparations were found to be effectively degraded following treatment with serum from healthy individuals, as demonstrated by dose- and time-dependent kinetics experiments (Supplementary Fig. 1A–C). In addition, the sera derived from the younger and the older HBD groups (HBD-1 and HBD-2, respectively) were found to manifest similar capacities for SNEC degradation and DNase1 activity with no statistical differences to each other (data not shown), therefore all HBD studied were considered as a single group in the comparative analyses. SNEC did not contain intrinsic degrading capacity, but underwent significant breakdown following treatment with recombinant DNase1 at the appropriate buffer (Supplementary Fig. 1D). In sera, the activity of DNase1 was found to be essential for the degradation of SNEC, as supported by the effective blockade by the DNase1 inhibitor G-actin [29] and compatible biochemical properties, such as heat-lability and dependence to calcium and magnesium (Supplementary Fig. 1E and data not shown).

3.2. Deficient capacity for degradation of SNEC and DNA by serum in SS and SLE patients

The SS and SLE patients studied (but not in those from RA) manifested several serological aberrations, including the presence of various autoantibodies and hypocomplementemias (Table 1). In addition, compared to sera from HBD, the sera of SS and SLE patients were found to display significantly reduced capacity for degradation of SNEC (both for p = 0.007, Fig. 1A and B), as well as of DNA (DNase1 activity; both for p < 0.0001, by the SRED assay; Fig. 1C and for p ≤ 0.004 by ELISA; Fig. 1D). There was a highly significant linear correlation between the serum DNase1 activity values of sera and the serum DNase1 activity (by the SRED assay; r = 0.454 p = 0.017, Fig. 1F). Despite the occurrence of reduced DNase1 activity in the patients with SS and SLE, the analysis of serum protein levels of DNase1 had not revealed any significant differences between specimens derived from patients with SS and SLE and those from HBD (Supplementary Fig. 2).

The levels of DNase1 activity in the SS and SLE patients studied correlated inversely and linearly with the disease activity of these disorders (ESSDAI; r = −0.445, p = 0.0001, and SLEDAI; r = −0.500, p = 0.013, respectively, Fig. 1G and H). Among SS patients, serum DNase1 activity was found to be significantly impaired among patients with type-I disease (median: 0.072, range: 0.023–0.097) compared to those without (median: 0.089, range: 0.054–0.121, p = 0.002), as well as among SS patients with cryoglobulinemia (median: 0.069, range: 0.045–0.097) compared to those without (median: 0.087, range: 0.054–0.118, p = 0.0005). In addition, the levels of serum DNase1 activity correlated positively with serum C1q (r = 0.501, p = 0.021), C4 (r = 0.469, p = 0.0005) and C3 complement levels (r = 0.387, p = 0.0038). The impaired capacity of serum for degradation of SNEC and DNA in SS patients would be expected to result in the accumulation of unprocessed nucleosomes and/or cell-free DNA in their serum which are thought to be
released into the blood circulation by dying cells. In support to this notion, significantly increased levels of circulating nucleosomes were observed in the sera of SS patients tested \((n = 23;\) median absorbance measurements at 450 nm: 0.339, range: 0.115–2.919) compared to HBD \((n = 8;\) median:0.209, range:0.106–0.463, \(p = 0.034)\) and were significantly more prevalent among non-degraders SS patients \((\text{median:1.016, range:0.104–2.919})\) compared to degraders \((\text{median: 0.213, range: 0.115–0.432, } p = 0.01)\). In addition, significantly increased amounts of circulating cell-free DNA were detected in the sera of patients with SS and SLE, as compared to those from HBD and RA patients \((\text{both for } p < 0.05, \text{Supplementary Fig. 3A})\). Importantly, also, a strong inverse correlation was observed between the levels of cell-free DNA and of DNAse1 activity in the sera studied \((r = −0.763, p < 0.0001, \text{Supplementary Fig. 3B})\).

### 3.3. Increased SNEC-phagocytosis by the peripheral blood monocytes and granulocytes of SS and SLE patients

The capacity of patients and controls for phagocytic ingestion of SNEC was comparatively assessed in whole blood assays. In preliminary experiments, the younger and the older HBD groups \((\text{HBD-1 and HBD-2, respectively})\) were found to manifest low but detectable SNEC-phagocytosis by monocytes and granulocytes with no statistical differences to each other \((\text{data not shown})\), therefore all HBD studied were considered as a single group in the comparative analyses. RA patients displayed similar SNEC-phagocytosis indices to HBD groups \((\text{Fig. 2A and B})\). In direct contrast, both SS and SLE patient groups were found to manifest highly significantly increased SNEC-phagocytosis by monocytes \((\text{both for } p < 0.0001)\), as well as by granulocytes \((\text{both for } p < 0.0001)\), compared to HBD, as well as to RA patients \((\text{Fig. 2A and B})\). There were no differences of SNEC-phagocytosis indices between the groups of SS and SLE patients, for either monocytes or granulocytes. In both the SS and the SLE patient groups studied, the occurrence of increased SNEC-phagocytosis was found to correlate with the severity and damage scores of these disorders, but not with the other clinical or serological parameters examined. In the SS patients, the degree of SNEC-phagocytosis by monocytes was found to positively correlate with the SSDDI disease severity scores \((r = 0.663, p = 0.001)\) and the occurrence of extraglandular disease \((\text{in glandular SS; median SNEC-Phi: 132, range: 25–250, in extraglandular SS; median SNEC-Phi: 309, range: 50–671, } p = 0.003)\), whereas in the SLE patients with the SLICC/ACR damage scores \((r = 0.551, p = 0.01)\),

#### 3.4. The enhanced SNEC-phagocytosis in the peripheral blood of SS and SLE patients owes to the aberrant function of both the phagocytes and the sera of patients

We hypothesized that the enhanced SNEC-phagocytosis observed in the peripheral blood of SS and SLE patients may be due to abnormal functions of patients’ phagocytes and/or their sera. To discern between these two main interpretations, we conducted two independent types of comparative SNEC-phagocytosis experiments employing isolated peripheral blood cells and/or sera from the various groups of patients and controls studied. First, we performed SNEC-phagocytosis assays under serum-free conditions \((\text{using plasma-depleted whole blood specimens})\). These experiments indicated, first, that in the absence of serum, healthy phagocytes can ingest ample amounts of SNEC and second, that the phagocytes of SS and SLE patients \((\text{but not those of RA})\) manifest significantly increased SNEC-phagocytosis, compared to those of HBD \((\text{for monocytes: SS: } p < 0.0001, \text{SLE: } p = 0.014, \text{for granulocytes: SS: } p = 0.0015 \text{ SLE: } p = 0.007, \text{Fig. 3A and B})\). In the second set of experiments, we performed comparative cross-admixture SNEC-phagocytosis assays that involved the incubation of healthy peripheral blood cells \((\text{from an HBD})\) and SNEC treated with sera derived from autoimmune patients or HBD. In these experiments, the sera from SS and SLE patients displayed a significant SNEC phagocytosis-inducing effect, compared to the sera from HBD and RA patients \((\text{Fig. 4A and B})\). Similar cross-admixture assays were also conducted using peripheral blood phagocytes from selected SS and SLE patients \((\text{who had showed increased uptake of SNEC in the whole blood studies, 3 SS and 8 SLE})\) in the presence of the homologous serum or an HBD serum. In these assays, the addition of the HBD serum resulted in significantly lower levels of SNEC-phagocytosis compared to that obtained in the presence of the homologous patient serum \((\text{median reduction; for monocytes: 50.0%, range: 26.0–90.0%, } p = 0.001, \text{granulocytes: 45.8%, range: 11.0–81.3%, } p = 0.01, \text{data not shown})\). Taken together, these results indicated that the increased SNEC-phagocytosis observed in the peripheral blood of SS and SLE patients owes both to the hyperfunctional activity of patients’ phagocytes and to the aberrant function of their sera.

#### 3.5. The increased SNEC phagocytosis-inducing effect of SS and SLE sera largely owes to deficient DNAse1 activity

Serum appears to play an important physiologic role in the prevention of SNEC ingestion by blood-borne phagocytes. The uptake of SNEC by isolated peripheral blood phagocytes from an HBD...
was found to be significantly amplified by reducing the concentration of HBD serum or plasma specimens used (Supplementary Fig. 4A and B). In particular, DNA degradation by serum DNase1 appears to have a major involvement in the aversion of SNEC-phagocytosis in the peripheral blood, as supported by the significant augmentation of SNEC-phagocytosis following the inhibition of DNase1 in serum samples from HBD by G-actin (Supplementary Fig. 4C). On this basis, the impaired activity of DNase1 in the sera of SS and SLE patients appears largely responsible for their significant SNEC phagocytosis-inducing effect, since deficient degradation would lead to increased amounts of SNEC available for uptake by phagocytes. In support to this notion, the degree of SNEC phagocytosis-inducing capacity of the various sera studied (shown above in Fig. 4A) was found to display strong inverse correlation with the DNase1 activity in those sera, as measured by the SRED assay ($r = -0.634$, $p < 0.0001$, Fig. 4C), as well as by ELISA ($r = -0.570$, $p = 0.001$, data not shown).

3.6. IgG anti-SNEC autoantibodies in the sera of SS and SLE patients enhance SNEC-phagocytosis by opsonization and by shielding SNEC against degradation

Besides DNase1 activity, the SNEC phagocytosis-inducing effect of the SS and SLE sera (shown above in Fig. 4A and B) was also found to be significantly amplified by reducing the concentration of HBD serum or plasma specimens used (Supplementary Fig. 4A and B). In particular, DNA degradation by serum DNase1 appears to have a major involvement in the aversion of SNEC-phagocytosis in the peripheral blood, as supported by the significant augmentation of SNEC-phagocytosis following the inhibition of DNase1 in serum samples from HBD by G-actin (Supplementary Fig. 4C). On this basis, the impaired activity of DNase1 in the sera of SS and SLE patients appears largely responsible for their significant SNEC phagocytosis-inducing effect, since deficient degradation would lead to increased amounts of SNEC available for uptake by phagocytes. In support to this notion, the degree of SNEC phagocytosis-inducing capacity of the various sera studied (shown above in Fig. 4A) was found to display strong inverse correlation with the DNase1 activity in those sera, as measured by the SRED assay ($r = -0.634$, $p < 0.0001$, Fig. 4C), as well as by ELISA ($r = -0.570$, $p = 0.001$, data not shown).

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to correlate with the presence of specific autoantibodies in those sera, namely the anti-Ro/SSA and anti-La/SSB antibodies in the sera of SS patients, and the anti-dsDNA, anti-histone and anti-chromatin antibodies in those of SLE (Table 2). In this context, we sought to address whether the occurrence of IgG autoantibodies against SNEC had also a role in the SNEC phagocytosis-inducing capacity of those sera. Purified serum IgG preparations from SS and SLE patients, but not those from RA patients and HBD were found to exhibit increased reactivity against SNEC (Fig. 5A). SNEC treated with serum IgG preparations from SS and SLE patients were found to exhibit increased reactivity against SNEC (Fig. 5B). Interestingly, the capacity of the various serum IgG preparations to bind to SNEC was found to correlate linearly with the aptitude of those IgG specimens to induce phagocytosis (Fig. 5C and D). Under serum-free conditions, SNEC treated with healthy IgG from SS and SLE patients were also found to be more avidly phagocytosed by healthy monocytes compared to those treated with healthy IgG (Fig. 5E). Interestingly, the capacity of the various serum IgG preparations to bind to SNEC was found to correlate linearly with the aptitude of those IgG specimens (as well as of the sera from which they originated) to support SNEC phagocytosis in the respective cross-admixture assays (Fig. 5C and D). Under serum-free conditions, SNEC treated with IgG from SS and SLE patients were also found to be more avidly phagocytosed by healthy monocytes compared to those treated with healthy IgG preparations, likely due to enhanced engulfment of opsonized necrotic debris (Supplementary Fig. 5A). Furthermore, in cross-admixture assays with isolated peripheral blood monocytes from SS patients, IgG preparations from SS patients had also a more pronounced SNEC phagocytosis-inducing effect, over HBD-derived IgG (Supplementary Fig. 5B).

4. Discussion

Dying cells are a rich source of oligo- and mono-nucleosomes that are generated intracellularly following the cleavage of chromatin by endonucleases [30,31]. Such structures may be released in the extracellular space when the dying cells enter late stages of apoptosis, i.e. secondary necrosis [32]. Under healthy conditions, the effective removal of apoptotic cells by phagocytes prevents the subsequent appearance of ample amounts of necrotic debris in the circulation that can elicit injurious inflammatory reactions [33]. In addition, the degradation of DNA and chromatin by the endonuclease DNase1 and complement factors provides a fail-safe mechanism for the rapid elimination of nuclear remnants from the blood and tissues [30]. In this context, overwhelming apoptosis and/or impaired removal of dying cells and necrotic cell debris are considered responsible for the increased amounts of circulating nuclear material (nucleosomes and DNA) that have been previously reported in the plasma of SLE patients [34–36]. In SLE, circulating nucleosomes are considered the major source of autoantigens that drive the generation of anti-DNA and anti-nucleoprotein autoantibodies and the formation of inflammatory immune complexes [37]. A portion of SLE patients has been previously shown to manifest impaired serum DNase1 activity that is thought to lead to defective degradation of nucleosomes and necrotic cell debris [38,39], as well as in the impaired dismantling of chromatin structures that are released by neutrophils against pathogens (the so-called neutrophil extracellular traps or NETs) [15]. Our findings corroborate the occurrence of deficient serum DNase1 activity and increased amounts of circulating nucleosomes and cell-free DNA in SLE patients and also demonstrate for the first time the occurrence of these aberrations in the SS patients. Importantly, the strong correlation between the impaired DNase1 activity and the circulating amounts of nucleosomes and cell-free DNA likely implies an etiologic association between these phenomena. Furthermore, our data also indicate a highly significant inverse correlation between serum DNase1 activity and the disease activity of the SS and SLE patients studied. Our findings appear to hold promising potentials for the elucidation of SS and SLE pathogenesis; however, the precise nature of decreased serum DNase1 activity in these patients is presently unclear. According to our findings, such deficient activity cannot be attributed to decreased DNase1 protein levels, and fractionation studies are in progress to assess whether the impairment is due to defective function of the enzyme and/or to the occurrence of inhibiting substances [15,39], such as the circulating nucleosomes.

### Table 2

<table>
<thead>
<tr>
<th>Serum specimens studied</th>
<th>Induction of SNEC phagocytosis in HBD phagocytes by SS and SLE sera</th>
<th></th>
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</thead>
<tbody>
<tr>
<td></td>
<td>HBD monocytes</td>
<td></td>
<td>HBD granulocytes</td>
<td></td>
</tr>
<tr>
<td></td>
<td>SNEC-PhI values</td>
<td>p-Value</td>
<td>SNEC-PhI values</td>
<td>p-Value</td>
</tr>
<tr>
<td></td>
<td>Median [range]</td>
<td></td>
<td>Median [range]</td>
<td></td>
</tr>
<tr>
<td><strong>Sera from SS patients:</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Anti-Ro/SSA status</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Positive (n = 20)</td>
<td>520 [90–2116]</td>
<td>0.02</td>
<td>178 [46–2870]</td>
<td>NS</td>
</tr>
<tr>
<td>Negative (n = 9)</td>
<td>240 [140–645]</td>
<td>0.03</td>
<td>120 [36–270]</td>
<td>NS</td>
</tr>
<tr>
<td>Anti-La/SSB status</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Positive (n = 9)</td>
<td>616 [90–1260]</td>
<td>0.03</td>
<td>160 [46–2193]</td>
<td>NS</td>
</tr>
<tr>
<td>Negative (n = 20)</td>
<td>298 [112–2116]</td>
<td>178 [36–2870]</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Sera from SLE patients:</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Anti-dsDNA status</td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Positive (n = 12)</td>
<td>1295 [460–4524]</td>
<td>0.006</td>
<td>605 [260–2219]</td>
<td>NS</td>
</tr>
<tr>
<td>Negative (n = 7)</td>
<td>736 [350–910]</td>
<td>0.01</td>
<td>167 [71–1363]</td>
<td>NS</td>
</tr>
<tr>
<td>Anti-histone status</td>
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<td></td>
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</tr>
<tr>
<td>Positive (n = 6)</td>
<td>2533 [780–4524]</td>
<td>0.01</td>
<td>1293 [260–2219]</td>
<td>NS</td>
</tr>
<tr>
<td>Negative (n = 11)</td>
<td>736 [350–1710]</td>
<td>336 [103–1363]</td>
<td></td>
<td></td>
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<tr>
<td>Anti-chromatin status</td>
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<td></td>
<td></td>
<td></td>
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<tr>
<td>Positive (n = 5)</td>
<td>2920 [1369–4524]</td>
<td>0.01</td>
<td>850 [260–2219]</td>
<td>NS</td>
</tr>
<tr>
<td>Negative (n = 12)</td>
<td>718 [350–3545]</td>
<td>362 [103–1078]</td>
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</table>
In direct agreement with previous reports \cite{11, 16}, our results indicate that the majority of SLE patients manifest markedly increased uptake of SNEC by peripheral blood granulocytes and monocytes, as compared to RA patients and healthy individuals. In addition, increased SNEC-phagocytosis was also shown to characterize several SS patients, in a manner and magnitude comparable to that observed in those with lupus. Further studies are needed to address the specific inflammatory consequences of massive phagocytic ingestion of SNEC in SS patients, as well as the efficiency of intracellular DNA-degrading enzymes such as DNase II and III, in patients’ phagocytes. Nevertheless, the detection of aberrant DNase1 activity and SNEC-phagocytosis in the blood of patients with SS and SLE conforms well to the several immunologic phenomena shared between these two disorders \cite{19}.

The cause of increased uptake of SNEC by blood-borne phagocytes of both SS and SLE patients was found to reside in the hyperfunctional activity of phagocytes, as well as in serologic abnormalities. Patients’ phagocytes were shown to manifest enhanced capacity for SNEC uptake and further studies are required to define whether it represents an activation-induced phenomenon or an intrinsic aberration. On the other hand, two serologic abnormalities of SS and SLE patients, namely the impaired degrading activity of serum DNase1 and the increased SNEC-binding capacity of serum IgG, appear also to contribute significantly in the increased SNEC-phagocytosis observed. In comparative cross-admixture assays, the treatment of SNEC with sera from SS and SLE patients (but not from HBD or RA) resulted in the induction of significant SNEC-phagocytosis by healthy monocytes. Most remarkably, the enzymatic activity of DNase1 in the serum of patients and controls was found to be inversely and linearly related to the eventual uptake of SNEC by blood-borne phagocytes. In fact, serum DNase1 activity appears to hold a key physiologic role in the prevention of SNEC-phagocytosis, as demonstrated by DNase1 inhibition in healthy sera. Thus, the impaired DNase1 activity in SS and SLE sera appears to be critically involved in the induction of SNEC-phagocytosis by allowing the encounter of phagocytes with the necrotic debris and their subsequent uptake. Furthermore, the positive correlation between DNase1 activity and the serum levels of C1q and C4 also underscores the role of complement system and other serum factors in the degradation of DNA and necrotic debris. The aberrant degradation of DNA and SNEC may have profound inflammatory and immunogenic consequences not only by fostering the exposure of the immune system to necrotic cells remnants, but also by allowing the pro-inflammatory ingestion of SNEC by phagocytes and the subsequent stimulation of intracellular sensors of DNA, RNA-associated nucleoproteins and alarmins \cite{8, 11, 40}. In fact, the activation of the proinflammatory type-I interferon pathway in SLE and SS has been thought to involve the internalization of immune complexes carrying nuclear autoantigens \cite{40, 41}.

Besides the impaired DNase1 activity, our data demonstrate that the SNEC phagocytosis-inducing effect of SS and SLE sera also largely owes to their content of IgG anti-SNEC autoantibodies. Serum IgG preparations from SS and SLE patients displayed increased aptitude to enhance SNEC-phagocytosis by healthy monocytes both under serum-free conditions, as well as when
added in the whole blood from healthy donors. In fact, IgG anti-SNEC antibodies were found to enhance the phagocytic uptake of necrotic cell debris not only via opsonization, but also through protection from enzymic digestion by serum. Although, the parallel occurrence of inhibitory anti-DNase1 antibodies cannot be excluded, our experiments indicate that the IgG of SS and SLE patients, by virtue of binding to SNEC, induce bulky SNEC aggregates and "shields" them from degradation by serum DNase1. In a similar fashion, anti-NET antibodies in the sera of SLE patients have been recently also shown to avert the enzymatic digestion of NETs [15]. Among SS and SLE patients, serum anti-nuclear autoantibodies may be reasonably expected to play such counter-degradation role by binding to necrotic cell remnants [11,15]. In fact, in similar previous studies, the occurrence of anti-dsDNA antibodies in sera from SLE patients has been reported to correlate with the induction of SNEC-phagocytosis by healthy phagocytes [11]. Our cross-admixture SNEC-phagocytosis experiments with lupus sera have essentially confirmed such correlation with the presence of anti-dsDNA, and to lesser extent that of anti-histone and anti-chromatin antibodies. In a similar manner, significantly increased uptake of SNEC by healthy monocytes was observed using sera of SS patients that were positive for anti-Ro/SSA and anti-La/SSB autoantibodies. In this context, we are currently conducting autoantibody depletion and antigen inhibition experiments to delineate the nature and role of particular IgG and IgM autoantibody specificities in the clearance of SNEC.

In conclusion, this study provides evidence that in a manner similar and comparable to SLE, SS patients are characterized by...
increased phagocytic uptake of SNEC in the peripheral blood that largely owes to the deficient degradation of necrotic debris by serum DNAse1. Importantly, these aberrations were found to correlate with clinical indices of disease activity and/or severity of SS and SLE, and thus may provide novel biomarkers for these disorders. Furthermore, our data indicate that blood-borne monocytes and granulocytes of SS and SLE patients manifest phagocytic hyperactivity for SNEC, whereas the occurrence of serum IgG anti-SNEC antibodies may have crucial immunomodulatory role through interference against degradation and opsonization (Table 3). Altogether, our data indicate that, upon cell necrosis the patients with SS and SLE are confronted with the pathophysiologic consequences of the aberrant exposure of their immune system to the necrotic debris and its massive uptake by phagocytes. Such aberrations may represent major causes of inflammatory and autoimmune reactions and thus, may hold key roles in the pathogenesis of the above disorders.

Conflict of interest
The authors declare no conflict of interest.

Acknowledgments
Authors are grateful to Drs Luiz Muñoz and Martin Herrmann at the University of Erlangen-Nuremberg, Germany, for valued help in establishing the SNEC-phagocytosis assay. The study was supported by grants from the Hellenic Society for Rheumatology (ERE141/10 to M.N.M), the Propondis Foundation (Athens, Greece, to A.G.V.) and the Lillian Voudouri Foundation (Ref No: 54/98).

Appendix A. Supplementary data
Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.jaut.2014.08.004.

References


