Impairment of neutrophil extracellular trap degradation is associated with lupus nephritis

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Systemic lupus erythematosus (SLE) is an autoimmune disease in which patients develop autoantibodies to DNA, histones, and often to neutrophil proteins. These form immune complexes that are pathogenic and may cause lupus nephritis. In SLE patients, infections can initiate flares and are a major cause of mortality. Neutrophils respond to infections and release extracellular traps (NETs), which are antimicrobial and are made of DNA, histones, and neutrophil proteins. The timely removal of NETs may be crucial for tissue homeostasis to avoid presentation of self-antigens. We tested the hypothesis that SLE patients cannot clear NETs, contributing to the pathogenesis of lupus nephritis. Here we show that serum endonuclease DNase1 is essential for disassembly of NETs. Interestingly, a subset of SLE patients' sera degraded NETs poorly. Two mechanisms caused this impaired NET degradation: (*i*) the presence of DNase1 inhibitors or (*ii*) anti-NET antibodies prevented DNase1 access to NETs. Impairment of DNase1 function and failure to dismantle NETs correlated with kidney involvement. Hence, identification of SLE patients who cannot dismantle NETs might be a useful indicator of renal involvement. Moreover, NETs might represent a therapeutic target in SLE.

DNase1 | systemic lupus erythematosus | autoimmunity | innate immunity

Systemic lupus erythematosus (SLE, or lupus) is a life-threatening, chronic, and severe autoimmune disease that affects multiple tissues and organs (1). SLE patients produce antibodies (Abs) against self; mainly against chromatin (2) and often to neutrophil proteins like lactoferrin (3), myeloperoxidase, proteinase-3 (4), and elastase (5). Abs against these granular proteins are known as antineutrophil cytoplasmic antibodies (ANCA) (6). Antigens and Abs form immune complexes that can be either formed in situ or deposited in kidneys and contribute to the pathogenesis of lupus nephritis, a frequent and dangerous organ manifestation in SLE.

Infections can initiate flares and are a major cause of mortality in SLE patients (7, 8). Neutrophils are recruited to infection sites, where they release antimicrobial, extracellular traps (NETs) (9–12). NETs are formed through a unique cell death program (13) and are composed of chromatin and neutrophil proteins (14). Because SLE patients make Abs against chromatin and ANCA antigens, which are NET components, we speculated that after inflammatory responses, the timely removal of NETs may be crucial for tissue homeostasis and to avoid presentation of self-antigens.

Defective clearance of apoptotic (15) and necrotic (16) cells is thought to be the major source of autoantigens in SLE. We, in addition, propose that inefficient NET degradation is directly linked to the pathogenesis of SLE. In the present study we found that serum DNase1 is responsible for NET degradation. We analyzed a cohort of SLE patients and controls and showed that a subset of the SLE patients had impaired NET degradation. This was due to the presence of DNase1 inhibitors or Abs that protected NETs from this endonuclease. Furthermore, we found that, in our cohort, lack of NET degradation, high anti-NET titers, and renal involvement are correlated.

Results

Serum DNase1 Degrades NETs. To analyze how NETs are degraded, we first analyzed whether there are neutrophil factor(s) that degrade NETs. In vitro they were stable for more than 90 h in the absence of serum (Fig. 1*A*), although they can be experimentally digested with nucleases such as micrococcal nuclease (MNase) (13). However, NETs were degraded after incubation with 10% serum isolated from healthy donors (Fig. 1*B*). We initially tested sera of 11 different healthy donors for NET degradation and did not observe a significant variation (Fig. S1). These data were confirmed by microscopy. We induced the formation of NETs, incubated them with sera, and later stained the NETs with a DNA dye and Abs that recognize myeloperoxidase and histones (Fig. 1*D*). NET degradation was dose and time dependent (Fig. 1 *B* and *C*), suggesting an enzymatic activity.

To identify the serum factor(s) responsible for NET disassembly, we investigated the requirement of divalent cations, a cofactor of several nucleases. EGTA, a calcium-specific chelator, prevented NET degradation, and this inhibition was reverted with addition of exogenous calcium (Fig. 1*E*). The calcium dependence of NET degradation suggested that serum DNase1 degraded the NETs. This extracellular, neutral endonuclease is mainly produced in the pancreas and secreted into the digestive system and the blood stream (17, 18). G-actin forms a complex with DNase1, thereby inhibiting its nuclease activity (19). The biological significance of this association is not yet understood (20). We used this property of G-actin and showed that it inhibited NET degradation in a dose-dependent manner (Fig. 1*F*), indicating that DNase1 degrades NETs. Anti-DNase1 Abs, but not an irrelevant Ab control, prevented serum-mediated NET degradation (Fig. 1*H*), confirming that DNase1 digests NETs. In addition, as controls, we showed that inhibitory concentrations of either G-actin (Fig. 1*G*) or anti-DNase1 Abs (Fig. 1*I*) block DNase1 but not MNase-mediated NET disintegration. These data indicate that serum DNase1 is essential for NET degradation.

Degradation of NETs is a unique function of DNase1. We speculated that the dysfunction of this enzyme could be linked to the immunopathogenesis of SLE. We hypothesized that insufficient NET degradation by DNase1 would allow NETs to

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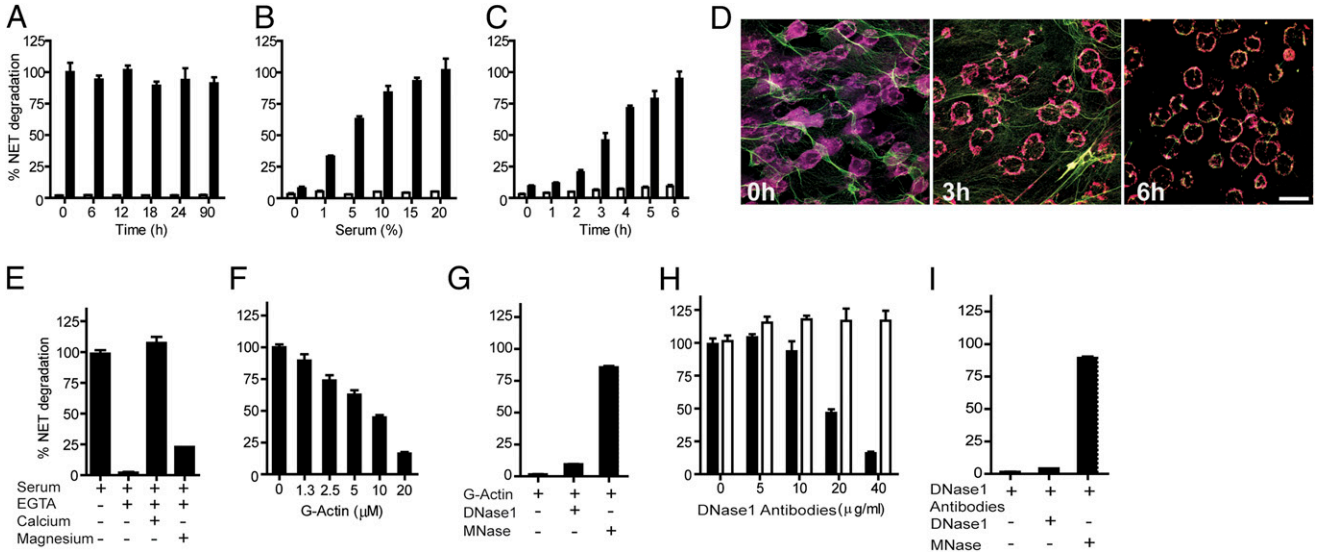

Fig. 1. Serum DNase1 degrades NETs. Human neutrophils were activated to form NETs and incubated in the indicated conditions before measurement of the digested and released NET DNA using the fluorescencent DNA dye Picogreen. (*A*) In the absence of serum (open bars) NETs are stable for at least 90 h in vitro. NET degradation with MNase for 10 min at each time point (black bars) represents the total NETs recovered. (*B*) Serum-mediated degradation of NETs is concentration and (*C*) time dependent when exposed to 10% serum, suggesting an enzymatic activity (open bars are medium controls). (*D*) Activated neutrophils that formed NETs were incubated in media with 10% serum for the indicated time points, fixed, and immunostained for myeloperoxidase (green) and histones (red). DNA (blue) was stained with Draq5. (Scale bar, 10 μm.) (*E*) Serum NET degradation requires calcium. NETs were incubated with 10% serum for 6 h. EGTA, a calcium chelator, inhibited degradation. Calcium, but not magnesium ions, restored the NET-degrading activity. (*F*) Inhibition of NET degradation by G-actin, a specific inhibitor of DNase1, is dose dependent. NETs were incubated with 10% serum for 6 h in the presence of the indicated concentrations of G-actin, and NET degradation was measured as described. (*G*) To control for the specificity of G-actin for DNase1, we incubated NETs with 20 μM of G-actin with either commercially available purified DNase1 or MNase to digest the NETs. G-actin blocked degradation by DNase1 but not MNase. (*H*) NETs were incubated with serum as described above and with the indicated concentrations of polyclonal anti-DNase1 (black bars) or irrelevant control Abs (open bars). Inhibition of NET degradation by anti-DNase1 Abs was specific and dose dependent. (*I*) Anti-DNase1 antibodies are specific. We incubated NETs in the presence of 40 μg/mL of anti-DNase1 Abs and incubated with purified DNase1 or MNase. The data shown are representative of experiments performed in triplicate and are presented as mean ± SD.

persist and thus to foster the presentation of chromatin-associated self-antigens, a process that may promote SLE.

Impaired NET Degradation in a Subset of SLE Patients. We analyzed the NET degradation activity of 145 sera collected from (*i*) 54 healthy, unrelated blood donors, (*ii*) 30 rheumatoid arthritis (RA) patients, and (*iii*) 61 unrelated patients with documented SLE (59 female, 2 male; female/male ratio 29:1) at different disease stages and activities (Table S1). We tested the NET degradation activity of these sera in a blinded experiment on NETs produced by neutrophils isolated from a healthy donor. The NET degradation activity of the sera from this donor was defined as 100%. We arbitrarily set 60% of NET degradation as cutoff and termed donors with higher values "degraders". Those with less than 60% NET degradation were termed "nondegraders". In the NET degradation assay, sera from healthy donors dismantled NETs efficiently, with a mean percentage degradation of 98.1% and an SD of ±8.2% (Fig. 2*A*). All but one of the sera from control RA patients degraded NETs normally (mean 91.3% ± 16.7%). Interestingly, we identified two populations in the SLE sera; 63.9% were "degraders" and 36.1% were "nondegraders". These results were confirmed by microscopy; accordingly, we observed that NETs were degraded by sera from healthy donors (Fig. 2*B*) and a subgroup of SLE patients (Fig. 2*C*). We also corroborated that a subgroup was unable to degrade NETs (Fig. 2*D*). Thus, a subpopulation of SLE patients displayed poor NET degradation in vitro.

Inhibitory Mechanisms of NET Degradation. To elucidate why some SLE sera cannot digest NETs, we exogenously added DNase1 to the sera. We anticipated two possible results: (*i*) "spiking" with exogenous DNase1 would restore the NET degradation, implying a nonfunctional DNase1; or (*ii*) "spiking" would not restore the serum activity, suggesting the presence of either DNase1 inhibitors or the physical protection of NETs from this enzyme. To further clarify the second option, we also "spiked" sera with MNase. Digestion with MNase of NETs that were not digested by DNase1 would indicate the presence of DNase1-specific inhibitors ("group 1"). In contrast, if MNase would not restore NET degradation activity, this would indicate the general "protection" of NETs from nucleases ("group 2").

Addition of DNase1 to nondegrader SLE sera did not restore NET degradation to the level of controls (Fig. 3*A*, green circles). These data suggested that mutations in DNase1 did not account for impaired NET degradation in our cohort (21, 22). Spiking these sera with MNase identified two groups of patient sera. In group 1, addition of MNase restored NET degradation (from median 29.7–95.2%), indicating the presence of DNase1-specific inhibitor(s) (Fig. 3*A*, green circles with black border). Further investigation will determine the nature of these inhibitors. In group 2, addition of MNase resulted only in a marginal increase in NET degradation (from median 10.5–20.9%; Fig. 3*A*, orange circles vs. orange circles with black border), indicating the presence of factor(s) that protect NETs from enzymatic degradation.

NET-Protecting Abs in SLE Sera Prevent DNase1 Degradation of NETs. We tested whether the sera in group 2 contained NET "protecting" Abs that block the access of nucleases to NETs. To analyze this, we depleted these sera of Abs using protein A/G beads. Fig. 3*B* shows that sera in group 2 efficiently digested NETs after Ab depletion (median 19.9% before and 78% after Abs depletion; orange circles). In contrast, NET degradation increased only slightly in group 1 sera (median 29% before and 43% after Abs depletion; green circles). These data indicate that sera of group 2 contain Abs

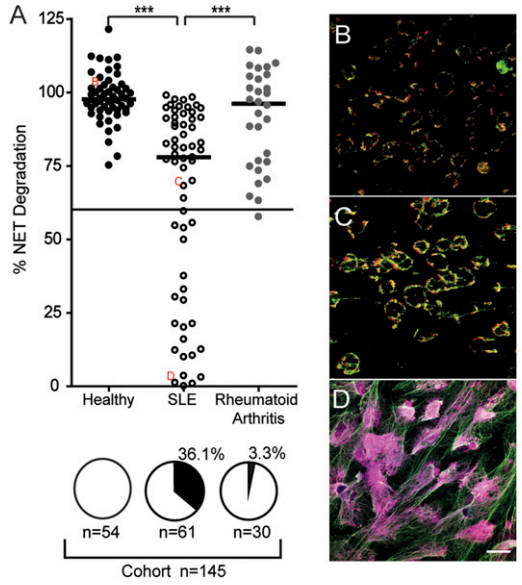

Fig. 2. NET degradation is impaired in a subset of SLE patients. (*A*) We activated neutrophils isolated from healthy donors to make NETs, incubated them with 10% sera from our cohort for 6 h, and quantified NET degradation. The cohort is described in *Materials and Methods* and in Table S1. Each circle corresponds to one individual donor. The samples are grouped into healthy donors, SLE patients, and RA patients as indicated. One hundred percent NET degradation was determined using the serum from the healthy donor of the neutrophils. We arbitrarily considered sera that degrade at least 60% of the NETs within 6 h as normal (horizontal line). Sera from all healthy donors (n = 54, black circles) degraded NETs normally; 36.1% of SLE patient sera (n = 61, open circles) and 3.3% of the RA sera (n = 30, gray circles) degraded NETs poorly. ***$P < 0.001$; Kruskal-Wallis test with Dunn's post hoc comparisons. (*B–D*) NETs were exposed to representative sera (labeled B, C, or D in *A*), fixed and immunostained for myeloperoxidase (green) and histones (red). DNA (blue) was stained with Draq5. Representative micrographs show efficiency of NET degradation, with serum from a healthy donor (*B*), from an SLE patient who degraded NETs (*C*), and from an SLE patient who did not disassemble NETs (*D*). (Scale bar in *D*, 25 μm for *B–D*.)

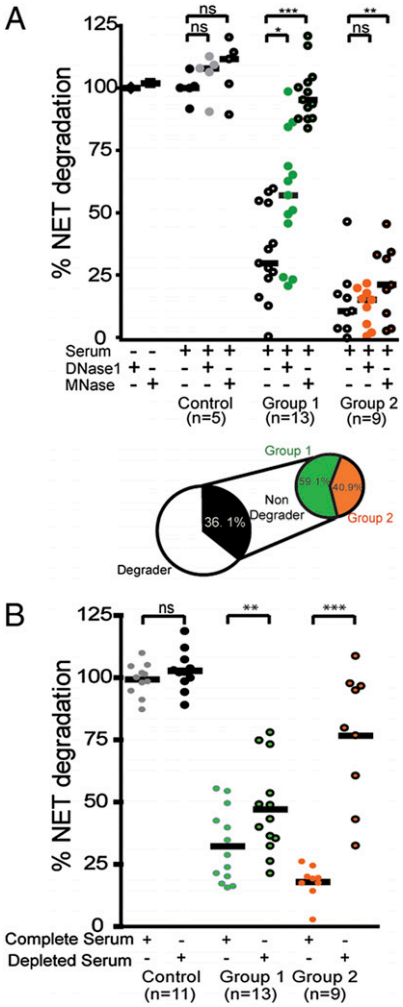

Fig. 3. Inhibitory mechanisms of NET degradation. (*A*) A subset of SLE sera contains DNase1 inhibitor(s). NETs incubated with sera from healthy donors (n = 5) or SLE patients who did not degrade NETs (n = 22) were spiked with exogenous DNase1 or MNase, and then we quantified NET degradation. Degradation of NETs by healthy sera was unaffected by the addition of the exogenous nucleases. The SLE nondegrader sera fell into two groups: in group 1, addition of MNase but not DNase1 fully restored NET degradation activity, suggesting the presence of specific DNase1 inhibitor(s). In group 2, neither DNase1 nor MNase completely restored NET degradation, suggesting a mechanism of NET protection. ***$P < 0.001$; **$P = 0.0013$; *$P < 0.05$; $P > 0.05$; ns, nonsignificant compared by Friedman's test with Dunn's post hoc comparison. The bar denotes the median of the group. Protecting Abs impair NET degradation. (*B*) Sera from NET degraders and nondegraders were depleted of antibodies with protein A/G Sepharose beads. The Ab-depleted sera were incubated with NETs for 6 h before quantification of NET degradation. Depletion of Abs increased the NET degradation of sera from group 1 only marginally. In contrast, sera from patients in group 2 degraded NETs efficiently after depletion. This indicates that sera from patients of group 2 contain Abs shielding NETs from degradation. ***$P < 0.0001$; **$P = 0.0056$; ns, nonsignificant using parametric paired t test, because the data followed a Gaussian distribution. Each circle in *A* and *B* represents the activity of a single serum and is the value of the mean in an experiment performed in triplicate. Bars denote the mean of the group.

that shield the NETs from nucleases. Taken together these data show that NET degradation is prevented either by inhibiting DNase1 (group 1) or by covering NETs with Abs and protecting them from endonuclease digestion (group 2).

Elevated Levels of Anti-NET Abs in Nondegraders. We proposed that inefficient NET degradation might be linked to high titers of anti-NET Abs in vivo. To test this we retrospectively quantified anti-NET Abs using a modified ELISA (as described in *Materials and Methods*) in the sera. SLE sera, in particular those from nondegraders, contained high levels of anti-NET Abs (Fig. 4*A*). Furthermore, consistent with the model that anti-NET Abs protect NETs from DNase1 degradation, these Abs were particularly abundant in group 2. We confirmed this by microscopy; sera from nondegraders, both from group 1 and 2, bound to NETs at the tested concentration (representative micrographs shown in Fig. S2). As controls, sera from healthy donors or SLE degraders did not recognize NETs either by ELISA or by immunofluorescence. These data indicate that inefficient NET degradation correlates with high levels of anti-NET Abs.

Impaired NET Degradation Correlates with Lupus Nephritis. We corroborated our findings with established clinical markers. Anti-dsDNA and anti-nuclear Abs (ANA) are hallmark tests for SLE diagnosis. Anti-dsDNA Abs correlate with renal disease, and increasing titers may indicate disease flares (23). Anti-dsDNA and ANA titers were determined at the same clinical visit when the serum samples for the NET degradation assays were taken. Fig. 4 *B* and *C* show that nondegraders have significantly higher anti-dsDNA and ANA titers than degraders. Consistently, we showed that an anti- dsDNA monoclonal Ab derived from an SLE patient (24) binds to NETs (Fig. S3). Interestingly, sera in

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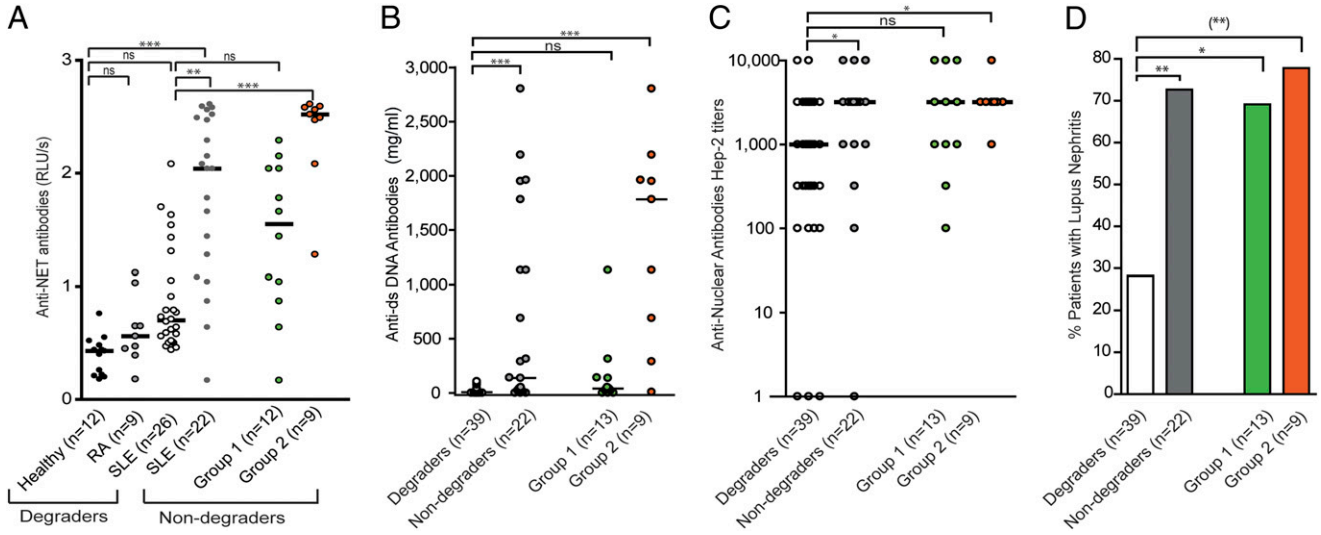

Fig. 4. Defective NET degradation correlates with high anti-NET Abs anti-dsDNA, ANA titers and increased risk of lupus nephritis. (*A*) Abs binding to NETs were quantified with a NET ELISA, whereby NETs are used as antigen, patient sera as primary Abs, and anti-human Cy3 coupled Abs as secondary Abs. Sera from healthy donors, from SLE degraders, or from patients with other autoimmune diseases did not contain anti-NET Abs. Most of the sera in group 2 contained high levels of anti-NET Abs. Sera in group 1, however, were heterogeneous, but as a group the concentrations of anti-NET antibodies were significantly higher than in the NET degraders. In *A* and *B* each circle represents the activity of a single serum and is the mean of an experiment performed in triplicate. Bars show the median of the group. (*B*) The concentrations of anti-dsDNA Abs were significantly higher in SLE nondegraders compared with degraders. (*C*) The titers of ANA detected by indirect immunofluorescence on fixed Hep2 cells were significantly higher in nondegraders compared with degraders. There was a significant difference between SLE degraders and group 2 but not with group 1. For *A*: ***, **, $*P < 0.05$; for *B* and *C*: $***P < 0.001$; $*P < 0.05$; ns, $P > 0.05$ using Kruskal Wallis test with post hoc Dunn's multiple comparison test. Each circle in *A* represents the mean of a triplicate experiment with patient serum. Bar denotes the median of the group. (*D*) We retrospectively analyzed the association between ineffective NET degradation with nephritis. There was a higher incidence of nephritis in SLE nondegraders than in degraders. Group 1 and group 2 showed a significantly higher risk of nephritis when compared with NETs degraders. The statistics for *D* are based on Fisher's exact test. The odds ratio with 95% confidence interval between nondegraders and degraders is 6.79 (2.108–21.86), $**P = 0.0012$; between degraders and group 1 is 5.73 (1.457–22.52), $*P = 0.0188$; and between degraders and group 2 is 8.909 (1.596–49.74), $(**)P = 0.0091$.

group 2 have higher Ab titers than those in group 1, consistent with their NET-protecting function.

A frequent and serious manifestation of SLE is glomerulonephritis, a condition that can cause proteinuria and progress to kidney failure. A retrospective correlation analysis showed that patients who do not degrade NETs developed lupus nephritis significantly more frequently than degraders (Fig. 4*D*). Notably, all nondegrader patients, regardless of belonging to group 1 or 2, were likely to develop lupus nephritis. These data indicate that impaired NET degradation correlates with renal disease, independently of the causative mechanism. Indeed, we observed IgG deposition on NETs in tubuli and glomeruli in the kidney of an SLE patient who degraded NETs poorly (Fig. S4). We detected NETs by staining with a DNA dye and an anti-myeloperoxidase (MPO) antibody and colocalized them to Ab deposits. Moreover, in our cohort impaired NETs degradation and high concentrations of anti-dsDNA Abs, a known risk factor (23), were both associated with lupus nephritis (Fig. 4*D* and Fig. S5). To confirm our observations, we tested seven patients not included in the original cohort who had biopsy-proven lupus. Five of the seven sera obtained around the time of kidney biopsies did not degrade NETs, supporting the correlation between lack of NET degradation and lupus nephritis (Fig. S6*A*). We did not observe a correlation between certain types of lupus nephritis (World Health Organization classification) with NET degradation (Fig. S6*B* and Table S2). A larger study should corroborate this direct association. Interestingly, as a control for other nephritis we showed that sera from patients with IgA nephropathy (25) degraded NETs (Fig. S7). This is consistent with the observations that these patients do not make antibodies against NET components or against NETs. Importantly, our results suggest that defective NET degradation contributes to SLE pathogenesis, especially glomerulonephritis.

Discussion

In SLE patients, infections are often associated with flares and mortality (7, 8, 26). Neutrophils are abundantly recruited to infection sites and have also been associated with SLE (3–5). Furthermore, SLE patients develop Abs against chromatin and neutrophil proteins (4, 5, 27), the components of NETs. We propose that defects in NETs clearance exacerbate the disease.

In this report we show that serum DNase1 is required to degrade NETs. We observed that the sera of some SLE patients (group 1) contain a specific DNase1 inhibitor. These findings are in line with reports showing that sera of some SLE patients contain DNase1 inhibitors (28, 29). The sera of other SLE patients (group 2) have high titers of Abs that bind to NETs and thus protect them from DNase1. This is in agreement with the observation that anti-DNA Abs protect DNA from DNase fragmentation in vitro (30). Interestingly, mutations (21) and polymorphisms in DNase1 (31) have been associated with SLE. However, we did not identify any patient with this mutation in our cohort. These mutations seem to be rare (22, 32) and were reported exclusively in a Japanese cohort (21). In the cohort we studied, we identified two mechanisms of impaired NET degradation in a subset of SLE patients. Regardless, it is interesting to speculate that the development of SLE in patients with mutations in DNase1 is also linked to a defect in NET degradation.

We propose that in addition to apoptotic (15) and necrotic cell remnants (16), NETs might be a source of autoantigens and immunostimulatory damage-associated molecular patterns in SLE. NETs are formed in response to infections and expose chromatin and neutrophil proteins at inflammatory sites. Nota-

bly, NET formation involves reactive oxygen species (13) that modify DNA and proteins, making them more immunogenic (33–35). It is possible that anti-NET Abs and persistent NETs could form "NET immunocomplexes", which might be relevant in the exacerbation of SLE. This is supported by the observation that IgG–chromatin complexes potentiate autoantibody production (36). Furthermore, these immune complexes could be pathogenic in the development of glomerulonephritis. Indeed, we find NET–IgG complexes in the tubuli and glomeruli in kidney biopsy of a patient who degraded NETs poorly. Notably, a decreased renal DNase1 expression was described as promoting lupus nephritis in NZB/NZW F1 mice (37).

The patients analyzed in this study were already diagnosed with SLE and presented with anti-NET antibodies. Future prospective studies could analyze the question of how the B cell clones producing anti-NET antibodies develop during the course of SLE pathogenesis.

Recombinant DNase1 was shown to be ineffective as a therapeutic agent in SLE patients (32). Our data suggest that this failure was, at least in part, due to the presence of DNase1 inhibitors and Abs that prevented NET degradation. It is possible that an alternative nuclease might be helpful in the treatment of patients who have DNase1 inhibitors. For patients with Abs that protect NETs from degradation, depletion of Abs using immunoadsorbant columns (38) could restore the NET degradation efficiency. It is tempting to speculate whether classifying patients according to NET degradation might facilitate a better treatment strategy; especially nondegraders may require more aggressive therapy because of their high risk of renal involvement. First, a prospective study should determine whether the NET degradation status can actually predict the risk of lupus nephritis.

Interestingly, we observed a strong correlation between the NET disassembly status, anti-NET Abs, anti-dsDNA titers, and lupus nephritis. We propose a model whereby, in healthy individuals, NETs are made at inflammatory sites and subsequently cleared by DNase1-mediated degradation. If the activity of this nuclease is prevented or impaired, NETs may persist and constitute a source of autoantigens. In SLE, persistence of NETs may lead to prolonged exposure to NET antigens in an immunostimulatory context. This fact may further exacerbate the autoimmune response and form a vicious cycle that leads to the formation of immune complexes, which are known to induce Ab production (36, 39). Indeed, we show the deposition of Ab on NETs in renal biopsy of a "nondegrader". This deposition was also observed in small-vessel vasculitis (40), suggesting that this pathway might also contribute to the pathogenesis of other autoimmune diseases. These immune complexes may get deposited within the kidneys, leading to lupus nephritis.

Our results provide a mechanistic link between impaired NET degradation and glomerulonephritis in SLE. This correlation might help in the development of better diagnostics and therapeutic interventions in SLE.

Materials and Methods

Donors, Patients, and Clinical Diagnosis. Sixty-one unrelated patients with SLE (59 female, 2 male; female/male ratio 29:1) from the Department of Internal Medicine 3, University Hospital of Erlangen-Nuremberg were randomly selected irrespective of severity or stage of disease. Additionally, 54 healthy unrelated blood donors, 30 patients with RA, and 4 patients with IgA nephropathy served as control groups. All SLE patients fulfilled the 1982 and 1997 revised criteria of the American College of Rheumatology for the diagnosis of SLE (41, 42). Patients had a median of 13 visits, varying from 1 to 54. Clinical data on disease manifestations of SLE, including proteinuria, nephritic sediments, creatinine clearance, results of kidney biopsies, and arthritis, as well as anti-dsDNA Ab concentrations, were retrospectively collected from the patient records. The clinical diagnosis of lupus nephritis was based on histological examination of kidney biopsies, nephritic urine sediments, and proteinuria. All patients with proven lupus nephritis, even if resolved or occurring after serum sampling (Table S2), were considered as having renal manifestation of SLE. The renal component of British Isles Lupus Assessment Group (BILAG) 2004 was used to grade the severity of kidney involvement (43). The study was approved by the ethics committee of the University Erlangen-Nuremberg, and informed consent was obtained from the patients and healthy blood donors. In addition, we selected seven patients who already had nephritis and a BILAG score of A and tested them for NET degradation.

Abs against dsDNA in human serum were quantified using an in vitro diagnostic radioimmunoassay (IBL). Abs titers against nuclear components in human serum were quantified using indirect immunofluorescence on fixed Hep2 cells (Euroimmun). Proteinuria and hematuria were semiquantitatively assessed using reagent strips for urine analysis. Urine sediments were analyzed by light microscopy. Proteinuria was quantified in urine collected over 24 h.

Neutrophils and Sera. Human neutrophils were isolated from blood obtained from the blood bank in a protocol approved by the ethics committee of the Charité Hospital, Berlin. We isolated neutrophils by density gradient separation (44). The cells were seeded onto tissue culture plates or on coverslips and activated with phorbol myristate acetate for NET formation. Serum was obtained from venous blood and aliquoted and stored at −20 °C until use. For Abs depletion sera were incubated with a protein A/G Ultra-Link Resin (Thermo Scientific) according to the manufacturer's instructions. The hybridoma producing the anti-dsDNA Abs was generated and purified as described previously (24).

Bioassay for NET Degradation. Wells containing NETs were incubated with 1 U/mL MNase or DNase1 (both from Worthington) for 10 min or 10% human serum for 6 h. We then added 2 mM EDTA to stop nuclease activity and collected the culture supernatants. Picogreen (Invitrogen), a DNA fluorescent DNA dye, was added, and the DNA content was quantified by fluorescence spectrometry (13). For inhibition experiments, G-actin (rabbit skeletal; Sigma Aldrich) or anti-DNase1 and irrelevant control Abs (Abcam) were used at the indicated concentrations. The amount of NET DNA released with MNase or DNase1 was considered as "100% NET degradation". For analysis of the cohort's sera, NET degradation by the serum of the healthy neutrophil donor was considered to be 100%. In some cases NETs were treated with 10% sera spiked with 1 U/mL DNase1 or MNase before quantification of NET degradation.

Immunofluorescence Microscopy. Fixed NETs were incubated with primary Abs to the anti-H2A–H2B–DNA complex (45), with antimyeloperoxidase, or human sera, and bound antibodies were detected with respective secondary Abs coupled to Cy2 or Cy3 (Jackson Laboratory). DNA was stained with Draq5 (Biostatus). Isotype-matched controls were used. The specimens were processed and analyzed as described previously (13). Sections of a paraffin-embedded biopsy was rehydrated, subjected to antigen retrieval, and incubated with the following antibodies: rabbit anti-MPO (Dako, A0398), mouse anti-H2A/H2B/DNA. Species-specific donkey secondary antibodies were used: dk anti-rabbit Cy2 and dk anti-ms Cy3. Additionally, dk anti-human IgG Cy5 was used to detect IgG complexes. The images are projections of a confocal stack.

NET ELISA. Neutrophils were activated to allow maximum NET formation and then washed and fixed. The NETs were incubated with 1/100 dilution of serum for 1 h at 37 °C. The secondary Ab, anti-human IgG coupled with Cy3, was added and incubated for 1 h. Thereafter the NETs were stained with Sytox Green. The fluorescence was measured with two channels, 518/590 nm and 485/518 nm. The Cy3 signal value was normalized to the Sytox signal, so the value was proportional to the amount of NETs in each well. The results were plotted as relative fluorescence light units.

Statistical Analysis. The normality of the data was checked by a Shapiro-Wilk normality test. For unpaired comparisons of two groups, a *t* test and, in the case of nonnormality, a nonparametric Wilcoxon test was performed. For more than two unpaired groups, a variance analytical approach was used, and comparison was done with an ANOVA and Kruskal-Wallis test in case of normality. To adjust the α level, Dunnett's post hoc tests were used. Paired comparisons of two groups were performed with a paired *t* test or with a paired Wilcoxon test in the case of nonnormality. An ANOVA with repeated measurements or a Friedman test were performed for a paired comparison of more than two groups, and Dunn's post test was used. For clinical data analysis the 95% confidence interval and the odds ratio were calculated. The overall level of significance was set at $P < 0.05$.

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