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Neutrophil Extracellular Traps That Are Not Degraded in Systemic Lupus Erythematosus Activate Complement Exacerbating the Disease

Jonatan Leffler,* Myriam Martin,* Birgitta Gullstrand,[†] Helena Tydén,[‡] Christian Lood,[‡] Lennart Truedsson,[†] Anders A. Bengtsson,[‡] and Anna M. Blom*

Ongoing inflammation including activation of the complement system is a hallmark of systemic lupus erythematosus (SLE). Antimicrobial neutrophil extracellular traps (NETs) are composed of secreted chromatin that may act as a source of autoantigens typical for SLE. In this study, we investigated how complement interacts with NETs and how NET degradation is affected by complement in SLE patients. We found that sera from a subset of patients with active SLE had a reduced ability to degrade in vitro-generated NETs, which was mostly restored when these patients were in remission. Patients that failed to degrade NETs had a more active disease and they also displayed lower levels of complement proteins C4 and C3 in blood. We discovered that NETs activated complement in vitro and that deposited C1q inhibited NET degradation including a direct inhibition of DNase-I by C1q. Complement deposition on NETs may facilitate autoantibody production, and indeed, Abs against NETs and NET epitopes were more pronounced in patients with impaired ability to degrade NETs. NET-bound autoantibodies inhibited degradation but also further increased C1q deposition, potentially exacerbating the disease. Thus, NETs are a potent complement activator, and this interaction may play an important role in SLE. Targeting complement with inhibitors or by removing complement activators such as NETs could be beneficial for patients with SLE. *The Journal of Immunology*, 2012, 188: 3522–3531.

Systemic lupus erythematosus (SLE) is a complex multifactorial autoimmune disorder caused by as yet not fully elucidated mechanisms. The incidence has been constant for the past decades with around 4–5 people affected per 100,000 each year and with the disease being more common in women than men (1, 2). Autoantibodies found in SLE patients indicate that the disease is “antigen driven” (3, 4) and are often directed against nuclear Ags such as dsDNA and histones. The main source of Ags has been suggested to be apoptotic/necrotic material that patients fail to dispose, but the actual cause of autoantibody production is still controversial. Recently, a new Ag source candidate has been identified by the discovery of neutrophil extracellular traps (NETs) (5), a mechanism used by neutrophils to

capture and destroy pathogens. NETs consist of chromatin covered with antimicrobial enzymes and can be secreted from activated neutrophils. Autoantibodies may be formed due to increased levels of Ags as a result of impaired degradation or clearance of antigenic material (6). Lower threshold for B cell activation has also been proposed as shown in one SLE mouse model (7). Others suggest that a lack of anti-inflammatory signals generated during clearance might be the cause and not the uncleared cells themselves (8). Numerous genes, mainly encoding proteins involved in inflammatory pathways, including complement, have been found to predispose to SLE (9).

Defects in the classical complement pathway are strongly associated with SLE, probably due to its ability to opsonize apoptotic material (10, 11). Additionally, the classical pathway is also associated with immune-modulatory effects, mainly focusing on induction of B cell anergy (12) (for a review, see Ref. 13) but also with inhibition of IFN- α production by plasmacytoid dendritic cells (14). Complement itself is a proinflammatory cascade due to the generation of anaphylatoxins. Inhibition of complement and especially C5a was shown to be a promising strategy in model diseases such as collagen-induced arthritis (15), making complement an interesting target for other autoimmune disorders such as SLE.

Chromatin released into circulation is normally degraded by serum endonucleases, such as DNase-I. Previous studies have shown a decreased activity of serum DNase-I in patients with SLE (16, 17). Rare mutations in the enzyme have additionally been described (18), but increased serum levels of DNase-I inhibitors, such as G-actin, might also explain decreased activity (19). Mice lacking DNase-I develop SLE-like symptoms (20). The strategy of treating SLE patients with DNase-I originated in the 1960s (21) followed by similar studies but without clear results (22). This could be attributed to presence of DNase-I inhibitors such as G-actin in serum. Application of G-actin-resistant DNase in a mouse model for SLE has showed promising results (23). Some complement proteins such as C1q (24) and C4b-binding protein

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Abbreviations used in this article: ACR, American College of Rheumatology; AF, Alexa Fluor; C4BP, C4b-binding protein; DGVB++, dextrose gelatine veronal buffer + Mg²⁺ and Ca²⁺; Hi-NHS, heat-inactivated normal human serum; MNase, micrococcal nuclease; NET, neutrophil extracellular trap; NHS, normal human serum; rhDNase-I, recombinant human DNase-I; SLE, systemic lupus erythematosus; SLEDAI, SLE disease activity index.

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(C4BP) (25) bind nuclear structures including DNA and histones. These interactions could potentially have an effect in degradation of DNA and chromatin.

It has been shown recently that sera from patients with SLE degrade NETs less efficiently than that by sera from healthy controls (26). This attenuated degradation correlated with occurrence of Abs that could bind to NETs and with lupus nephritis. In the current study, we confirmed impaired NET degradation in sera from SLE patients using an independent patient cohort. We further discovered that the decreased NET degradation is not a constant characteristic of a given patient but changes with disease activity. Furthermore, we revealed that sera from SLE patients, which are not able to degrade NETs, showed signs of increased complement consumption, and we found that NETs activate the classical complement pathway due to interaction with C1q, and thus consumed complement. Decreased NET degradation and increased complement activation were related to presence of NET-specific autoantibodies. These antibodies could prevent degradation of NETs at high concentrations but could additionally recruit and affect the effects of C1q on NET. Furthermore, we showed that complement C1q effectively inhibits NET degradation, giving complement a potential new role in the pathogenesis of SLE.

Materials and Methods

Sera, proteins, and Abs

The 94 SLE patients included in the study were recruited from 1985 to 2005 at the Department of Rheumatology, Lund University (Lund, Sweden). There were 9 men and 85 women in the study, all fulfilling four or more American College of Rheumatology (ACR) classification criteria (27). Disease activity was evaluated using SLEDAI-2K (28). For each patient, the time point of highest SLE Disease Activity Index (SLEDAI) score was identified, and this time point was defined as "flare." SLEDAI score varied between 2 and 28 at flare. We studied the patients at two time points: one point at flare and another point at lower clinical disease activity compared with that of the flare, here defined as remission. Routine laboratory tests were used to assess disease activity including concentration of complement components (C3, C4, and C1q) and presence of Abs against dsDNA, C1q, cardiolipin, SSA, and SSB. Sera from 54 healthy individuals were included as controls.

Sera from patients in remission or in flare were collected and stored at -80°C until tested. Most renal biopsies (for clinical classification) were taken at the time of sampling. All patients gave written consent to the study, which was approved by the Regional Ethical Review Board in Lund according to the Declaration of Helsinki.

Normal human serum (NHS) was prepared and pooled from blood from healthy individuals as previously described (29) and approved by the ethical committee of Lund University. C1q-deficient serum and control serum were both purchased from Quidel. C1q (30) and C4BP (31) were purified from human serum. Bovine DNase-I (Sigma-Aldrich), recombinant human DNase-I (rhDNase-I; Bioworld), micrococcal nuclease (MNase; Worthington Biochemical), rabbit skeletal G-actin (Sigma-Aldrich), and histones 1–4 (Roche Diagnostics) were all from commercial sources.

The following Abs were used: rabbit anti-human C1q (Dako), rabbit anti-human C1q F(ab')₂ fragments (homemade), rabbit anti-human C3d (Dako), mouse anti-human C5a (Hycult), allophycocyanin-labeled mouse anti-human CD16 (Immunotools), goat anti-human IgG–Alexa Fluor (AF) 647 (Invitrogen), goat anti-rabbit IgG–AF647 (Invitrogen), goat anti-rabbit IgG–AF488 (Invitrogen), HRP-conjugated goat anti-rabbit IgG (Dako) and goat anti-mouse IgG (Dako), and alkaline phosphatase-conjugated goat anti-human IgG F(ab')₂ fragments (Sigma-Aldrich). Antibodies from patient or control serum were purified using a Hi-Trap protein G column (GE Healthcare) according to the manufacturer's instructions.

Neutrophil isolation

For neutrophil purification, blood was collected from healthy individuals using NH Vacutainer (Becton Dickinson) tubes followed by separation by centrifugation on Histopaque-1119 (Sigma-Aldrich) according to Ref. 32. The neutrophil-rich phase was collected, washed with 0.5% human serum albumin (Sigma-Aldrich) in PBS, and separated from erythrocytes on a 65–85% Percoll gradient (GE Healthcare). Neutrophils were then collected from the 70–75% layer, washed, and resuspended in phenol red-deficient RPMI-1640 (Invitrogen) supplemented with 10 mM HEPES. Purity of

neutrophils (>70%) was determined with a CyFlow Space flow cytometer (Partec) using staining with anti-CD16.

NET degradation assay

Neutrophils (50,000 per sample) were stimulated to induce NETs using 20 nM PMA (Sigma-Aldrich) in RPMI-1640 medium for 4 h at 37°C and 5% CO_2 . Under these conditions, the majority (>90%) of neutrophils produced NETs as judged by confocal microscopy. After induction, NETs were incubated with DNase-I and/or human serum in 10 mM Tris-HCl pH 7.5, 10 mM MgCl_2 , 2 mM CaCl_2 , and 50 mM NaCl (DNase buffer) for 45–60 min at 37°C . For NET degradation in patient serum, 10% serum was incubated for 60 min. For DNase-I reconstitution assay, 5% serum with 2 mU rhDNase-I was incubated for 60 min. Degraded NETs were, during this time, released into solution. The solution was, after incubation, transferred to a new plate, and EDTA was added to a final concentration of 2 mM. The DNA content in the solution was quantified using PicoGreen (Invitrogen). We ascertained that amounts of DNA measured were within linear range of the assay. In some experiments, an intra-assay control was used to minimize variations due to neutrophil preparations, exact cell number, and so forth; for that, NETs were incubated with 50 mU MNase for 10 min at 37°C in DNase buffer to assess the quantity of NETs generated for each experiment. NET degradation in those assays is given as the ratio to the MNase standard. Unstimulated cells generated no signal in the PicoGreen assay upon incubation with serum. For complement assays, complement components were added either before (followed by washing) or together with serum. Inhibiting or purified antibodies were added directly to serum. For IgG predeposition, IgG was deposited from 2% patient sera diluted in PBS with 4 mM EDTA followed by washing with PBS before addition of 3% NHS with or without 20 $\mu\text{g}/\text{ml}$ C1q. DNA content was measured in solution after incubation with patient sera to confirm that NETs were not degraded during this step. NET-bound IgG was also quantified as described later to confirm deposition.

Type I IFN quantification

Type I IFN activity was measured as described previously (33). Briefly, WISH cells (CCL-25; American Type Culture Collection) were cultured for 6 h with patient sera after which lysis mixture (Panomics) was added. The cell lysates were analyzed on a Luminex 100 (Luminex Corporation) for mRNA expression of three housekeeping genes (GAPDH, PPIB, and B2M) and six type I IFN-regulated genes (LY6E, MX1, OAS1, ISG15, IFIT1, and EIF2AK2) using the QuantigenePlex 2.0 assay as described by the manufacturer (Panomics). The IFN score was calculated as the relative type I IFN expression compared with WISH cells cultured with medium.

Complement deposition assay

NETs were induced as described (in the section *NET degradation assay*) on optical bottom microtiter plates (Nunc) coated with poly-D-lysine (Sigma-Aldrich), washed in 2.5 mM veronal buffer with 0.1% gelatin, 1 mM MgCl_2 , 0.15 mM CaCl_2 , and 2.5% D-glucose dextrose gelatine veronal buffer + Mg^{2+} and Ca^{2+} (DGVB++). NHS diluted to 0.2–10% in DGVB++ was then incubated with NETs for 40 min at 37°C . For isolated proteins, C1q or AF488-labeled C4BP were incubated with NETs in 10 mM HEPES pH 7.4, 150 mM NaCl, 5 mM KCl, 1 mM MgCl_2 , and 2 mM CaCl_2 . For NETs preincubated with IgG, IgG was deposited from 2% patient serum in PBS with 4 mM EDTA before washing with PBS and addition of 0.2% NHS as above. After incubation with serum, NETs were washed, and complement was detected using either rabbit anti-C1q or rabbit anti-C3d with AF488- or AF647-labeled goat anti-rabbit secondary Ab. NETs were visualized using either propidium iodide (Sigma-Aldrich) or SytoxGreen and analyzed as for IgG deposition.

Hemolytic assay

NETs were induced as for the NET degradation assay and treated with 0.1 U/sample DNase-I for 30 min at 37°C followed by incubation with DGVB++ for 20 min and addition of NHS for additional 20 min. The supernatant was then added to amboceptor (Dade Behring) coated sheep erythrocytes in DGVB++ and incubated for 1 h at 37°C . Erythrocytes were removed by centrifugation for 3 min at $800 \times g$, and free hemoglobin was measured in supernatants at 405 nm in a Cary50 Bio UV spectrophotometer connected to a 50MPR microplate reader (Varian).

C5a generation assay

Samples were generated as for the hemolytic assay. NETs, DNase-degraded NETs, or DNase alone were incubated with 10% NHS in DGVB++ for 30 min at 37°C , and thereafter complement activation was stopped by addition of 10 mM EDTA. Samples were then frozen and later subjected to SDS-

PAGE and blotted onto a polyvinyl difluoride membrane where C5a was detected using mouse anti-C5a, HRP-conjugated goat anti-mouse and quantified using ImageGauge software (Fujifilm).

DNase-I activity

pStalker plasmid vector (a generous gift from Dr. Brad Spiller, Cardiff University, U.K.) purified using Plasmid Midi Kit (Qiagen) was mixed in duplicate with either C1q or G-actin in 2 mM CaCl₂ and 5 mM MgCl₂ with added PicoGreen. DNase-I (2 mU) was added to one of the duplicates, and PicoGreen fluorescence was measured over time at 37°C in a Victor² multilabel counter (Wallac). Samples without added DNase-I were used as a reference.

C1q-DNase-I binding ELISA

rhDNase-I was coated onto Maxisorp microtiter plates (Nunc) in 75 mM Na₂CO₃ pH 9.6, blocked with washing solution 50 mM Tris-HCl, 150 mM NaCl, 2 mM CaCl₂, and 0.1% Tween 20 supplemented with 3% fish gelatin (Quench), incubated with C1q in 10 mM HEPES pH 7.4, 150 mM NaCl, 5 mM KCl, 1 mM MgCl₂, and 2 mM CaCl₂, washed with washing solution, incubated with rabbit anti-C1q, washed, incubated with HRP-conjugated goat anti-rabbit, and developed with OPD tablets (Dako) according to the manufacturer's instructions and analyzed for absorbance at 490 nm in a Cary50 Bio UV spectrometer connected to a 50MPR microplate reader (Varian).

Anti-histone ELISA

A mixture of histones 1–4 was coated onto Maxisorp microtiter plates (Nunc) in PBS pH 7.2, blocked with 1% BSA in PBS, incubated with patient sera in PBS-Tween 20 with 0.1% BSA, washed with PBS-Tween 20, incubated with alkaline phosphatase-conjugated goat anti-human IgG (ab')₂ fragments, washed and developed with disodium-*p*-nitrophenyl phosphate (Sigma-Aldrich) according to instructions, and analyzed for absorbance at 405 nm in a Multiskan Plus (Labsystems).

Anti-NET IgG deposition

NETs were induced as for the complement deposition assay but washed in PBS and incubated with 2% serum in PBS supplemented with 4 mM EDTA to prevent degradation. NETs were washed, blocked with 1% BSA, incubated with AF647-labeled goat anti-human IgG, washed, and then stained with SytoxGreen (Invitrogen). The ratio of IgG to NETs was measured using an Infinite M200 (Tecan) multimode reader. SytoxGreen solution was then removed before addition of fluorescent mounting media (Dako) for confirmation of IgG deposition in an LSM 510 Meta Confocal microscope (Zeiss).

Statistical analysis

Patient data were analyzed using JMP 7 software (SAS) using Wilcoxon rank sum test to calculate significance of differences observed between two groups of continuous patient data and Kruskal-Wallis test followed by Dunn's multiple comparison test when more than two groups were analyzed. For nominal data, the χ^2 test was used. For experimental assays, Prism5 (GraphPad) was used, and significance of differences was calculated using ANOVA followed by Bonferroni posttest for experiments with more than one parameter or group. For experiments with two groups and one parameter, unpaired *t* test was used. Correlation was calculated according to Spearman.

Results

Sera from SLE patients degrade NETs less efficiently compared with that by sera from healthy controls

In the current study, we analyzed serum samples obtained from 94 SLE patients. The mean age of the patients was 42 y (range, 14–75 y), and 90% of the patients were women. The patient characteristics and classification criteria according to ACR describe the clinical phenotypes of the patients. These are summarized in Table I and were typical for a SLE patient cohort. Sera from the patients were taken at two time points, of which the one with higher disease activity was referred to as flare, and the one with lower disease activity was referred to as remission. To analyze whether NET-degrading ability in SLE patients is a characteristic of each patient or changes with disease activity, NET degradation *in vitro* was determined in sera taken from the same SLE patient both in

Table I. Patient characteristics according to ACR criteria

ACR Criteria	Patient Number (%)
Malar rash	62 (66.0)
Discoid rash	28 (29.8)
Photosensitivity	60 (63.8)
Oral ulcer	29 (30.9)
Arthritis	78 (83.0)
Serositis	53 (56.4)
Nephritis	43 (45.7)
Neurological disorder	8 (8.5)
Hematological disorder	53 (56.4)
Immunological disorder	71 (75.5)
Antinuclear Abs	92 (97.9)

remission and during flare and compared with that of sera from healthy controls (Fig. 1A). All sera were compared with degradation by an MNase as an intra-assay control. To distinguish between “low-degrading” and “high-degrading” patient sera, a cutoff was set below 3 SDs of the mean value for the healthy controls. Almost one-third (29%) of the patient sera taken during SLE flare failed to degrade NETs and were hence low-degrading. Only 12% of the sera taken during remission failed to degrade NETs. All but two patients that had low-degrading sera in remission also had low-degrading sera during flare. The majority of low-degrading sera in flare were high-degrading in remission (Fig. 1B). This indicates that the ability to degrade NETs is not constant for a particular individual and varies with the disease activity. DNase-I is responsible for NET degradation in serum, and upon addition of rhDNase-I, the activity of all low-degrading sera could be increased, although these did not completely reach the levels of those of the healthy controls (Fig. 1C). In the following experiments, associations of ability to degrade NETs with disease activity markers were analyzed for patients in flare, unless stated otherwise. Patients with low-degrading sera generally had a more active disease when assessed with the SLEDAI (Fig. 1D), which describes the clinical manifestations at the time point of sample collection. The correlation coefficient between the SLEDAI score and NET degradation was calculated to -0.51 according to Spearman. The SLEDAI score mostly increased for each patient between remission and flare but in some exceptional cases remained constant (Fig. 1E). According to SLEDAI score, kidney involvement, especially SLE nephritis type IV, as well as pleuritis was strongly associated with low NET degradation (Table II). Low-degrading sera from patients with a flare also more often had low complement levels and expression of anti-dsDNA Abs compared with high-degrading sera from patients with a flare. Arthritis and rash, manifestations considered as a milder form of SLE, were seen less frequently in patients with low-degrading sera. This illustrates the association between decreased NET degradation and a more severe disease. Medications differed between patients, but no correlation with NET-degrading ability and different therapies was observed (Table III).

Increased type I IFN activity in low-degrading sera

Type I IFNs have been shown to prime neutrophils to produce NETs more easily. Furthermore, Ab-coated NETs have also been shown to be potent inducers of IFN- α production by plasmacytoid dendritic cells (34, 35). We therefore measured the type I IFN activity in the patient sera (Fig. 2). Patients with low-degrading sera showed a significantly higher type I IFN activity in their sera compared with that of patients with high-degrading sera. Thus, the impaired degradation of NETs might be an important mechanism for the ongoing production of type I IFNs in SLE patients.

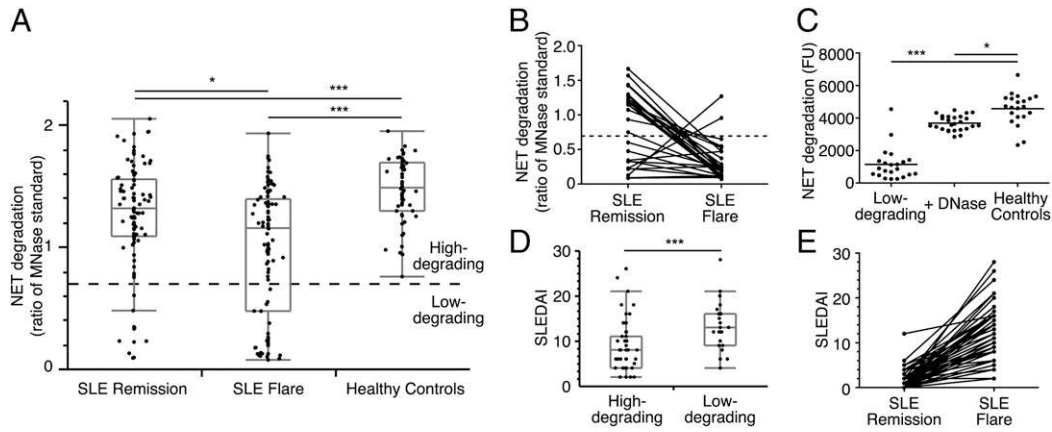


FIGURE 1. A subset of SLE patients fails to degrade NETs. **(A)** NETs were incubated with 10% serum from SLE patients ($n = 94$) at both in remission and in flare and from healthy controls ($n = 54$). Degraded NETs for each sample were quantified by PicoGreen and compared with the MNase intra-assay standard included on each plate to minimize assay variability. At both states, a subset of SLE patient sera degraded NETs less efficiently compared with that by sera from healthy controls, but the subset was larger at flare compared with remission. Patient sera degrading NETs below 3 SDs from the mean of healthy controls were defined as “low-degrading.” **(B)** NET degradation for patients with low-degrading sera connected for remission and flare. Most patients with low-degrading sera in remission still could not degrade NETs in flare. Two low-degrading sera in remission gained degrading abilities during flare. **(C)** Low-degrading sera (5%) in flare ($n = 22$) were spiked with 2 mM rhDNase-I ($n = 26$), which greatly increased degradation, although not completely to the level of controls ($n = 21$). **(D and E)** SLEDAI score was compared for low-degrading ($n = 27$) and high-degrading ($n = 67$) sera during flare (D) and between remission and flare (E). Patients with low-degrading sera had higher SLEDAI values compared with those of high degraders. Generally, SLEDAI value increased going from remission to flare. Significance of differences between the groups was calculated using Kruskal–Wallis test followed by Dunn’s multiple comparison test for (A) and (C) and Wilcoxon rank sum test in (D). * $p < 0.05$, *** $p < 0.001$.

NETs activate the complement system

Complement is a potent inducer of inflammation and plays a major role in the inflammatory processes in SLE. To investigate whether there was a difference in complement activity and consumption between patients with a flare and either high- or low-degrading sera, levels of serum C1q, C4, and C3 were measured. There was no significant difference of C1q levels in high- and low-degrading sera (Fig. 3A), whereas C4 and C3 levels (Fig. 3B, 3C) were markedly

reduced in low-degrading sera compared with those in high-degrading sera. This indicates ongoing activation of the classical or lectin pathway in these sera, which consumes C4 and C3.

C1q initiates classical pathway activation, and DNA is one of its ligands (24). To confirm that C1q binds to NETs and thereby activates complement, NETs were incubated with purified C1q and analyzed by confocal microscopy. Clear binding of C1q to NETs was observed (Fig. 4A), whereas no binding of the soluble

Table II. SLEDAI-qualifying symptoms during the time of sample collection for patients with SLE flare

Symptom	High Degrading ($n = 67$); Number (%)	Low Degrading ($n = 27$); Number (%)	p Value (Pearson)	SLEDAI Score
Seizure	1 (1.5)	0 (0)	0.5	8
Psychosis	0 (0)	0 (0)	—	8
Organic brain syndrome	0 (0)	0 (0)	—	8
Visual disturbance	5 (7.5)	2 (7.5)	0.99	8
Lupus headache	2 (3)	0 (0)	0.4	8
Cerebrovascular accident	1 (1.5)	1 (3.5)	0.5	8
Vasculitis	8 (12)	3 (11)	0.9	8
Arthritis	23 (34)	2 (7.5)	0.008*	4
Myositis	2 (3)	0 (0)	0.4	4
Glomerulonephritis	16 (24)	16 (59)	0.001*	4–16
Type II ^a (biopsy)	2 (3)	2 (8)	0.3	—
Type III ^a (biopsy)	0 (0)	1 (4)	0.1	—
Type IV ^a (biopsy)	8 (13)	9 (36)	0.01*	—
Type V ^a (biopsy)	2 (3)	1 (4)	0.8	—
Rash	25 (37)	4 (15)	0.03*	2
Alopecia	6 (9)	1 (3.7)	0.4	2
Mucosal ulcers	5 (7.5)	2 (7.5)	0.99	2
Pleuritis	6 (9)	7 (26)	0.03*	2
Pericarditis	4 (6)	3 (11)	0.4	2
Low complement	30 (45)	22 (81)	0.001*	2
dsDNA Abs	20 (30)	25 (93)	<0.0001*	2
Fever	2 (3)	3 (11)	0.1	1
Thrombocytopenia ^b	3 (5)	0 (0)	0.3	1
Leukopenia ^b	6 (9)	4 (15)	0.4	1

*Significant difference with the increased proportion in boldface.

^aDefined according to the World Health Organization criteria. Biopsy results were not available for six patients of whom four had high-degrading and two low-degrading sera.

^bData were not available for four patients of whom three had high-degrading and one low-degrading sera.

Table III. Treatment received by patients in flare stratified according to NET-degrading ability at the time of sample collection

Treatment Received	High Degrading (n = 67); Number (%)	Low Degrading (n = 27); Number (%)	p Value (Pearson)
Antimalaria treatment	24 ^a (35.8)	11 (40.7)	0.20
Hydroxychloroquine	19 (28.4)	11 (40.7)	0.25
Chloroquine phosphate	6 (9.0)	0 (0)	0.11
Any prednisolone	44 (65.7)	20 (76.9)	0.29
1–5 mg	11 (16.7)	1 (3.7)	0.09
6–10 mg	14 (20.9)	8 (29.6)	0.37
11–15 mg	5 (7.5)	4 (15.4)	0.25
16–20 mg	4 (6.0)	3 (11.1)	0.39
21–30 mg	5 (7.5)	2 (7.4)	0.99
31–40 mg	5 (7.5)	2 (7.4)	0.99
Immunosuppressants	26 (38.8)	12 (44.4)	0.61
Rituximab	—	—	—
Cyclophosphamide	3 (4.5)	2 (7.4)	0.57
Mycophenolate mofetil	5 (7.5)	2 (7.4)	0.99
Azathioprine	12 (17.9)	6 (22.2)	0.63
Cyclosporin A	4 (6.0)	2 (7.4)	0.80
Methotrexate	2 (3.0)	0 (0)	0.36

The p values are calculated by Pearson's χ^2 test. $p < 0.05$ is considered significant.

^aOne patient received both treatments.

classical pathway inhibitor C4BP could be detected (Fig. 4B). Complement activation on NETs should result in deposition of C3b. To confirm this, NETs were incubated with NHS or heat-inactivated normal human serum (Hi-NHS), and an Ab against C3d, an epitope available on C3b, was used to detect bound C3b. C3b was deposited on NETs (Fig. 4C), and C3b deposition was substantially lower using Hi-NHS (Fig. 4D). This indicates deposition of C3b through complement activation and not unspecific binding. Because patients that failed to degrade NETs had lower levels of complement in their sera, we investigated if NETs have the ability to consume complement in serum. Thus, NHS was incubated with NETs or DNase-I-treated NETs and then evaluated in a hemolytic assay. The DNase-I-treated sample was used to make sure that the neutrophil remains did not cause complement consumption but that the effect was DNA/NET specific. A significantly decreased hemolytic activity of serum incubated with NETs was observed in comparison with that of serum incubated with DNase-I-treated NETs (Fig. 4E). To confirm complement activation in this setting with a direct assay, C5a was detected with Western blot and quantified. An increase of C5a generation was seen in NHS incubated with NETs compared with that of NHS incubated with DNase I-treated NETs (Fig. 4F). This further indicates a strong complement-activating activity by NETs.

C1q interferes with NET and DNA degradation

Nondegraded NETs activated complement, but it was unclear whether deposited complement in turn affected the degradation of

NETs. We first confirmed that NET degradation was decreased in Hi-NHS (Fig. 5A). This loss of activity could be restored by addition of physiological concentrations of rhDNase-I and was therefore related to heat sensitivity of DNase-I and not lack of complement activation. Therefore, complement activation is not required for NET degradation, although complement proteins might still affect degradation. To assess if C1q affects degradation, NHS was spiked with additional C1q, which decreased NET degradation (Fig. 5B). Spiking NHS with C4BP did not affect degradation. The inhibiting effect of C1q could be reversed by addition of rhDNase-I (data not shown). Blocking of endogenous C1q with anti-C1q F(ab')₂ fragments (Fig. 5C) caused an increase of NET degradation, whereas addition of Abs against C3d had no effect. An inhibitory effect of C1q on NET degradation was also observed when C1q was deposited on NETs before addition of NHS (Fig. 5D). Inhibition was also seen when NETs were incubated with purified C1q and DNase-I in the absence of complete serum (data not shown). In an even simpler setup using a plasmid as a DNA source, degradation by DNase-I was followed over time. Degradation was inhibited in a concentration-dependent manner by increasing amounts of C1q and also by G-actin, a well-known inhibitor of DNase-I (36) (Fig. 5E). A microtiter plate-based binding assay showed direct binding of C1q to DNase-I (Fig. 5F). In comparison, no binding of C4BP to DNase-I could be detected (data not shown). Quench, a blocking agent containing fish gelatin, was used as a negative control. These combined results show that C1q inhibits NET degradation most likely due to its ability to bind to DNA and directly to inhibit DNase-I.

Autoantibodies against NETs are more common in low-degrading sera

NETs consist of Ags, which are commonly targeted by autoantibodies in SLE. Autoantibodies can affect complement, and complement can also increase the immunogenicity of targets due to its interaction with complement receptor 2 (CD21) on B cells (37). We therefore measured levels of Abs against the NET Ags, histones, and dsDNA in patients with a flare. Patients with low-degrading sera displayed significantly higher levels of Abs against dsDNA and histones (Fig. 6A, 6B). Notably, patients with low-degrading sera also displayed significantly higher levels of Abs against C1q (Fig. 6C). Furthermore, Abs against SSB were more common in patients with low-degrading sera (22.2%) compared with high-degrading

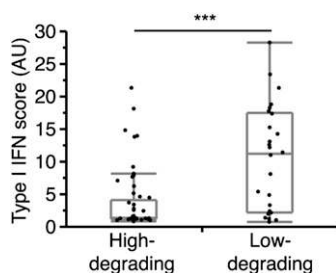
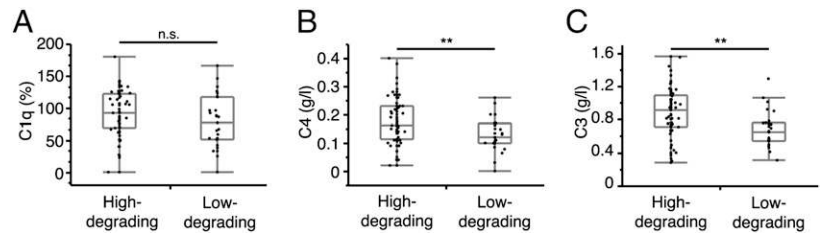


FIGURE 2. Low-degrading sera have more type I IFN activity. The type I IFN activity in sera from patients with high- and low-degrading sera was compared. Low-degrading sera showed an increase in activity compared with high-degrading sera. Significance of differences between the groups was calculated using Wilcoxon rank sum test. *** $p < 0.001$.

FIGURE 3. Complement is consumed in patients with low-degrading serum. Serum levels of complement factors C1q (A), C4 (B), and C3 (C) were measured in patient sera during flare. Both C4 and C3 levels were significantly decreased in low-degrading sera, whereas no difference in C1q levels was observed. Significance of differences between the groups was calculated using Wilcoxon rank sum test. ** $p < 0.01$.



sera (6.1%; $p = 0.02$). No difference was, however, observed for having Abs against SSA ($p = 0.21$) or cardiolipin ($p = 0.32$).

Levels of specific Abs against NETs were also determined by measuring IgG deposition from patient sera on NETs (Fig. 6D). Patients in flare had significantly higher levels of Abs against NETs compared with both those when they were in remission and those of healthy controls. Among the patients with flare, patients with low-degrading sera displayed higher levels of Abs against NETs compared with those of patients with high-degrading sera (Fig. 6E). These data were also confirmed by confocal microscopy (Fig. 6F). Among patients with flare and high (defined as 2 SDs above mean) IgG titers directed against NETs, all but five patients displayed anti-dsDNA Abs. Of these five, two had high titers of Abs directed either against histones or C1q; the remaining three had Abs toward other epitopes presented on NETs (data not shown).

Deposited autoantibodies affect complement activation and NET degradation

To investigate whether autoantibodies can inhibit NET degradation or whether they are a consequence of stimulation of the adaptive immune system by nondegraded NETs, sera from SLE patients were incubated with NETs in the presence of EDTA. EDTA prevents degradation by inhibiting the Ca^{2+} - and Mg^{2+} -dependent DNase-I while allowing autoantibody binding. C1q is known to bind Abs, and we therefore analyzed how the interaction affected NET degradation. Therefore, after washing the NETs with de-

posited autoantibodies, degradation by NHS or NHS spiked with C1q as well as IgG deposition were analyzed (Fig. 7A). NET degradation by NHS correlated negatively only weakly with the amounts of deposited IgGs (Fig. 7A). This indicates that IgG at high concentrations can affect degradation. However, NHS spiked with additional C1q generally showed a decreased degradation but interestingly also a stronger negative correlation to IgG deposition (Fig. 7A). This indicates that high concentrations of C1q might work in synergy with autoantibodies to prevent NET degradation. To confirm deposition of C1q on autoantibody-coated NETs, C1q was also measured in this setting (Fig. 7B). IgG-dependent C1q deposition was only evident from low concentrations (0.2%) of NHS where a strong correlation was observed (Fig. 7B). At a higher NHS concentration (3%), C1q deposited independently of autoantibodies (data not shown). C1q deposition was further confirmed by confocal microscopy (Fig. 7C). Antibodies only weakly correlated with decreased degradation (Fig. 7A), and to test if this effect was concentration dependent, Abs were purified from a low-degrading SLE patient serum and added to NHS (Fig. 7D). At high Ab concentrations, a decrease of NET degradation could be observed compared with that when the same amount of Abs from a healthy individual was added. NET-specific Abs can hence prevent NET degradation at high concentrations but also recruit complement to NETs at lower concentrations. To test if the inhibitory effect of purified Abs was C1q dependent, the Abs were added to C1q-deficient sera and to C1q-deficient sera reconstituted with C1q and assayed for NET degradation (Fig. 7E). No differ-

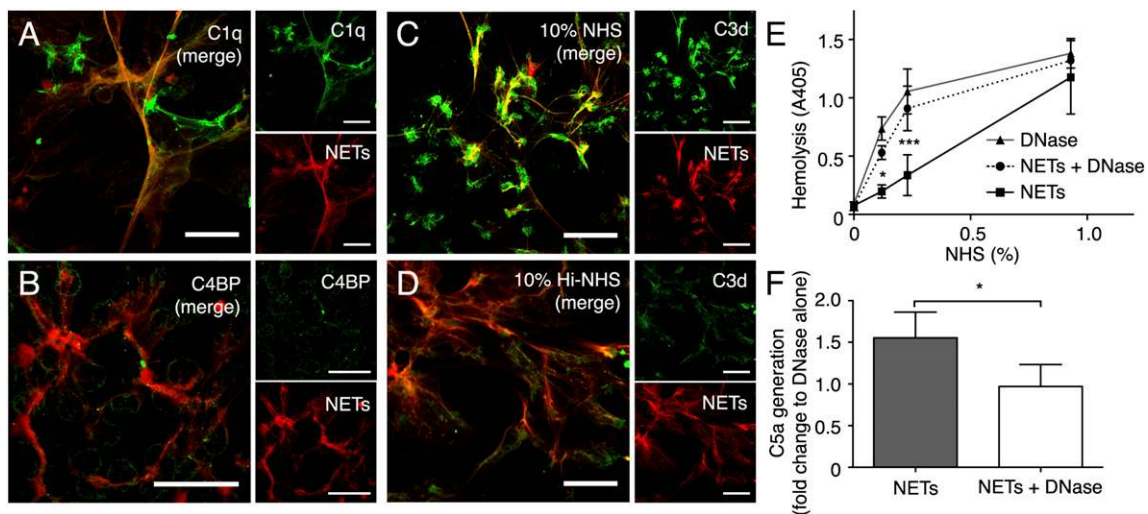


FIGURE 4. Complement becomes activated and consumed by components of NETs. (A and B) Purified C1q (green) (A) or AF488-labeled C4BP (green) (B) was incubated with NETs stained with propidium iodide (red). C1q was found to bind NETs, whereas no binding of C4BP to NETs was detected. (C) C3b deposited on NETs during incubation with 10% serum. (D) Little or no deposition of C3b from Hi-NHS (lacking active complement) could be observed. In (C) and (D), C3d is stained in green and NETs are stained in red with propidium iodide. (E) Serum preincubated with NETs showed less hemolytic activity in lysing sensitized sheep erythrocytes compared with that of serum preincubated with DNase-I-treated NETs or DNase-I-treated control. (F) Complement activation was confirmed in NHS incubated with NETs as judged by C5a detection by Western blot, which was quantified and compared with DNase-I-treated NETs and is displayed as fold change to DNase-I-treated control. In (A)–(D), representative images of three independent experiments are shown; for (E) and (F), data are presented as mean values of duplicates \pm SD; $n = 3$. Significance of differences was evaluated using two-way ANOVA with Bonferroni posttest for (E) and unpaired t test for (F). Scale bars, 50 μm . * $p < 0.05$, *** $p < 0.001$.

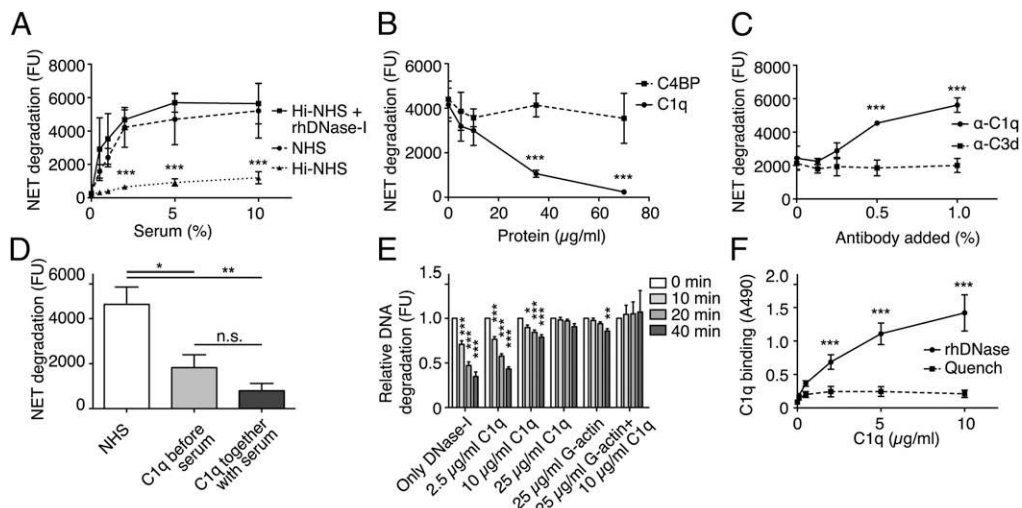


FIGURE 5. C1q binds to and affects degradation of NETs and DNA by DNase-I. (A) NETs were subjected to degradation by NHS, Hi-NHS, or Hi-NHS reconstituted with rhDNase-I in concentrations corresponding with serum. Degradation of NETs was decreased by heat inactivation but could be restored upon addition of DNase-I. (B) Addition of C1q to 1% NHS decreased NET degradation in a dose-dependent manner, whereas no effect was seen upon addition of C4BP. (C) Addition of anti-C1q F(ab')₂ fragments to 0.5% NHS increased NET degradation, whereas no effect was seen upon addition of an anti-C3d Ab. Both Ab solutions had similar IgG concentrations. (D) NETs were degraded by 1% NHS either after C1q incubation or in presence of C1q. Addition of C1q significantly inhibited degradation in both cases compared with NHS alone. No significant difference was detected on degradation between samples with C1q added followed by NHS and C1q added together with NHS. (E) The inhibiting effect of C1q was tested in a general DNA degradation system where DNase-I degrades plasmid DNA. DNA degradation was inhibited by addition of C1q or G-actin measured at several time points. Degradation is displayed as change in intact DNA at time point 0 min. (F) Direct binding of C1q to recombinant DNase-I was measured in an ELISA with clear concentration-dependent binding. NET degradation is displayed in fluorescent units (FU). Data are presented as mean values of duplicates [single values for (E)] ± SD; *n* = 3. Significance of differences was calculated using two-way ANOVA with Bonferroni posttest for all except (D) where one-way ANOVA followed by Bonferroni multiple comparison test was used. **p* < 0.05, ***p* < 0.01, ****p* < 0.001.

ences in degradation could be observed compared with that of control serum. Taken together, this indicates a complex and concentration-dependent interplay of C1q and NET-specific Abs in

binding to NETs where a local production of C1q could have a potentially detrimental effect on NET degradation and outcome for the patient.

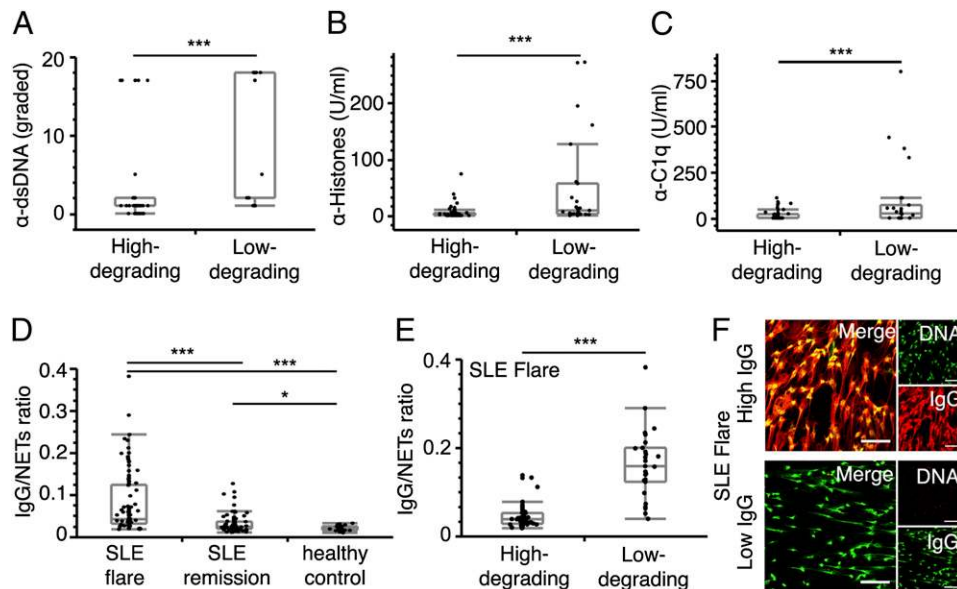


FIGURE 6. Low-degrading sera contain higher levels of autoantibodies compared with those of high-degrading sera. (A–C) Abs against dsDNA (A), histones (B), and C1q (C) were measured in high- and low-degrading sera from patients with flare (*n* = 94). Low-degrading sera had higher levels of Abs against both Ags. (D) IgG deposition on NETs from patient sera were measured and displayed as a ratio of IgG signal to the amounts of NETs in each sample. Patients with flare (*n* = 94) showed higher levels of NET Abs compared with those when in remission (*n* = 93) and with those in healthy controls (*n* = 29). (E) IgG deposition on NETs from patient sera with flare was divided into high and low degrading. Low-degrading sera showed significantly more IgG deposition on NETs compared with that of high-degrading sera. (F) Binding of NET Abs to NETs was confirmed by confocal microscopy. IgGs were stained with AF647 (red) and NETs visualized using SytoxGreen (green). Representative images are shown for high and low levels of IgG; scale bars, 100 µM. Significance of differences was calculated using Wilcoxon rank sum test in (A)–(C) and (E) and Kruskal–Wallis test followed by Dunn's multiple comparison in (D). **p* < 0.05, ****p* < 0.001.

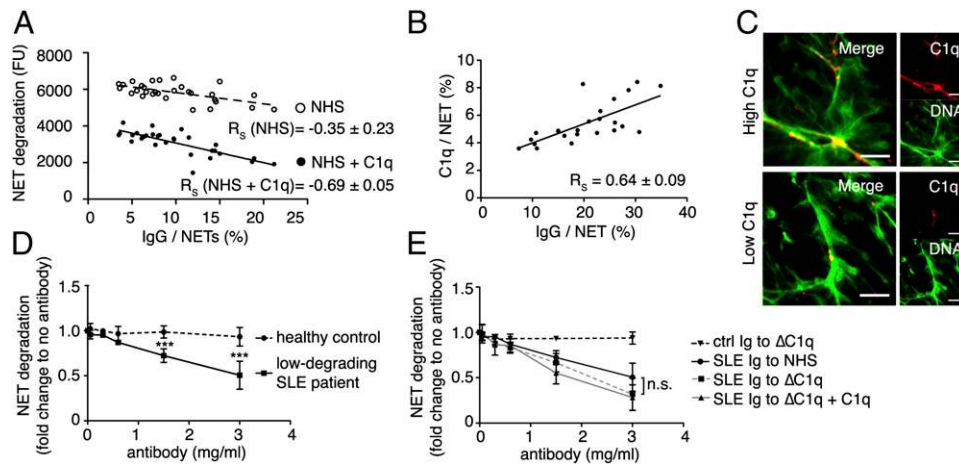


FIGURE 7. IgG deposition on NETs does not prevent degradation but causes more C1q deposition. **(A)** NETs were incubated with 2% patient sera ($n = 30$) containing high anti-NET IgG levels (defined as above 2 SDs of the mean anti-NET IgG titers) followed by degradation in 2% NHS or NHS with addition of 20 $\mu\text{g}/\text{ml}$ C1q. For NHS alone, weak negative correlation was observed for NET degradation and the amount of IgG deposited on the NETs. For NHS followed by C1q, a stronger negative correlation was observed. **(B and C)** NETs were incubated with sera containing high anti-NET IgG levels ($n = 24$) followed by 0.2% NHS and stained for C1q and IgG deposition. NETs were visualized with SytoxGreen. Deposition was displayed as ratio of C1q or IgG to NETs. C1q deposition correlated strongly with IgG deposition **(B)**. **(C)** C1q deposition was confirmed using confocal microscopy where C1q was visualized in red and NETs in green. **(D)** NETs were degraded by NHS incubated with increasing amounts of Abs purified from healthy control or low-degrading SLE patient serum. At high concentrations, Abs from the patient inhibited degradation of NETs compared with control Abs. **(E)** NETs were degraded in NHS, C1q-deficient sera, or C1q-deficient sera reconstituted with C1q. Purified Abs (Ig) were added to the different sera. No difference in inhibition by SLE Abs was observed between NHS and C1q-deficient serum. Data are presented as mean of two **(A)** or three **(B, D, E)** independent experiments \pm SD. For **(C)**, representative images are shown from three independent experiments with high (upper quartile) and low (intermediate quartiles) C1q deposition; scale bar, 50 μM . Correlations were calculated according to Spearman's test and are shown as mean \pm SD of two **(A)** or three **(B)** independent experiments. Significance of difference in **(D)** and **(E)** was calculated using two-way ANOVA followed by Bonferroni posttest. *** $p < 0.001$.

Discussion

In this study, we investigated how complement interacts with NETs and how these interactions could contribute to the pathogenesis of SLE. We showed in accordance with a recent study (26) that a substantial fraction of SLE patient sera failed to degrade NETs. This phenomenon appeared to be a variable characteristic of each patient, as failure to degrade NETs was more common during flare than when the same patients were in remission. It may be beneficial for individuals to block NET degradation in some circumstances, for example to enhance antimicrobial activity of NETs during infections. This hypothetical mechanism could explain observed variations in NET degradation. Variable serum concentrations of a DNase-I inhibitor or blocking antibodies are likely explanations. Inflammation has been suggested to lead to release of G-actin from damaged cells, which inhibits DNase-I. However, we were not able to detect G-actin in any of the patient sera we tested, possibly due to low assay sensitivity (data not shown). An efficient actin scavenging system exists in serum consisting of Gc-globulin, also known as vitamin D-binding protein (38). The system was previously shown to become saturated in liver disease (39) but has, to our knowledge, not been studied in human SLE.

In the recent report investigating NET degradation (26), addition of DNase-I to low-degrading sera restored NET degradation only in a subset of patients. In our cohort, NET degradation could be restored for all sera. The amount of DNase added might be an important difference because excessive degradation renders small NET fragments undetectable using the PicoGreen method, which was also used by Hakkim et al. (26). Another difference was that the initial study used bovine DNase-I, whereas we used rhDNase-I.

Sera from patients in flare that could not degrade NETs had lower levels of complement C4 and C3, which are typical signs of classical pathway activation and consumption. To confirm the

complement activation potential of NETs in vitro, we showed that complement interacted with NETs and that C1q bound well and C3b deposited on NETs. Notably, we did not detect binding of C4BP to NETs, and thus not every protein with a potential to bind DNA interacts with NETs.

Complement not only seemed to be activated by nondegraded NETs but also was shown to be directly involved in the degradation of NETs by DNase-I. C1q binds DNA (24) and was previously found to promote degradation of necrotic cell-derived chromatin (40). In our experimental setup, C1q inhibited degradation of NETs in both serum and in a system composed of purified C1q and DNase-I. The mechanism for this is still unclear but could be due either to the direct interaction between C1q and DNase-I, which we have demonstrated in the current study, or to steric hindrance. C1q is a large molecule of ~ 500 kDa, and its binding to NETs could potentially inhibit DNase-I access. Our data also confirmed that C1q bound to NETs did inhibit their degradation. We showed that the inhibitory effect of endogenous C1q could be reversed by addition of anti-C1q $\text{F}(\text{ab}')_2$ fragments. However, C1q-deficient serum did not degrade NETs more efficiently compared with that by NHS. This indicates that C1q does not inhibit degradation at physiological concentrations in serum, but the observed inhibition might be a phenomenon important at local sites of C1q production.

C1q deficiency, even though extremely rare, is one of the strongest risk factors for developing SLE, and patients with severe SLE sometimes have low levels of C1q due to C1q consumption. Thus, the inhibitory effect on NET degradation is puzzling. While opsonization of apoptotic cells and immune complexes as well as immune-modulating effects of C1q might be of great importance for preventing development of SLE, the inhibitory effects on degradation of NETs and DNA might be a trade-off for efficient opsonization to ensure non-inflammatory clearance. The effect

might be more important at local sites where NETs are cleared and where C1q can be directly produced by immune cells and its concentration exceeds that of serum.

Patients with low-degrading sera had more IgG specific for NETs. These Abs were previously suggested to inhibit NET degradation (26). When NETs were preincubated with sera from SLE patients to allow deposition of autoantibodies, prior to degradation by NHS, only a weak correlation with decreased degradation and IgG deposition was observed. When purified Abs from a low-degrading SLE patient serum were added to NHS, a decrease of degradation occurred at high Ab concentrations. Hence, the deposition probably did not generate high enough Ab concentrations for such inhibition. Alternatively, anti-DNA Abs have been described to cross-react with DNase-I, which could potentially explain inhibition by Abs in solution but not by deposited Abs (41).

Deposited IgG should recruit more C1q to NETs, but such effect was, however, only evident at low serum concentrations. Notably, the weak correlation between deposited IgG and NET degradation could be improved upon spiking with additional C1q during degradation. This shows that deposited Abs might recruit more C1q to NETs, which together with the Abs prevent NETs from being degraded. Abs directed against NETs could also independently prevent degradation at higher concentrations (Fig. 6E).

Several studies on the involvement of NETs in SLE have shown that antimicrobial peptides bound to NETs can form nondegradable complexes. These complexes were more readily found in patients with SLE and could also stimulate production of IFN- α by plasmacytoid dendritic cells (42). In our study, we could confirm that patients with low-degrading sera showed a higher type I IFN activity compared with that of patients with high-degrading sera. The increased type I IFN activity seen in the low-degrading sera could be due to impaired degradation of NETs. This may lead to phagocytosis of NETs in complexes with anti-NET Abs by plasmacytoid dendritic cells inducing production of type I IFNs (35). Furthermore, high levels of IFN- α could increase the susceptibility of neutrophils to produce NETs. This phenomenon has been observed by others (34, 43). In the latter study, the authors investigated a less mature form of neutrophils present in SLE patients that was more prone to generate NETs. They were also able to detect NETs in skin and kidneys from SLE patients. In other studies, Abs against ribonucleoprotein (35) and antimicrobial peptides (42) were shown to activate neutrophils in SLE patients but not in healthy controls. Taken together, these studies show that in SLE, both an imbalance in NET generation and clearance occur. Complement deficiencies correlate strongly with SLE; therefore, how NETs covered with complement components could affect their perception by the cellular immune system would be of particular future interest. Previous studies related to this topic showed that C1q had an inhibitory role for production of inflammatory cytokines such as IFN- α by plasmacytoid dendritic cells (14).

In conclusion, we showed that sera from a substantial subgroup of SLE patients failed to degrade NETs. NETs can activate complement, which in turn induces inflammation and modulates adaptive immune responses resulting in the production of NET-specific Abs. Decreased degradation of NETs led to deposition of complement and to deposition of autoantibodies, recruiting more complement and creating a vicious circle of decreased degradation and proinflammatory reactions. Removal of NETs by a DNase resistant to a potential inhibitor could break this circle with a positive outcome for the patient and warrants further investigation.

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Disclosures

The authors have no financial conflicts of interest.

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