Factor VII-activating protease: Mechanism and regulation of nucleosome release from dead cells
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Inhibition of nucleosome releasing activity in serum of patients with systemic lupus erythematosus


* These authors contributed equally to this paper

Submitted for publication
Abstract

Objectives: Systemic lupus erythematosus (SLE) is a chronic, inflammatory and multifactorial autoimmune disease. Inefficient removal of dying cells and a loss of self-tolerance due to a dysregulated immune response seem to be involved in the pathogenesis of SLE. Plasma proteins have been reported to play a role in the removal of dead cells. Inadequately removed apoptotic cells expose the immune system to intracellular contents, which may result in the formation of autoantibodies. Serum removes nucleosomes from late apoptotic and necrotic cells. The plasma serine protease factor-VII activating protease (FSAP) is responsible for this activity. Since nucleosomes play a prominent role in the pathogenesis of SLE, we investigated nucleosome release by serum from SLE patients.

Methods: Late apoptotic Jurkat cells were incubated with serum of SLE patients and healthy controls and nucleosome release was analyzed by flow cytometry.

Results: Nucleosome release by sera of SLE patients with high disease activity is significantly decreased as compared to healthy subjects (p<0.01). This decrease cannot be explained by decreased FSAP serum levels or disturbed activation of FSAP. Decreased nucleosome releasing activity correlates with SLEDAI (r=0.52, p<0.001) and anti-DNA antibodies (r=0.55, p<0.001). Removal of IgG and IgM antibodies from sera of SLE patients restored nucleosome releasing activity. Addition of monoclonal antinuclear antibodies to sera from healthy subjects resulted in inhibition of nucleosome release.

Conclusion: SLE patients in exacerbation show decreased nucleosome release which is mainly due to antinuclear antibodies. This inhibition may contribute to the impaired clearance of dead cells and propagation of disease.
Introduction
Systemic lupus erythematosus (SLE) is a chronic inflammatory autoimmune disease, characterized by exacerbations and remissions with variable clinical manifestations. The disease is characterized by an antigen-driven T-cell-dependent immune response, B cell hyperactivity, the production of autoantibodies and formation of immune complexes (1;2). Different mechanisms are suggested to cause SLE, indicating a multifactorial etiology. Among others, inefficient removal of dying cells and loss of self-tolerance due to a dysregulated immune response seem to be critically involved in the pathogenesis of SLE. Plasma proteins, e.g components of the classical complement pathway have been reported to play a crucial role in the removal of dead cells. Defects in this pathway are strongly associated with the development of SLE (3). On the other hand, the activation of complement is known to play a major role in the inflammatory process in SLE (4).

One of the main characteristics of SLE is the formation of antinuclear antibodies (ANAs) directed against ubiquitous intracellular antigens, e.g. double stranded (ds)-DNA and histones. These ANAs form immune complexes which can contribute to the pathogenesis of SLE upon deposition. Remarkably, a number of these immunogenic endogenous proteins are damage-associated molecular pattern molecules (DAMPs). Several DAMPs such as DNA, RNA, histones and the DNA-binding protein HMGB1 are known to stimulate Toll-like receptors (TLRs) and are critically involved in the pathogenesis of SLE models (5-12). Hence, circulating nucleosomes might function as endogenous danger signals. High levels of circulating nucleosomes and anti-nucleosome antibodies have been found in patients with SLE (13-15). Furthermore, nucleosome-specific autoantibodies are detectable in murine lupus models before the onset of other autoantibody specificities (15).

We have previously shown that serum removes nucleosomes from late apoptotic and necrotic cells (16;17). The serum factor responsible for this nucleosome release is factor VII-activating protease (FSAP) (17). FSAP, also known as plasma hyaluronic acid binding protein 2 (HABP2), is a serine protease circulating as an inactive single-chain molecule of 78 kDa in plasma. It is proteolytically converted in its active two-chain form consisting of a 50 kDa heavy and a 28 kDa light chain, connected by a disulfide bond (18). FSAP has been described to become activated upon contact with either late apoptotic or necrotic cells (19) and circulating histones (20). Since nucleosomes play a central role in the antinuclear antibody response in SLE and FSAP has been shown to release nucleosomes from late apoptotic cells, we investigated whether the nucleosome releasing activity of serum is disturbed in patients with SLE.

Materials and Methods
Patient sera
Twenty-seven SLE patients were randomly selected of whom the mean age was 38 years
(range, 18-64 years) and 89% of the patients were woman. Sera were taken at two time points, of which the one with the higher Systemic Lupus Erythematosus Disease Activity Index (SLEDAI) score (21), at least ≥ 4, was referred to as “high disease activity” and the one with low SLEDAI score (SLEDAI ≤ 2) was referred to as “low disease activity”. Patient characteristics and prevalence of the specific American College of Rheumatology classification criteria for SLE are summarized in Table 1. Antibodies to ds-DNA were measured using EliA dsDNA from Phadia (fluoroenzym-immunoassay on the ImmunoCAP instrument). Complement levels C3 and C4 were detected using nephelometry. Sera were taken from healthy donors and all donors were homozygous for the wild-type form of FSAP. All sera have been obtained as anonymized samples from the diagnostic laboratory, according to the Dutch rules and regulation for the use of patient material.

Table 1 Disease characteristics of SLE patients

<table>
<thead>
<tr>
<th></th>
<th>exacerbation</th>
<th>remission</th>
</tr>
</thead>
<tbody>
<tr>
<td>SLEDAI</td>
<td>8 (7-11)</td>
<td>0 (0-2)</td>
</tr>
<tr>
<td>Anti-dsDNA, Farr (IU/ml)</td>
<td>66.5 (33-699)</td>
<td>14 (6.5-52)</td>
</tr>
<tr>
<td>C3, g/l</td>
<td>0.57 (0.45-0.97)</td>
<td>0.94 (0.75-1.14)</td>
</tr>
<tr>
<td>C4, g/l</td>
<td>0.08 (0.06-0.13)</td>
<td>0.17 (0.12-0.21)</td>
</tr>
<tr>
<td>ACR criteria, n (%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Malar rash</td>
<td>7 (26%)</td>
<td></td>
</tr>
<tr>
<td>Discoid rash</td>
<td>9 (33%)</td>
<td></td>
</tr>
<tr>
<td>Photosensitivity</td>
<td>9 (33%)</td>
<td></td>
</tr>
<tr>
<td>Oral ulcers</td>
<td>4 (15%)</td>
<td></td>
</tr>
<tr>
<td>Arthritis</td>
<td>14 (52%)</td>
<td></td>
</tr>
<tr>
<td>Serositis</td>
<td>10 (37%)</td>
<td></td>
</tr>
<tr>
<td>Renal disorder</td>
<td>14 (52%)</td>
<td></td>
</tr>
<tr>
<td>Neurologic disorder</td>
<td>4 (15%)</td>
<td></td>
</tr>
<tr>
<td>Haematologic disorder</td>
<td>21 (78%)</td>
<td></td>
</tr>
<tr>
<td>Immunologic disorder</td>
<td>25 (93%)</td>
<td></td>
</tr>
<tr>
<td>Antinuclear antibody</td>
<td>27 (100%)</td>
<td></td>
</tr>
<tr>
<td>Prednisolone use, n (%)</td>
<td>11 (41%)</td>
<td>22 (81%)</td>
</tr>
<tr>
<td>Daily mean dose, mg</td>
<td>7.5 (5.2-22.5)</td>
<td>10 (6.3-27.5)</td>
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<tr>
<td>Hydroxychloroquine use, n (%)</td>
<td>10 (37%)</td>
<td>12 (44%)</td>
</tr>
<tr>
<td>Daily mean dose, mg</td>
<td>400 (400-400)</td>
<td>400 (400-400)</td>
</tr>
<tr>
<td>Azathioprine use, n (%)</td>
<td>5 (19%)</td>
<td>7 (26%)</td>
</tr>
<tr>
<td>Daily mean dose, mg</td>
<td>100 (75-150)</td>
<td>125 (112.5-137.5)</td>
</tr>
</tbody>
</table>

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

**Reagents**

Mouse monoclonal antibodies to FSAP (17), α₂-antiplasmin (AAP20), kappa light chain and monoclonal antinuclear antibodies (ANAs) (22) were prepared at our department. IMDM was obtained from BioWhittaker Europe (Verviers, Belgium). FCS was obtained from Bodinco BV (Alkmaar, The Netherlands). Penicillin and streptomycin were obtained from Gibco/Invitrogen (Groningen, The Netherlands). RNase A, β-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 was 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).

**Cell culture and induction of apoptosis**

Jurkat cells were cultured in Iscove’s Modified Dulbecco’s Medium (IMDM) containing 5% (v/v) fetal calf serum (FCS), penicillin (100 IU/ml), streptomycin (100 µg/ml) and 50 µM β-mercaptoethanol. Before apoptosis induction, cells were washed with serum-free IMDM by centrifugation at 360 x g for 10 minutes and resuspended in serum-free IMDM. Cells (1x10⁶ cells/ml) were incubated for 48 hours with etoposide in a final concentration of 200 µM to induce apoptosis.

**Nucleosome release**

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 minutes at 37°C. Subsequently cells were added to the sera and incubated for another 30 minutes at 37°C. To examine the release of nucleosomes, cells were stained with propidium iodide (PI) diluted in HN buffer (3 µg/ml) to detect the remaining amount of DNA. Samples were analyzed by flow cytometry. The median fluorescence intensity of PI 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% nucleosome release.

**FSAP ELISA**

FSAP antigen levels were determined by ELISA as recently described (17). Briefly, a monoclonal antibody (anti-FSAP4) which recognizes the light chain of FSAP was used as a catching antibody. Biotinylated anti-FSAP2 recognizing the heavy chain of FSAP in combination with HRP labeled streptavidin was used for detection.
FSAP-$\alpha_2$-antiplasmin complex ELISA

FSAP-$\alpha_2$-antiplasmin (FSAP-AP) complex levels were determined by ELISA as recently described (19). Briefly, a monoclonal antibody (AAP20) 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 Sepharose 4B-coupled protein G with 1 ml of serum overnight at 4°C. IgG-depleted serum was recovered after centrifugation and IgG depletion was verified by ELISA (23).

IgM was depleted from serum by incubating 2 ml of an anti-IgM fragment coupled to NHS-activated Sepharose 4 Fast Flow with 0.5 ml of serum for 4 hours at RT. IgM-depleted serum was recovered after centrifugation and IgM depletion was verified by ELISA (24).

Fab fragments

To produce F(ab')2 fragments, 0.8 mg/ml antibody was incubated with 20 μg/ml pepsin for 24hr 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 hours at 4°C. After centrifugation, the F(ab')2 fragments were recovered and subjected to 10 mM dithiotreitol for 30 minutes to make monovalent Fab fragments. Free thiol groups were blocked with 20mM of N-ethylmaleimide and Fab fragments were dialyzed to PBS.

Statistics

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

Results

Nucleosome releasing activity in serum of SLE patients

Late apoptotic cells were incubated with sera of 27 SLE patients during exacerbation of the disease as well as in remission and 30 healthy donors for 30 minutes and cells were stained with propidium iodide (PI) to detect the amount of DNA that remains in the cells. The nucleosome releasing activity of a reference serum of a healthy donor was defined as 100% nucleosome release. A significant number of SLE sera showed a decreased nucleosome releasing activity compared to the sera of healthy donors. Interestingly, nucleosome releasing activity was significantly lower during exacerbation compared to low disease activity (Figure
Decreased nucleosome release by serum of SLE patients

1). We correlated the decrease in nucleosome releasing activity with two established disease activity parameters, the SLEDAI score and levels of anti-dsDNA antibodies. These analyses showed that there was a correlation with SLEDAI score ($r = 0.54$ $P<0.001$). Interestingly, the decrease in nucleosome releasing activity correlated strongly with the levels of anti-DNA antibodies ($r = 0.55$ $P<0.001$). To investigate a possible difference in complement consumption between patients with either normal or decreased nucleosome release, C3 and C4 levels were measured. Both C3 and C4 levels were reduced in the sera with decreased nucleosome release (Figure 2). This indicates that in those patients there is more activation and consumption of complement.

Factor VII-activating protease in serum

To elucidate why some SLE sera cannot release nucleosomes from late apoptotic cells, we first tested whether levels of FSAP, the factor in serum that releases nucleosomes, were comparable to healthy donors. All patients have normal levels of FSAP in the sera as measured by ELISA (Figure 3). To examine whether FSAP is less susceptible for activation in the SLE
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Sera with decreased nucleosome release, activation of FSAP was detected by measuring the complex formation of FSAP with its inhibitor α₂-antiplasmin (AP) after incubation with late apoptotic cells. FSAP-AP complexes could be detected in the SLE sera, which indicates that FSAP present in SLE serum becomes activated upon contact with apoptotic cells, comparable

Figure 2: C3 and C4 levels in serum of SLE patients
Serum levels of complement factors C3 (A) and C4 (B) were measured in sera of SLE patients with low disease activity and in high disease activity. C3 and C4 levels were compared for normal and reduced nucleosome releasing sera. Patient sera with a nucleosome release below 3 SDs from the mean of healthy controls were defined as sera with reduced nucleosome release. * indicates a P < 0.05.

Sera with decreased nucleosome release, activation of FSAP was detected by measuring the complex formation of FSAP with its inhibitor α₂-antiplasmin (AP) after incubation with late apoptotic cells. FSAP-AP complexes could be detected in the SLE sera, which indicates that FSAP present in SLE serum becomes activated upon contact with apoptotic cells, comparable
Decreased nucleosome release by serum of SLE patients

**Inhibitory mechanism of nucleosome release**

Since no alteration in FSAP levels or activation was observed in the SLE sera, we hypothesized that an inhibitor of nucleosome release could be present in the sera. We investigated the presence of an inhibitor in two patient sera that completely lacked nucleosome releasing activity. Serum of a healthy donor with normal nucleosome releasing activity was preincubated with serum of the SLE patients and subsequently incubated with late apoptotic cells. The nucleosome releasing activity of the healthy donor was set at 100%. After preincubation of the serum of the healthy donor with the SLE sera, no nucleosome release could be detected, indicating that an inhibitor in the SLE sera can inhibit the FSAP activity of the healthy donor. Preincubation with serum of a healthy control did not show this effect. Since there is a strong inverse correlation between decrease in nucleosome releasing activity and anti-DNA antibodies in the sera, we tested whether antibodies were involved in this inhibition. SLE sera were depleted for IgG and again incubated with serum of a healthy donor prior to incubation.

**Figure 3: FSAP antigen levels in serum of SLE patients**

Levels of FSAP antigen were measured in sera of SLE patients by means of ELISA. FSAP levels were expressed as AU/ml. Serum of 30 healthy donors was taken as a control.

to FSAP in healthy donor serum (data not shown). These data suggest that neither changes in FSAP levels nor inhibition of the activation accounted for the decrease in nucleosome releasing activity.
with late apoptotic cells. After IgG depletion, the serum of patient 1 was not able to inhibit the nucleosome release by the healthy donor serum, suggesting that serum IgG is responsible for the decrease in nucleosome releasing activity in this patient. In patient 2 however, inhibition of the healthy donor serum is still present after IgG depletion, suggesting that the inhibition is not induced by IgG antibodies in this patient. Notably, 80% of the anti-DNA antibodies in patient 2 were of the IgM class (25). To investigate whether IgM antibodies have an inhibitory effect on nucleosome release in patient 2, serum of patient 2 was depleted for IgM and tested for inhibition. Indeed, after IgM depletion, serum of patient 2 was not able to inhibit the nucleosome release by the healthy donor serum (Figure 4). This shows that in addition to IgG antibodies, IgM antibodies are also able to inhibit nucleosome release by healthy donor serum.

**Nucleosome-masking antibodies prevent nucleosome release**

Since antibodies in the serum are responsible for the inhibition of nucleosome release, antinuclear antibodies (ANAs) are likely candidates. To test this, late apoptotic cells were incubated with mouse monoclonal ANAs prior to incubation with healthy donor serum. Subsequently, cells were stained with propidium iodide and nucleosome release was determined. Preincubation of the cells with ANAs inhibited the nucleosome release by serum.
Decreased nucleosome release by serum of SLE patients

**Figure 5:** Inhibition of nucleosome release by incubation with intact monoclonal antinuclear antibodies or Fab fragments

(A) After induction of apoptosis, cells were incubated with monoclonal antinuclear antibodies (12.5 µg/ml) prior to incubation with 20% serum of a healthy donor. ANA58 recognizes nucleosomes, ANA60 recognizes histone H3 and ANA123 recognizes double-stranded DNA. Cells were stained with propidium iodide (3 µg/ml) and analyzed by flow cytometry. The nucleosome releasing activity of 20% serum of the healthy donor was defined as 100% nucleosome release. (B) After induction of apoptosis, cells were incubated with intact ANA58 or ANA58 Fab fragments. Next, cells were washed and incubated with buffer or 100 µg/ml anti-light chain antibody and incubated with 20% serum of a healthy donor. Cells were stained with propidium iodide (3 µg/ml) and analyzed by flow cytometry. The nucleosome releasing activity of 20% serum of the healthy donor was defined as 100% nucleosome release. Isotype controls did not show any inhibition of nucleosome release.

of a healthy donor (Figure 5A). These results illustrate the inhibitory role of antinuclear antibodies on nucleosome release by serum.

To investigate whether ANAs block a specific target required for nucleosome release or form large immune complexes that are unable to be released, Fab fragments were made of the ANAs and used in our assay. Due to the decreased affinity of the created Fab fragments as compared to intact antibodies, only Fab fragments of ANA58 still showed normal binding to the cells (data not shown). However, Figure 5B shows that these Fab fragments were unable to inhibit nucleosome release. In contrast, when late apoptotic cells were incubated with ANA58 Fab fragments followed by incubation with a monoclonal anti-light chain antibody in order to mimic the crosslinking of intact antibodies, nucleosome release was inhibited (Figure 5B). This suggests that the previously described inhibition of nucleosome release by ANAs is due to crosslinking of target antigen and the formation of large immune complexes which cannot be released by FSAP.
Discussion

We have previously shown that the serum protease FSAP removes nucleosomes from late apoptotic and necrotic cells. In this study we demonstrate that serum of a subset of SLE patients, with high disease activity and high anti-DNA levels, show a disturbed nucleosome releasing activity.

Delayed removal of early apoptotic cells results in the appearance of late apoptotic cells. Late apoptotic cells have lost their membrane integrity and expose the immune system to their intracellular contents. This may induce an autoreactive immune response, lead to the formation of autoantibodies and contribute to the pathogenesis of SLE.

FSAP removes nucleosomes from late apoptotic and necrotic cells (17;19). In this study we demonstrate that serum of some SLE patients show a disturbed nucleosome releasing activity. This disturbance in nucleosome release appeared to be unrelated to changes in FSAP levels and activation. The decrease in nucleosome release correlated with disease activity and more strongly with anti-DNA levels in the sera. We observed that antibodies in the sera cause the reduced nucleosome release. These antibodies are probably directed against (parts of) nucleosomes since a number of SLE sera interfered with the detection of nucleosomes by ELISA (data not shown).

In order to investigate whether ANAs, e.g. anti-nucleosome antibodies can interfere with nucleosome release we tested the effects of mouse monoclonal ANAs on nucleosome release by serum. ANAs with different specificities all inhibit the nucleosome releasing activity of serum. Our data shows that Fab fragments of monoclonal ANAs did not inhibit nucleosome release, suggesting that not a specific target of FSAP is blocked. Another possibility is that ANAs are able to cross-link nucleosomes in/on cells, thereby preventing nucleosome release. Indeed, by artificially crosslinking Fab fragments through a monoclonal anti-light chain antibody, inhibition of nucleosome release is restored again. This supports the notion that crosslinking of DNA e.g. nucleosomes is the main mechanism through which ANAs in SLE patients inhibit the release of nucleosomes by FSAP.

In absence of serum, nucleosomes remain bound to late apoptotic cells (26). Circulating phagocytes may not be very efficient in taking up these large fragments of cellular material. The removal of nucleosomes from late apoptotic cells may help in phagocytosis of the cell remnants and prevent exposition of the immunogenic intracellular content. The nucleosomes that are released into the circulation are rapidly cleared by hepatocytes (27). When FSAP is not able to remove the nucleosomes from the apoptotic cells, the nucleosomes, which stay attached to the cells, persist longer in the circulation and can play an immunogenic and pathogenic role in the development of lupus nephritis. This may lead to a vicious cycle leading to more anti-nucleosome autoantibodies and exacerbation of the disease.

Recent data indicates that DAMP-mediated TLR activation may drive the immune response...
and autoantibody production in several SLE models (5-12). Urbaniaviciute et al. demonstrated that HMGB1 remains bound to nucleosomes released from late apoptotic cells and induced a pro-inflammatory response and anti-dsDNA/anti-histone IgG responses in a TLR2–dependent manner in vitro (28). HMGB1–nucleosome complexes were also detected in plasma from SLE patients. HMGB1-free nucleosomes from viable cells did not induce cytokine production or dendritic cell activation. Nucleosomes that are removed from late apoptotic cells by FSAP might lack the HMGB1 and thereby become less immunogenic. It would be interesting to investigate whether the nucleosomes released from late apoptotic cells by recombinant/purified FSAP still contain HMGB1.

Recently, it has been shown that serum of a group of SLE patients lacks the ability to degrade neutrophil extracellular traps (NETs) (29). These NETs consist of DNA, histones and antimicrobial enzymes and can be secreted by neutrophils (30). Two mechanisms are causative in this impaired NET degradation: (i) the presence of DNase I inhibitors or (ii) anti-NET antibodies that prevent DNase I access to NETs (29). The second mechanism might be comparable with the mechanism that is causing the decreased nucleosome release from late apoptotic cells. 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 (31). The complement system seems to play a central role in SLE, which is illustrated by the fact that deficiencies in the classical complement pathway are strongly associated with the development of SLE (3). On the other hand SLE is associated with complement activation and deposition of complement proteins in inflamed tissues. We show significant lower levels of C3 and C4 in the serum of SLE patients with reduced nucleosome release. It is tempting to speculate, that in analogy to the undegraded NETs that activate complement, late apoptotic cells that have not been disposed of their nucleosomes can activate complement. Therefore it would be interesting to investigate complement activation by late apoptotic cells of which the nucleosomes are released by FSAP and cells which still contain their nucleosomes.

In summary, we showed that serum of a subset of SLE patients show an impaired nucleosome releasing activity. This reduced nucleosome release is caused by the presence of antibodies which may exert their inhibitory effect through crosslinking of nuclear antigen. Impairment of nucleosome release correlated with anti-DNA antibodies and activation and consumption of complement factors C3 and C4 which might lead to pro-inflammatory responses. Impaired nucleosome release may result in persistence of the nucleosomes in the circulation leading to a vicious cycle of more anti-nucleosome autoantibodies and exacerbation of the disease.

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References

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