Factor VII-activating protease

Unraveling the release and regulation of dead cell nuclear damps

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CHAPTER 4

FSAP-mediated nucleosome release from late apoptotic cells is inhibited by autoantibodies present in SLE

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ABSTRACT

Inefficient clearance of apoptotic cells and the subsequent exposure of the immune system to nuclear contents are crucially involved in the pathogenesis of systemic lupus erythematosus (SLE). Factor VII-activating protease (FSAP) is activated in serum upon contact with dead cells, and releases nucleosomes from late apoptotic cells into the extracellular environment. We investigated whether FSAP-mediated nucleosome release from late apoptotic cells is affected in SLE patients. Nucleosome release in sera of 27 SLE patients and 30 healthy controls was investigated by incubating late apoptotic Jurkat cells with serum and analyzing the remaining DNA content by flow cytometry. We found that nucleosome release in sera of SLE patients with high disease activity was significantly decreased when compared with that in SLE sera obtained during low disease activity or from healthy individuals. Upon removal of IgG/IgM antibodies from SLE sera, nucleosome release was restored. Similarly, monoclonal anti-nuclear antibodies inhibited nucleosome release in healthy donor serum or by plasma-purified FSAP. This inhibition was lost when Fab fragments were used, suggesting that antigen crosslinking is involved. In conclusion, FSAP-mediated nucleosome release from late apoptotic cells is greatly impaired in SLE patient sera, possibly hampering the clearance of these cells and thereby propagating inflammation.

INTRODUCTION

Systemic lupus erythematosus (SLE) is a chronic inflammatory autoimmune disease, characterized by intermitting periods of active disease with variable clinical manifestations. The disease is outlined by an antigen-driven, T-cell-dependent immune response, B cell hyperactivity and the production of antinuclear antibodies (ANAs) directed against ubiquitous nuclear antigens, such as double stranded (ds)-DNA and histones[1]. These ANAs can form immune complexes with their respective antigens, which upon deposition in tissues can lead to activation of complement, induction of local inflammation and consequently progression of disease[2].

Inefficient removal of apoptotic cells and loss of self-tolerance due to a dysregulated immune response are critically involved in the pathogenesis of SLE[3–8]. When apoptotic cells are not cleared timely, progression into late apoptosis or secondary necrosis occurs. Similar to apoptotic cells, late apoptotic cells expose phosphatidylserine on the outer plasma membrane and contain a fragmented nucleus with condensed chromatin[9]. However, late apoptotic cells have in addition lost their plasma membrane integrity[10]. This exposes potentially immunogenic cytosolic, but also nuclear constituents such as nucleosomes, containing histones, dsDNA and HMGB1, to the extracellular environment[11].

Collectively, these newly exposed intracellular components are known as damage-associated molecular pattern molecules (DAMPs). Several nuclear DAMPs such as dsDNA, histones, RNA and HMGB1 are known to stimulate Toll-like receptors (TLRs) and were critically involved in the pathogenesis of SLE in murine models[12–18] and human disease[19,20]. Circulating nucleosomes and anti-nucleosome antibodies have been found in patients with SLE[21], and their levels appear to inversely correlate with each other[22,23]. Furthermore, nucleosome-specific autoantibodies were detectable in murine lupus models before the occurrence of autoantibodies with other specificities, suggesting an important role for nucleosomes in disease pathogenesis[24].

We have previously shown that incubation of late apoptotic or necrotic cells with serum releases the chromatin from these cells[25,26] and this phenomenon has recently also been described by others[27]. After release, the chromatin can be found as nucleosomes in the extracellular environment[25] and in blood[28]. We have found that the plasma protein responsible for this

chromatin removal is the Factor VII-activating protease (FSAP), also known as hyaluronic acid binding protein 2 (HABP2)[29]. FSAP is a serine protease circulating in plasma as a 78 kDa inactive single-chain molecule. Upon activation, FSAP is auto-proteolytically converted into its active two-chain form consisting of a 50 kDa heavy and a 28 kDa light chain, interconnected by a disulfide bond[30]. It has been found that FSAP gets activated upon contact with either late apoptotic or necrotic cells[28] and circulating histones[31], and that its activation is required for nucleosome release. Notably, blocking FSAP activation in serum with a monoclonal anti-FSAP antibody fully inhibits the nucleosome releasing activity of serum. In the blood FSAP is only transiently active as it is rapidly bound by inhibitors including α2-antiplasmin (AP)[30]. Since nucleosomes play a central role in the antinuclear antibody response in SLE, we investigated whether the FSAP-mediated release of nucleosomes from late apoptotic cells is affected in serum of patients with SLE.

METHODS

Patient sera

Twenty-seven SLE patients were randomly chosen with a mean age of 38 years (range, 18-64 years) and 89% was female. Two serum samples were taken from each individual patient at different time points. One sample was taken during flair-induced hospitalization as indicated by a high Systemic Lupus Erythematosus Disease Activity Index (SLEDAI) score[32] of at least ≥ 4, referred to as "high disease activity". Another sample with low SLEDAI score (SLEDAI ≤ 2) was drawn during a regular outpatient visit preceding the flair and was referred to as "low disease activity". Patient characteristics and presence of the specific American College of Rheumatology classification criteria for SLE are summarized in Table 1. Antibodies to ds-DNA were measured using the RIA Farr assay according to the manufacturer's protocol (Siemens Healthcare Diagnostics, the Hague, the Netherlands). Complement protein C3 and C4 levels were detected using nephelometry. Sera were taken from healthy donors and all donors were known to be homozygous for the wild-type form of FSAP[33]. All sera were obtained as anonymous samples from the diagnostic laboratory, in accordance with the Dutch guidelines and regulations for the use of patient material.

Reagents

Mouse monoclonal antibodies against FSAP[29] and α_2-antiplasmin (AAP-20), anti-murine kappa light chain antibodies and monoclonal antinuclear antibodies (ANAs)[34] were prepared at our department. The anti-nucleosome antibody recognizes a complex formed by histone H2a, H2b and dsDNA. Iscove's Modified Dulbecco's Medium (IMDM) was obtained from BioWhittaker Europe (Verviers, Belgium). Heat-inactivated fetal calf serum (FCS) was obtained from Bodinco BV (Alkmaar, The Netherlands). Penicillin and streptomycin were obtained from Gibco/Invitrogen (Groningen, The Netherlands). RNase A, ß-mercaptoethanol, pepsin and N-ethylmaleimide were obtained from Sigma (Zwijndrecht, The Netherlands). High performance ELISA buffer (HPE) and poly-HRP labeled streptavidin were obtained from Sanquin (Amsterdam, The Netherlands). HRP labeled streptavidin and sepharose 4B-coupled Protein A were obtained from GE Healthcare (Diegem, Belgium). (3,5,3',5')-tetramethylbenzidine (TMB) and dithiotreitol were obtained from Merck Millipore (Nottingham, UK). Anti-IgM fragment coupled to NHS-activated Sepharose 4 Fast Flow (CaptureSelect IgM affinity Matrix) was obtained from Invitrogen (Groningen, the Netherlands). Plasma-purified active FSAP was prepared as described previously[29].

Table 1 Disease characteristics of SLE patients

	exacerbation	remission
SLEDAI	8 (7-11)	0 (0-2)
Anti-dsDNA, Farr (IU/ml)	66.5 (33-699)	14 (6.5-52)
C3, g/l	0.57 (0.45-0.97)	0.94 (0.75-1.14)
C4, g/l	0.08 (0.06-0.13)	0.17 (0.12-0.21)
ACR criteria, n (%)		
Malar rash	7 (26%)	
Discoid rash	9 (33%)	
Photosensitivity	9 (33%)	
Oral ulcers	4 (15%)	
Arthritis	14 (52%)	
Serositis	10 (37%)	
Renal disorder	14 (52%)	
Neurologic disorder	4 (15%)	
Haematologic disorder	21 (78%)	
Immunologic disorder	25 (93%)	
Antinuclear antibody	27 (100%)	
Prednisolone use, n (%)	11 (41%)	22 (81%)
Daily mean dose, mg	7.5 (5-22.5)	10 (6.3-27.5)
Hydroxychloroquine use, n (%)	10 (37%)	12 (44%)
Daily mean dose, mg	400 (400-400)	400 (400-400)
Azathioprine use, n (%)	5 (19%)	7 (26%)
Daily mean dose, mg	100 (75-150)	125 (112.5-137.5)

Unless otherwise indicated, data are expressed as median (interquartile range). SLEDAI = Systemic Lupus Erythematosus Disease Activity Index; ACR = American College of Rheumatology

Cell culture and induction of apoptosis

Jurkat cells were cultured in IMDM containing 5% (v/v) FCS, penicillin (100 IU/ml), streptomycin (100 µg/ml) and 50 µM ß-mercaptoethanol. Prior to apoptosis induction, cells were washed by centrifugation at 360 x *g* for 10 minutes with, and resuspended in serum-free IMDM. Cells ($1x10^6$ cells/ml) were incubated for 48 h with etoposide in a final concentration of 200 µM to induce apoptosis.

Nucleosome release

Nucleosome release was determined as described previously[29]. Briefly, late apoptotic Jurkat cells were washed with HN buffer (50 mM Hepes, 100 mM NaCl, pH 7.4) and treated with RNase (10 Units/ml) for 30 min at 37°C to remove RNA which we have previously found to interfere with DNA staining of apoptotic cells by propidium iodide (PI). The incubation with 10 U/ml RNase did not affect FSAP activity (data not shown). Where indicated, ANAs or ANA-derived Fab fragments followed by an anti-light chain antibody were incubated with the late apoptotic cells. Subsequently, serum or plasma-purified active FSAP was added to the cells and incubated for 30 minutes at 37°C. To examine the release of nucleosomes, cells were washed three times with HN buffer and stained with PI (3 µg/ml) or DAPI (100 ng/ml) to detect the remaining amount of DNA after serum incubation. Samples were analyzed on a BD LSRII flow cytometer. The median fluorescence intensity of PI or DAPI was measured and quantified using the FACS Diva Software (Becton Dickinson, Mountain view, CA, USA). The nucleosome releasing activity of a reference serum (20%) of a healthy donor was defined as 100%. Although we measure loss of DNA content in this study, it should be noted that this can be reliably used to measure the release of nucleosomes[25].

FSAP ELISA

FSAP antigen levels were determined by ELISA as recently described[29]. Briefly, a monoclonal antibody (anti-FSAP4) which recognizes the light chain of FSAP was used as a catching antibody. Biotinylated anti-FSAP2 monoclonal antibody recognizing the heavy chain of FSAP in combination with HRP labeled streptavidin were used for detection.

FSAP-α_2-antiplasmin complex ELISA

FSAP-α_2-antiplasmin (FSAP-AP) complex levels were determined by ELISA as recently described[28]. Briefly, a monoclonal antibody (AAP-20) recognizing AP was used as a catching antibody. Biotinylated anti-FSAP4 which recognizes the light chain of FSAP in combination with Poly-HRP labeled streptavidin was used for detection.

IgG and IgM depletion of serum

IgG was depleted from serum by incubating 2 ml of protein G-coupled Sepharose 4B with 1 ml of serum overnight at 4°C. IgG-depleted serum was recovered after centrifugation and IgG depletion was verified by specific ELISA[35].

IgM was depleted from serum by incubating 2 ml of NHS-activated Sepharose 4 Fast Flow with a coupled anti-IgM fragment with 0.5 ml of serum for 4 h at RT. IgM-depleted serum was recovered after centrifugation and IgM depletion was verified by specific ELISA[36].

Fab fragments

To produce F(ab')$_2$ fragments, 0.8 mg/ml antibody was incubated with 20 µg/ml pepsin for 24 hr at 37°C in 0.1 M sodium citrate, pH 4.1. F(ab')$_2$ fragments were dialyzed against phosphate buffered saline (PBS). Undigested antibody was removed by incubation with Sepharose 4B-coupled Protein A for 2 h at 4°C. After centrifugation, the F(ab')$_2$ fragments were recovered and incubated with 10 mM dithiotreitol for 30 minutes to make monovalent Fab fragments. Free thiol groups were blocked with 20 mM of N-ethylmaleimide and Fab fragments were dialyzed to PBS.

Statistics

Results are presented as mean ±SEM. Differences between groups were compared using the Man-Whitney Rank Sum test. Paired samples were compared by means of Wilcoxon rank sum test.

RESULTS

Nucleosome release from late apoptotic cells is impaired in SLE sera obtained during active disease

To study extracellular nucleosome release we incubated late apoptotic Jurkat cells with sera from 27 SLE patients from whom we obtained sera both during active disease as well as during remission, and sera from 30 healthy donors. After incubation for 30 min the cells were stained with propidium iodide (PI) to detect the amount of DNA that remains in the cells. The decrease in DNA content of late apoptotic cells after incubation with a reference serum from a healthy donor was defined as 100% nucleosome release, as previously described[25,29]. A significant number of SLE sera obtained during active disease showed a reduced release of nucleosomes compared to healthy donor sera (**Figure 1A**). Interestingly, this difference in nucleosome release was not seen when late apoptotic cells were incubated with sera from the same patients obtained during low disease activity. We then studied the correlation between nucleosome release and an established disease activity parameter, the level of anti-dsDNA antibodies. This analysis showed that anti-dsDNA antibody levels were significantly elevated in samples obtained during high disease activity (**Figure 1B**). Moreover, the levels of anti-dsDNA antibodies inversely correlated with extracellular nucleosome release ($r = -0.63$ $P<0.0001$) (**Figure 1C**). Furthermore, we found that complement C3 and C4 levels were significantly reduced in the sera that had shown impaired nucleosome release from late apoptotic cells (**Supporting Information Figure 1**). This is indicative of enhanced activation and consumption of complement proteins during active disease in these patients.

FSAP levels and activation are normal in SLE

We have previously shown the critical importance of FSAP and its activation in efficient nucleosome release into serum, and therefore determined levels of FSAP in SLE patient sera and healthy donor sera. All patients had normal levels of FSAP antigen in sera as measured by ELISA (**Figure 2A**). To determine FSAP activation, we detected complexes of FSAP with its inhibitor α_2-antiplasmin (AP). FSAP-AP complexes were elevated in sera obtained during low disease activity compared to sera obtained during high disease activity (**Figure 2B**). To determine whether FSAP in the SLE sera was still susceptible to activation and

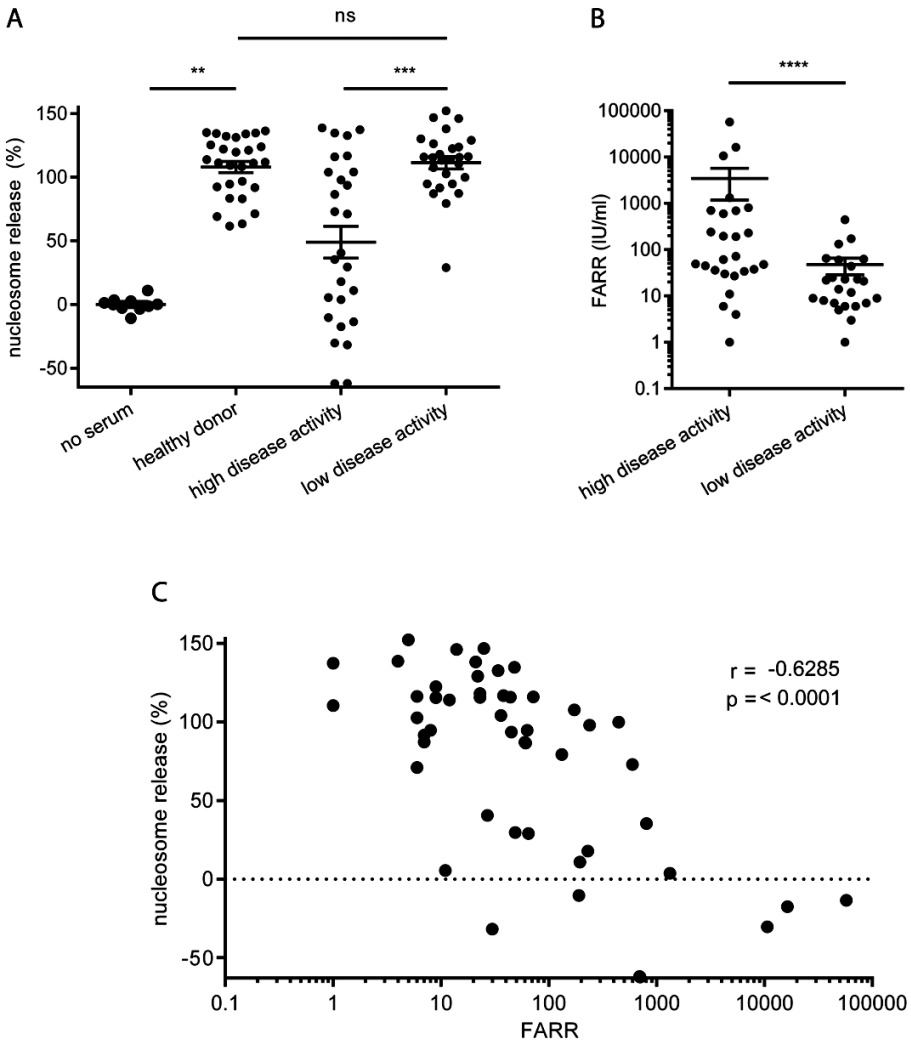

Figure 1. Nucleosome release from late apoptotic cells using serum from SLE patients and correlation with anti-dsDNA antibody level

(A) After induction of apoptosis, cells were incubated with 20% serum from SLE patients (n=27), obtained during both low disease activity and high disease activity, and from healthy donors (n=30). Cells were stained with propidium iodide (3 µg/ml) and analyzed by flow cytometry. The nucleosome release observed using a healthy donor reference serum was defined as 100%. (B, C) Anti-dsDNA levels were (B) determined in SLE sera during low and high disease activity by the Farr assay and (C) inversely correlated with nucleosome release. Each symbol represents an individual donor and bars represent mean ± SEM. Asterisks indicate significant differences between the groups indicated by brackets. Significance was determined using a two-tailed Student's t-test, Wilcoxon paired rank test for paired samples. For correlation analysis, Spearman's rank correlation coefficient was determined, *** $P < 0.001$, ** $P < 0.01$

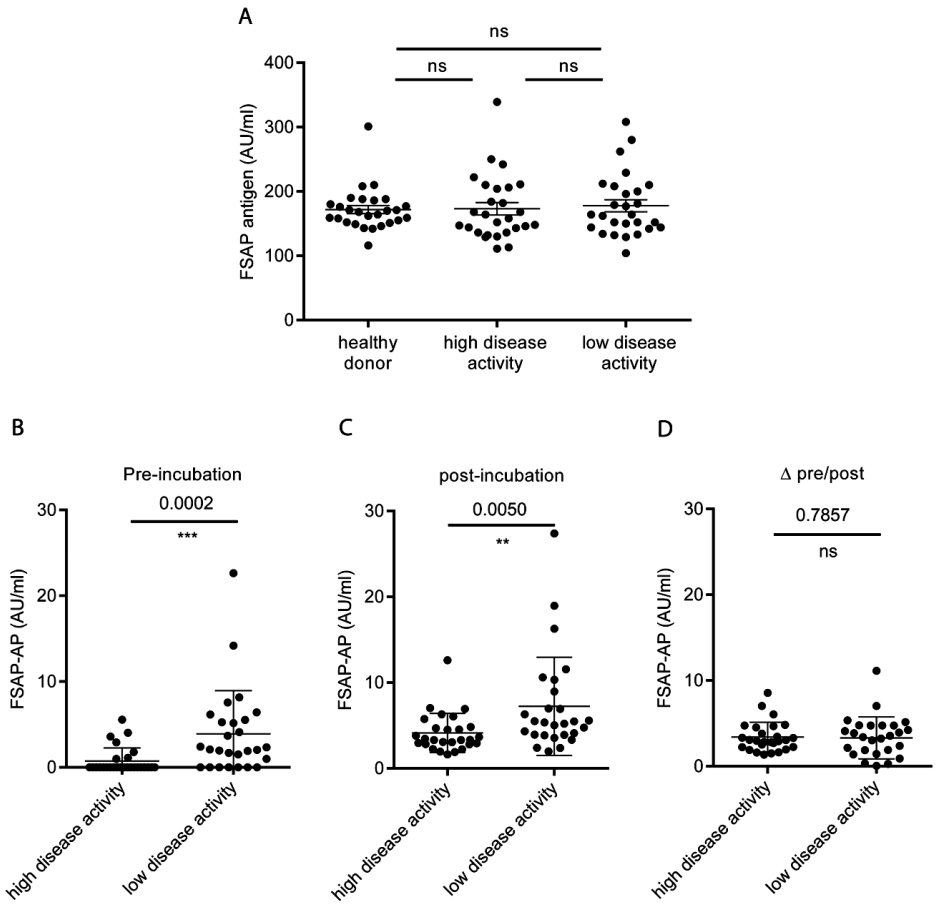

Figure 2. FSAP antigen and FSAP-AP complexes in serum from SLE patients

(A) Levels of FSAP antigen were measured in sera from SLE patients by ELISA. FSAP levels are expressed as AU/ml. Serum from 30 healthy donors were taken as a control. (B, C) Levels of FSAP-AP complexes were measured in sera from SLE patients (B) pre- and (C) post-incubation with late apoptotic cells. FSAP-AP levels are expressed as AU/ml. (D) Delta (Δ) expresses the difference in FSAP-AP levels pre- and post-incubation with late apoptotic cells. All measurements were performed in triplicate; each symbol represents an individual donor and bars represent mean ± SEM. Asterisks indicate significant differences between the groups indicated by brackets. Significance was determined using a two-tailed Student's t-test, Wilcoxon paired rank test for paired samples, *** $P < 0.001$, ** $P < 0.01$.

complex formation, FSAP-AP complexes were also measured upon incubation of sera with late apoptotic cells, as previously described[28]. Upon incubation with late apoptotic cells, all sera showed a near equal increase in FSAP-AP complexes, indicating that FSAP activation had occurred to a similar degree in the groups with low and high disease activity (**Figure 2C-D**). These data suggest that neither changes in FSAP antigen levels nor inhibition of FSAP activation/complex formation accounted for the decrease in nucleosome release from late apoptotic cells observed using these sera.

IgG and IgM antibodies in SLE sera inhibit nucleosome release from late apoptotic cells

Since no alteration in FSAP levels or susceptibility to FSAP activation was observed in the SLE sera, we hypothesized that an inhibitor of nucleosome release may be present in the sera. We investigated the presence of an inhibitor in patient sera that showed decreased nucleosome release from late apoptotic cells. Serum from a healthy donor with normal nucleosome release was mixed and pre-incubated with serum from SLE patients and subsequently added to late apoptotic cells. The level of nucleosome release observed using healthy donor serum was set at 100%. An impaired release of nucleosomes was detected when healthy donor serum was mixed with SLE serum (**Figure 3**), indicating that an inhibitor present in the SLE sera inhibits the nucleosome releasing activity in the healthy donor serum.

Because of the strong correlation between decreased nucleosome release and the presence of anti-DNA antibodies in the sera, we tested whether antibodies were involved in this inhibition. SLE sera were depleted for IgG and then incubated with serum from a healthy donor prior to the addition to late apoptotic cells. The observed inhibition of nucleosome release disappeared in six out of seven patient sera upon IgG depletion (**Figure 3**), suggesting that serum IgG is responsible for the inhibition of nucleosome release in these patient samples. In serum from one patient however, inhibition of the healthy donor serum was still detectable after IgG depletion, suggesting that in this patient sample the inhibition was not mediated by IgG antibodies. Notably, 80% of the anti-DNA antibodies in this patient were of the IgM class[37]. To investigate whether IgM antibodies had an inhibitory effect on nucleosome release by serum of this patient, it was depleted for IgM and tested for inhibition

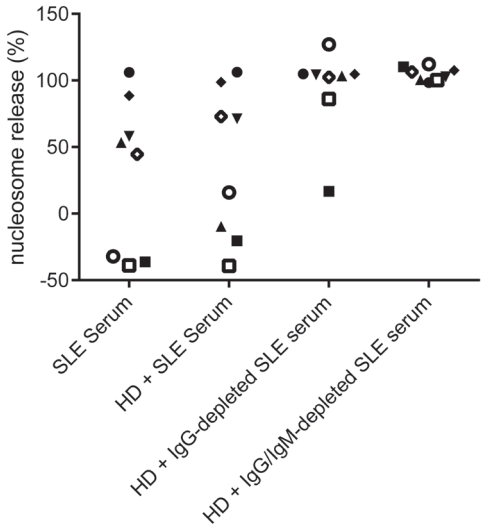

Figure 3. Antibodies in sera of SLE patients inhibit nucleosome release by healthy donor serum

After induction of apoptosis, cells were incubated with 10% serum from a healthy control or 10% serum from seven SLE patients with high disease activity. In addition, 10% serum of a healthy donor (HD) was pre-incubated with 10% serum, IgG-depleted serum or IgG+IgM-depleted serum of the seven SLE patients and a control. Cells were stained with propidium iodide (3 µg/ml) and analyzed by flow cytometry. The nucleosome releasing activity by 10% serum of the healthy control was defined as 100%. Closed circles indicate results obtained with the healthy control serum. Each of the other seven unique black symbols indicate results obtained with serum from one SLE patient and is shown as the mean of triplicates pooled from three independent experiments.

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of nucleosome release. Indeed, after IgM depletion, serum of this patient was no longer able to inhibit nucleosome release (**Figure 3**). This shows that in addition to IgG antibodies, IgM antibodies from SLE sera are able to inhibit nucleosome release by healthy donor serum.

Anti-nuclear antibodies inhibit nucleosome release from late apoptotic cells

Because antibodies present in the serum from SLE patients mediated the inhibition of nucleosome release, monoclonal anti-nuclear antibodies (ANAs) derived from mice with SLE-like disease[34] were screened for their effect on extracellular nucleosome release. To test this, late apoptotic cells were incubated with three different mouse monoclonal ANAs, recognizing histone H3, dsDNA and an epitope formed by a complex of histone 2A, 2B and DNA (anti-nucleosome), prior to incubation with healthy donor serum. Afterwards, cells were stained with DAPI and nucleosome release was determined. Pre-incubation of the cells with all three different ANAs resulted in a decreased nucleosome release compared to the isotype control (**Figure 4A**). To investigate whether this inhibition was due to a direct inhibitory effect on FSAP-mediated

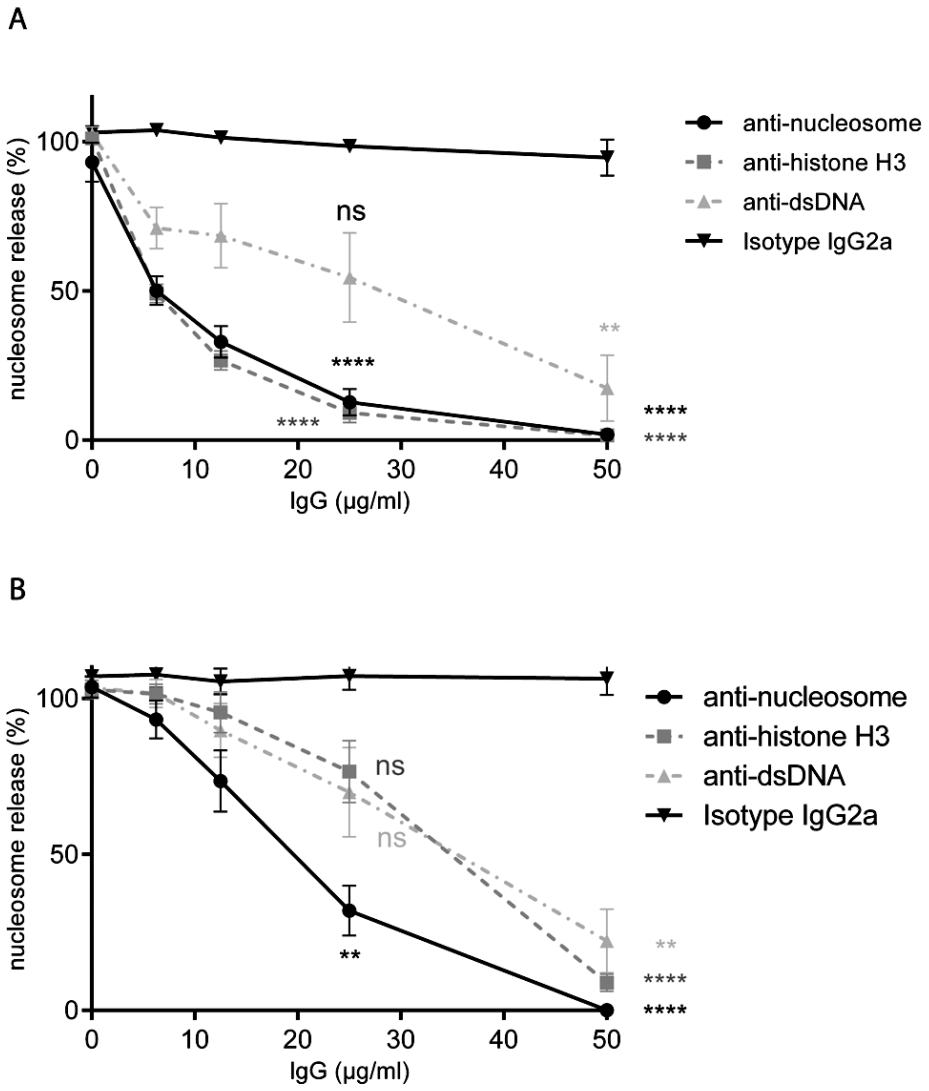

Figure 4. Inhibition of nucleosome release upon incubation with monoclonal anti-nuclear antibodies and with Fab fragments

(A, B) After induction of apoptosis, cells were incubated with monoclonal anti-nuclear antibodies (6.25-50 µg/ml) prior to incubation with (A) 10% serum of a healthy donor or (B) 10 U/ml plasma-purified tcFSAP. Cells were stained with propidium iodide (3 µg/ml) and analyzed by flow cytometry. The nucleosome release observed using 10% healthy donor serum was defined as 100%. 10 U/ml plasma-purified active FSAP corresponds to 10% serum. Data are shown as mean ± SEM of triplicates pooled from three independent experiments. Significance was calculated in relation to the isotype control using a two-tailed unpaired Student's t-test, ** $P < 0.01$, **** $P < 0.0001$, ns not significant.

nucleosome release, late apoptotic cells were incubated with the ANAs prior to incubation with plasma-purified active FSAP. Indeed, all three ANAs showed similar inhibition of nucleosome release by plasma-purified active FSAP as using serum (**Figure 4B**). These results illustrate the inhibitory effect of antinuclear antibodies on FSAP-mediated nucleosome release from late apoptotic cells.

To investigate whether ANAs block an FSAP specific target required for nucleosome release, Fab fragments were made of the ANAs and used in our assay. Due to the commonly observed decrease in affinity of created Fab fragments as compared to intact antibodies, only the Fab fragment of the anti-nucleosome antibody showed sufficient binding to the cells to allow for further analysis (data not shown). In **Figure 5** it can be seen that in a similar assay as described above, these Fab fragments were unable to inhibit nucleosome release. In contrast, when late apoptotic cells were incubated with anti-nucleosome Fab fragments followed by incubation with a monoclonal anti-light chain antibody in order to mimic antigen crosslinking by intact antibodies, nucleosome release was inhibited (**Figure 5**). This suggests that the observed inhibition of nucleosome release by ANAs is brought about by crosslinking of target antigen and the formation of large immune complexes, which cannot be released by FSAP.

DISCUSSION

Nucleosomes, or their constituents DNA and histones, are major intracellular auto-antigens in SLE. We have previously shown that the plasma protein factor VII-activating protease (FSAP) releases chromatin from late apoptotic and necrotic cells and that this chromatin can be detected extracellularly as nucleosomes[28,29]. In this study we demonstrate that sera from SLE patients show a decreased ability to release nucleosomes from late apoptotic cells during active disease (**Figure 1A**). This decreased ability correlates with both disease activity as well as anti-DNA levels (**Figure 1C**) in the sera and is unrelated to changes in FSAP levels (**Figure 2A**) or its activation (**Figure 2B-D**). Interestingly, we have found that serum FSAP-AP levels were elevated during low disease activity compared to high disease activity (**Figure 2B**). However, induction of FSAP activation through incubation of SLE sera with late apoptotic cells showed an equal increase of FSAP-AP levels in the low and high

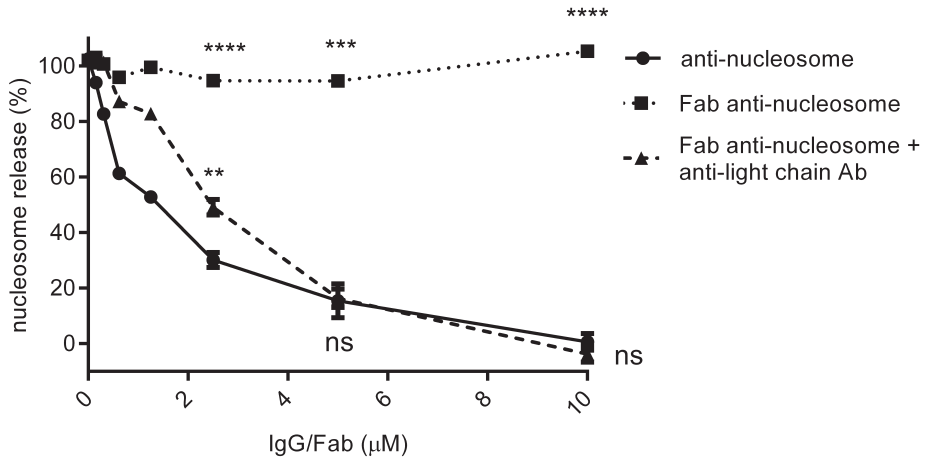

Figure 5. Inhibition of nucleosome release by anti-nuclear antibodies
After induction of apoptosis, cells were incubated with either intact anti-nucleosome antibodies or anti-nucleosome Fab fragments. Next, cells were washed and incubated with buffer or 100 μg/ml anti-light chain antibody and incubated with 10% serum from a healthy donor. Cells were stained with propidium iodide (3 μg/ml) and analyzed by flow cytometry. The nucleosome release observed using 10% serum of the healthy donor was defined as 100%. Isotype controls did not show inhibition of chromatin removal. Data are shown as mean ± SEM of triplicates pooled from three independent experiments. Significance was calculated in relation to the anti-nucleosome condition using a two-tailed unpaired Student's t-test, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$, ns not significant.

disease activity groups. This indicates that activation of FSAP, or alternatively its complex formation with AP is not inhibited during active disease, nor is the detection of FSAP-AP complexes hindered in these samples. Since the molecular mechanism responsible for FSAP activation *in vivo* has not been clearly identified yet, it is unclear whether the decreased activation of FSAP is a result of a decreased availability of its activator during active disease.

Late apoptotic cells have lost the integrity of their plasma membrane, which may expose the immune system to intracellular chromatin. Removal of chromatin may facilitate an immunologically silent clearance of late apoptotic cells in two ways. Firstly, it may prevent prolonged exposure of the immune system to potentially immunogenic chromatin and assist in its clearance. Indeed, nucleosomes that are released into the circulation in mice are very rapidly cleared by hepatocytes ($t_{½}$ = 10-30 min)[38]. Secondly, the presence of chromatin may influence the uptake of large cellular fragments by circulating phagocytes. We hypothesize that the release of nucleosomes from late apoptotic cells by FSAP may aid in the phagocytosis of cell remnants.

In support of this concept Liang *et al*. have shown that incubation of late apoptotic cells with serum leads to a decrease in DNA content and may lead to an increase in phagocytosis efficiency[27]. Notably, an impairment of phagocytosis of apoptotic cells brought about by an unidentified component in SLE serum has previously been observed when studying phagocytosis using mice macrophages[39]. However, it is unclear whether the observed impaired phagocytosis in that study may have been caused by the presence of anti-nuclear antibodies. Although nucleosome release by FSAP may contribute to efficient clearance of late apoptotic cells and nucleosomes themselves, circulating nucleosomes may also efficiently trigger the immune system or even have direct detrimental effects on cells[40].

In order to investigate whether anti-nuclear antibodies (ANAs) *e.g*. anti-nucleosome antibodies can interfere with nucleosome release, we tested the effects of mouse monoclonal ANAs on nucleosome release by both serum and plasma-purified FSAP. ANAs with different specificities all inhibited the release of nucleosomes. Furthermore, nucleosome release by plasma-purified FSAP was also inhibited, indicating that ANA's exert a direct effect on FSAP function. Our data shows that Fab fragments of a monoclonal ANA did not inhibit nucleosome release, suggesting that inhibition of nucleosome release is not likely to be attributed to blockage of a specific target for FSAP. Another possibility is that ANAs are able to cross-link nucleosomes in/on cells, thereby preventing their release. Indeed, by artificially crosslinking ANA Fab fragments through a monoclonal anti-light chain antibody, nucleosome release could be inhibited again. This supports the notion that crosslinking of nucleosomes and the formation of immune complexes form the main mechanism through which ANAs in SLE patients hinder the release of nucleosomes by FSAP from dead cells. In support of our observations that ANAs inhibit nucleosome release, others have found that circulating nucleosome levels in SLE patients inversely correlate with the level of anti-dsDNA antibodies[22,23]. The formation of immune complexes between ANAs and nucleosomes may lead to increased clearance of these complexes and thus lower levels of circulating nucleosomes. Additionally, the inhibition of nucleosome release by ANAs may account partly for decreased levels of circulating nucleosomes in patients with high ANA levels.

Recently, it has been shown that neutrophil extracellular traps

(NETs) are inefficiently degraded in sera from a group of SLE patients[41]. These NETs consist of DNA, histones and antimicrobial enzymes and can be secreted by neutrophils upon stimulation[42]. Two mechanisms are suggested to be causative in this impaired NET degradation: (i) the presence of DNaseI inhibitors or (ii) anti-NET antibodies that prevent DNaseI access to DNA in NETs. The second mechanism shows parallels with the anti-nuclear antibodies that inhibit nucleosome release from late apoptotic cells in our present study, as complex formation between ANAs and nucleosomes may prevent FSAP access to chromatin whilst it does not influence FSAP activation. Leffler *et al.* demonstrated that NETs activate complement *in vitro*, and SLE patients that have reduced ability to degrade NETs displayed lower levels of complement proteins C3 and C4 [43]. We found significantly lower levels of C3 and C4 in the serum of SLE patients with reduced nucleosome release (**Figure S1**). It is tempting to speculate that, in analogy to the non-degraded NETs that activate complement and the chromatin binding properties of C1q, late apoptotic cells that have not disposed of their nucleosomes can activate complement.

In summary, we showed that FSAP in serum obtained from SLE patients during active disease is unable to release nucleosomes from late apoptotic cells and that this impairment correlates with disease activity. The reduced nucleosome release is likely caused by the presence of antibodies which may exert their inhibitory effect through crosslinking of nuclear antigen (**Figure 6**).

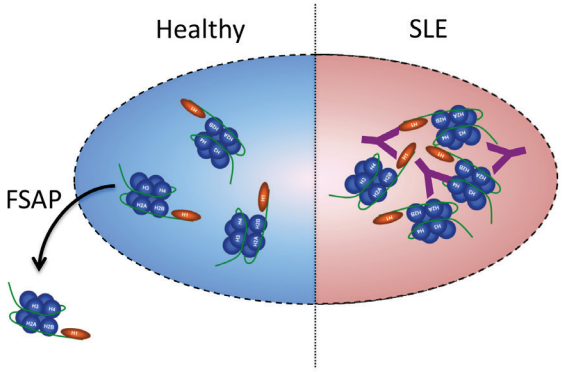

Figure 6. Mechanism of nucleosome release inhibition by anti-nuclear antibodies
In healthy individuals, FSAP is activated by late apoptotic and necrotic cells and releases nucleosomes from late apoptotic cells into the extracellular space, which may assist in the clearance of both nucleosomes as well as dead cell remnants. In SLE patients, FSAP is activated upon contact with late apoptotic cells, but nucleosome release is inhibited by the presence of anti-nuclear antibodies. These antibodies likely exert their inhibitory effect through cross-linking of nuclear antigen.

Impairment of nucleosome release correlates with the presence of anti-DNA antibodies and the consumption of complement factors C3 and C4, which may substantiate the ongoing pro-inflammatory response. Impaired nucleosome release may result in the persistence of DAMP-containing immune-complexes in dead tissue, thereby driving inflammation, the autoimmune response and progression of disease. These results reveal a new mechanism by which ANAs may contribute to impaired clearance of late apoptotic cells in SLE and contribute to the propagation of disease.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

REFERENCES

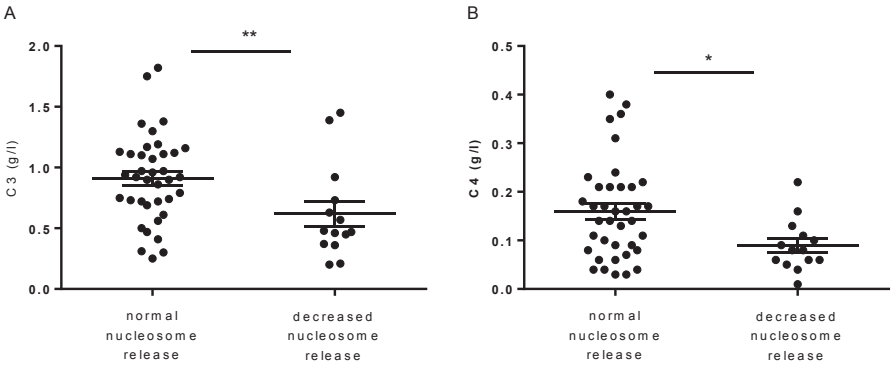
1. Dörner, T., Jacobi, A. M., Lee, J. & Lipsky, P. E. Abnormalities of B cell subsets in patients with systemic lupus erythematosus. *J. Immunol. Methods* **363,** 187–97 (2011).
2. Kalaaji, M. *et al.* Glomerular apoptotic nucleosomes are central target structures for nephritogenic antibodies in human SLE nephritis. *Kidney Int.* **71,** 664–72 (2007).
3. Yurasov, S. *et al.* Defective B cell tolerance checkpoints in systemic lupus erythematosus. *J. Exp. Med.* **201,** 703–11 (2005).
4. Gaipl, U. S. *et al.* Inefficient clearance of dying cells and autoreactivity. *Curr. Top. Microbiol. Immunol.* **305,** 161–76 (2006).
5. Muñoz, L. E., Lauber, K., Schiller, M., Manfredi, A. a & Herrmann, M. The role of defective clearance of apoptotic cells in systemic autoimmunity. *Nat. Rev. Rheumatol.* **6,** 280–289 (2010).
6. Fehr, E.-M. *et al.* Apoptotic-cell-derived membrane vesicles induce an alternative maturation of human dendritic cells which is disturbed in SLE. *J. Autoimmun.* **40,** 86–95 (2013).
7. Biermann, M. H. C. *et al.* The role of dead cell clearance in the etiology and pathogenesis of systemic lupus erythematosus: dendritic cells as potential targets. *Expert Rev. Clin. Immunol.* **10,** 1151–64 (2014).
8. Detanico, T., Guo, W. & Wysocki, L. J. Predominant role for activation-induced cytidine deaminase in generating IgG anti-nucleosomal antibodies of murine SLE. *J. Autoimmun.* **58,** 67–77 (2015).
9. Silva, M. T. Secondary necrosis: the natural outcome of the complete apoptotic program. *FEBS Lett.* **584,** 4491–9 (2010).
10. Vanden Berghe, T. *et al.* Necroptosis, necrosis and secondary necrosis converge on similar cellular disintegration features. *Cell Death Differ.* **17,** 922–30 (2010).
11. Urbonaviciute, V. *et al.* Induction of inflammatory and immune responses by HMGB1-nucleosome complexes: implications for the pathogenesis of SLE. *J. Exp. Med.* **205,** 3007–18 (2008).
12. Christensen, S. R. *et al.* Toll-like receptor 9 controls anti-DNA autoantibody production in murine lupus. *J. Exp. Med.* **202,** 321–31 (2005).
13. Wu, X. & Peng, S. L. Toll-like receptor 9 signaling protects against murine lupus. *Arthritis Rheum.* **54,** 336–42 (2006).
14. Christensen, S. R. *et al.* Toll-like receptor 7 and TLR9 dictate autoantibody specificity and have opposing inflammatory and regulatory roles in a murine model of lupus. *Immunity* **25,** 417–28 (2006).
15. Savarese, E. *et al.* Requirement of Toll-like receptor 7 for pristane-induced production of autoantibodies and development of murine lupus nephritis. *Arthritis Rheum.* **58,** 1107–15 (2008).
16. Demaria, O. *et al.* TLR8 deficiency leads to autoimmunity in mice. *J. Clin. Invest.* **120,** 3651–62 (2010).

17. Summers, S. A. *et al.* TLR9 and TLR4 are required for the development of autoimmunity and lupus nephritis in pristane nephropathy. *J. Autoimmun.* **35,** 291–8 (2010).
18. Urbonaviciute, V. *et al.* Toll-like receptor 2 is required for autoantibody production and development of renal disease in pristane-induced lupus. *Arthritis Rheum.* **65,** 1612–23 (2013).
19. Papadimitraki, E. D., Tzardi, M., Bertsias, G., Sotsiou, E. & Boumpas, D. T. Glomerular expression of toll-like receptor-9 in lupus nephritis but not in normal kidneys: implications for the amplification of the inflammatory response. *Lupus* **18,** 831–5 (2009).
20. Chen, R., Kang, R., Fan, X.-G. & Tang, D. Release and activity of histone in diseases. *Cell Death Dis.* **5,** e1370 (2014).
21. Li, T. *et al.* Anti-nucleosome antibodies outperform traditional biomarkers as longitudinal indicators of disease activity in systemic lupus erythematosus. *Rheumatology (Oxford).* **54,** 449–57 (2015).
22. Amoura, Z. *et al.* Circulating plasma levels of nucleosomes in patients with systemic lupus erythematosus: correlation with serum antinucleosome antibody titers and absence of clear association with disease activity. *Arthritis Rheum.* **40,** 2217–25 (1997).
23. Jørgensen, M. H., Rekvig, O. P., Jacobsen, R. S., Jacobsen, S. & Fenton, K. A. Circulating levels of chromatin fragments are inversely correlated with anti-dsDNA antibody levels in human and murine systemic lupus erythematosus. *Immunol. Lett.* **138,** 179–86 (2011).
24. van der Vlag, J. & Berden, J. H. M. Lupus nephritis: role of antinucleosome autoantibodies. *Semin. Nephrol.* **31,** 376–89 (2011).
25. Zeerleder, S. *et al.* A plasma nucleosome releasing factor (NRF) with serine protease activity is instrumental in removal of nucleosomes from secondary necrotic cells. *FEBS Lett.* **581,** 5382–8 (2007).
26. Stephan, F. *et al.* Cooperation of factor VII-activating protease and serum DNase I in the release of nucleosomes from necrotic cells. *Arthritis Rheumatol. (Hoboken, N.J.)* **66,** 686–93 (2014).
27. Liang, Y. Y. *et al.* Serum-dependent processing of late apoptotic cells for enhanced efferocytosis. *Cell Death Dis.* **5,** e1264 (2014).
28. Stephan, F. *et al.* Activation of factor VII-activating protease in human inflammation: a sensor for cell death. *Crit. Care* **15,** R110 (2011).
29. Zeerleder, S. *et al.* Nucleosome-releasing factor: a new role for factor VII-activating protease (FSAP). *FASEB J.* **22,** 4077–84 (2008).
30. Etscheid, M., Hunfeld, A., König, H., Seitz, R. & Dodt, J. Activation of proPHBSP, the zymogen of a plasma hyaluronan binding serine protease, by an intermolecular autocatalytic mechanism. *Biol. Chem.* **381,** 1223–31 (2000).
31. Yamamichi, S. *et al.* Extracellular histone induces plasma hyaluronan-binding protein (factor VII activating protease) activation in vivo. *Biochem. Biophys. Res. Commun.* **409,** 483–8 (2011).
32. Bombardier, C., Gladman, D. D., Urowitz, M. B., Caron, D. & Chang, C. H. Derivation of the

SLEDAI. A disease activity index for lupus patients. The Committee on Prognosis Studies in SLE. *Arthritis Rheum.* **35,** 630–40 (1992).

33. Roemisch, J., Feussner, A, Nerlich, C., Stoehr, H. & Weimer, T. The frequent Marburg I polymorphism impairs the pro-urokinase activating potency of the factor VII activating protease (FSAP). *Blood Coagul. Fibrinolysis* **13,** 433–41 (2002).
34. van Nieuwenhuijze, A. E. M., van Lopik, T., Smeenk, R. J. T. & Aarden, L. A. Time between onset of apoptosis and release of nucleosomes from apoptotic cells: putative implications for systemic lupus erythematosus. *Ann. Rheum. Dis.* **62,** 10–4 (2003).
35. Meilof, J. F., Van der Lelij, A., Rokeach, L. A., Hoch, S. O. & Smeenk, R. J. Autoimmunity and filariasis. Autoantibodies against cytoplasmic cellular proteins in sera of patients with onchocerciasis. *J. Immunol.* **151,** 5800–9 (1993).
36. Souwer, Y. *et al.* B cell receptor-mediated internalization of salmonella: a novel pathway for autonomous B cell activation and antibody production. *J. Immunol.* **182,** 7473–81 (2009).
37. Aarden, L. A., de Groot, E. R. & Feltkamp, T. E. Immunology of DNA. III. Crithidia luciliae, a simple substrate for the determination of anti-dsDNA with the immunofluorescence technique. *Ann. N. Y. Acad. Sci.* **254,** 505–15 (1975).
38. Gauthier, V. J., Tyler, L. N. & Mannik, M. Blood clearance kinetics and liver uptake of mononucleosomes in mice. *J. Immunol.* **156,** 1151–6 (1996).
39. Licht, R., Dieker, J. W. C., Jacobs, C. W. M., Tax, W. J. M. & Berden, J. H. M. Decreased phagocytosis of apoptotic cells in diseased SLE mice. *J. Autoimmun.* **22,** 139–45 (2004).
40. Decker, P., Wolburg, H. & Rammensee, H. G. Nucleosomes induce lymphocyte necrosis. *Eur. J. Immunol.* **33,** 1978–1987 (2003).
41. Hakkim, A. *et al.* Impairment of neutrophil extracellular trap degradation is associated with lupus nephritis. *Proc. Natl. Acad. Sci. U. S. A.* **107,** 9813–8 (2010).
42. Brinkmann, V. *et al.* Neutrophil extracellular traps kill bacteria. *Science* **303,** 1532–5 (2004).
43. Leffler, J. *et al.* Neutrophil extracellular traps that are not degraded in systemic lupus erythematosus activate complement exacerbating the disease. *J. Immunol.* **188,** 3522–31 (2012).

SUPPORTING INFORMATION



Supporting Information Figure 1: C3 and C4 levels in serum from SLE patients
Serum levels of complement factors C3 (A) and C4 (B) were measured in sera from SLE patients. C3 and C4 levels were compared between sera that displayed normal or decreased nucleosome release; Sera with a nucleosome releasing activity of >3 SDs above the mean nucleosome releasing activity were defined as sera with normal nucleosome releasing capabilities. Asterisks indicate significant differences (unpaired Student's t test) between the groups indicated by brackets, * $P < 0.05$.