

Original Article

Loading of nuclear autoantigens prototypically recognized by systemic lupus erythematosus sera into late apoptotic vesicles requires intact microtubules and myosin light chain kinase activity

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Summary

Most cases of systemic lupus erythematosus (SLE) are characterized by an impaired clearance of apoptotic cells in various tissues. Non-cleared apoptotic waste is considered an immunogen driving the autoimmune response in patients with SLE. During the execution of apoptosis, membrane blebs are formed and filled with cellular components. Here, we evaluate the cytoskeletal pathway(s) responsible for the loading of SLE prototypic nuclear autoantigens into the apoptotic cell-derived membranous vesicles (ACMV) generated during late phases of apoptosis. HeLa cells expressing a fusion protein of histone H2B with green fluorescent protein (GFP) were irradiated with ultraviolet (UV)-B to induce apoptosis. The appearance and trafficking of chromatin-derived material was monitored by fluorescence microscopy. Specific inhibitors of cytoskeletal pathways were employed to identify the motile elements involved in translocation and trafficking of the nuclear components. We observed that immediately after their appearance the ACMV did not contain histone H2B^{GFP}; in this phase the fluorescence was contained in the nuclear remnants and the cytoplasm. Within consecutive minutes the ACMV were loaded with chromatin-derived material, whereas the loading of simultaneously created ACMV with histone H2B^{GFP} was not uniform. Some ACMV were preferentially filled and, consequently, showed a remarkably higher histone H2B^{GFP} accumulation. Inhibitors of the cytoskeleton revealed that functional microtubules and myosin light chain kinase are required for nuclear shrinkage and loading of nuclear material into the ACMV, respectively.

Keywords: apoptosis, blebbing, nuclear autoantigens, systemic lupus erythematosus

Introduction

Anti-nuclear autoantibodies appear in high titres in certain systemic rheumatic autoimmune diseases, such as systemic sclerosis [1], mixed connective tissue disease [2], juvenile idiopathic arthritis [3], chronic iridocyclitis [4] and, especially, systemic lupus erythematosus (SLE) [5]. These chronic inflammatory diseases are characterized by the presence of autoantibodies and immune complex deposition with activation of complement in various tissues causing acute or/and chronic inflammation and tissue damage [6,7]. This suggests an autoantigen-driven immune response to intracellular components altered and presented during cell death. It has been shown that the defective clearance of apoptotic cells and the accumulation of debris are crucial for the aetiopathogenesis of systemic autoimmunity [8].

Apoptosis is a regulated/programmed physiological process of cell death which plays an essential role in tissue turnover and homeostasis and has to be distinguished from the accidental, traumatic and un-regulated form of cell death usually referred to as necrosis [9]. The former occurs as a response to either noxious stimuli (extrinsic pathway) or to extracellular ligands activating the death receptors [Fas, tumour necrosis factor receptor (TNFR)-1], or is induced by the lack of survival signals, respectively [10].

Histones are basic nuclear chromatin proteins of eukaryotic cells. They operate like spools on which the cellular DNA is organized into nucleosomes; thus, they play a crucial role in condensing and stabilizing the chromatin and in regulating the transcription of genes. Five major classes of histones are known. H2A, H2B, H3 and H4 form a core structure referred to as nucleosome that serves as framework for the DNA double-strand helix. The linker histones H1/H5 are localized between the individual nucleosomes [11]. During apoptosis, the effector caspases 3, 6 and 7 are activated and cleave certain target proteins. This causes the breakdown of structures essential for the integrity of the cellular and subcellular architecture [12]. The cleavage of A- and B-type lamins, actin and ICAD (inhibitor of caspase activated DNase, thereby freeing caspase activated DNase, CAD) causes the disassembly of the nucleus [13], the cytoskeleton [14] and chromatin [15], respectively. CAD initially generates high molecular weight DNA fragments by cleaving few internucleosomal sites of the chromatin, followed by the generation of mono- and oligonucleosomes, which occur as a so-called 'DNA ladder' in agarose gel electrophoresis [16,17]. The early fragmentation and extranuclear accumulation of chromatin fragments is a feature discriminating the apoptotic process from primary necrosis, where nuclei initially remain unaffected. A further characteristic of apoptosis is their swift non-phlogistic clearance by phagocytes, especially by macrophages [18]. These cells take up apoptotic cells and immediately degrade their macromol-

ecules into small recyclable units such as nucleosides and amino acids. This highly efficient process avoids uncontrolled contact of the immune system with the corpses of dying cells, the challenge of tolerance and finally autoimmunity [19].

Indeed, studies employing monocyte-derived macrophages of patients with SLE revealed an impaired clearance of apoptotic cell material in several patients [20]. This observation, the highly increased incidence of SLE in complement-deficient individuals, and several animal models of clearance deficiency (MFG-E8^{KO} [21], DNase 2^{KO} [22], sIgM^{KO} [23], lupus-prone mouse strains [24]), suggest that a deficient or impaired clearance of apoptotic cells is causally involved in the aetiology and the pathogenesis of SLE [7,25]. The major autoimmunogen of SLE is discussed to be secondary necrotic material evolved from apoptotic cells that had escaped proper clearance.

Late apoptosis and secondary necrosis are rare events in healthy subjects and the metabolism of nuclear autoantigens in late apoptotic cells remains elusive. Thus, to assess the fate of nuclear material during the stages of apoptosis, we employed HeLa cells expressing the fluorescent histone H2B^{GFP} fusion protein and monitored localization and trafficking of nuclear material in the dying cells. We analysed the cellular and nuclear remnants and various apoptotic cell-derived membranous vesicles (ACMV) during late apoptosis in the presence and absence of inhibitors of cell dynamics *in vitro*.

Here we report that the small (< 1 µm) ACMV^S and medium-sized (1–3 µm) ACMV^M formed during the early stages of apoptosis never contained histone H2B^{GFP}. In contrast, in some of the large (> 3 µm) ACMV^L formed during later stages of cell death, histone H2B^{GFP} can be detected readily. Importantly, the vesicles do not contain histone H2B^{GFP} *in statu nascendi*, but some vesicles are loaded within a couple of minutes. Although the ACMV^L are seemingly morphologically homogeneous, they differ in their load of nuclear autoantigens.

These findings corroborate the hypothesis that the loading of cleaved nuclear material into the ACMV^L during late stages of apoptosis is not just a passive random process, but an active and regulated process. Experiments with inhibitors for cytoskeletal remodelling revealed that microtubules and myosin light chain kinase (MLCK) are required for nuclear shrinkage and for the sorting of degraded chromatin into the ACMV^L, respectively.

Materials and methods

Stable transfection

Human HeLa cells were transfected with the pBOS-H2B^{GFP} plasmid for expression of a histone H2B-GFP fusion protein (BD Pharmingen, Heidelberg, Germany) using FuGENE® transfection reagent (Promega, Mannheim,

Table 1. Ultraviolet (UV)-B-induced apoptosis in the presence of inhibitors of cellular dynamic and motility.

Target	Inhibitor	Action	Inhibitor (conc.)	Observations
F-actin	Jasplakinolide	Stabilizer of F-actin	50 nM	Cells disaggregate immediately into small exocytotic vesicles. Weak contraction of cell body. Late ACMV ^L contain H2B ^{GFP}
G-actin	Latrunculin A	Inhibitor of G-actin	5 μM	Intermediate exocytotic vesicles. Plastic nuclei, plane periphery with small motile branches. Late ACMV ^L contain H2B ^{GFP}
p160ROCK	(R)-(+)-trans-N-(4-Pyridyl)-4-(1-aminoethyl)-cyclohexanecarboxamide (Y27632)	Inhibitor of Rho-associated protein kinase (p160ROCK)	5 μM	Some cells: strong contraction, extensive blebbing and chromatin condensation. Other cells cannot contract properly, sporadic late ACMV ^L contain H2B ^{GFP}
Myosin S1 ATPase	N-Benzyl- <i>p</i> -toluenesulphonamide (BTS)	Inhibitor of myosin S1-ATPase	125 μM	Cells bring their morphology into a round figure: strong contraction and condensation of chromatin. Late ACMV ^L contain H2B ^{GFP}
MLCK	1-(5-chloronaphthalene-1-sulphonyl)homopiperazine, HCl (ML-9)	Inhibitor of myosin light chain kinase (MLCK)	10 μM	No characteristic morphological changes during apoptosis. Strong condensation of chromatin and shrinkage of nuclei. No H2B ^{GFP} in late ACMV ^L
Tubulin (β-subunit)	Baccatin III N-benzyl-β-phenylisoserine Ester, Taxol [®] (Paclitaxel)	Stabilizer of microtubules	25 μM	No characteristic morphological changes during apoptosis. Faint condensation of chromatin, no/weak shrinkage of nuclei. No H2B ^{GFP} in late ACMV ^L

For each condition a total of approximately 50 cells was observed and analysed accurately in fluorescence microscopy. ACMV = apoptotic cell-derived membranous vesicles; H2B^{GFP} = histone 2-green fluorescent protein; ML-9 = 1-(5-chloronaphthalene-1-sulphonyl)-1H-hexahydro-1,4-diazepine. MLCK = myosin light chain kinase; ROCK = Rho-associated protein kinase.

Germany), according to the manufacturer's instructions. Limiting dilution was performed twice consecutively in 96-well plates for 2 weeks each in culture medium supplemented with the antibiotic blasticidin (2 μg/ml) (Invivogen, Toulouse, France), against which pBOS-H2B^{GFP} confers resistance. Selection of stable single clones was carried out by fluorescence microscopy.

Cell culture and induction of apoptosis

Cell culturing of the H2B^{GFP} positive HeLa cell line was performed routinely at 37°C and 5-5% CO₂ in R10 medium, consisting of RPMI-1640 medium supplemented with 10% fetal calf serum (FCS), 10% glutamine, 1% penicillin-streptomycin (G1BCo/Invitrogen, Karlsruhe, Germany) and 1% HEPES (10 mM, pH 7.2) (Calbiochem/Merck, Darmstadt, Germany). To restrain the growth of HeLa cells not containing the H2B^{GFP} expression plasmid, blasticidin (2 μg/ml) was added to the R10-medium. Apoptosis was induced by irradiation with ultraviolet-B (UV-B) (900 mJ/cm²).

Inhibition of cytoskeletal plasticity and induction of apoptosis

Adherent HeLa cells were cultured in six-well plates in R10 medium to a density of 3 × 10⁴ cells/cm². Ten min after the addition of one of the inhibitors for cytoskeletal plasticity displayed in Table 1 (all from

Calbiochem/Merck, Darmstadt, Germany) cells were irradiated with UV-B and prepared for fluorescence microscopy.

Apoptosis in the absence of the inhibitors served as control. The cells were monitored for 24 h employing fluorescence microscopy (Axiovert 200 microscope; Zeiss, Göttingen, Germany; the ORCA-HRC4742-95 camera was supplied by Hamamatsu Photonics, Hamamatsu, Japan) and morphometry. The latter was performed using Adobe Photoshop CS5 to measure ACMV, nuclear areas and mean fluorescence intensity (MFI). Because several factors (e.g. photograph bleaching, exposure time, etc.) may affect the MFIs, all fluorescence microscopy pictures were taken under the same conditions. For morphometric analyses, a total of 25 cells were analysed for each condition. Before performing the experiment, it was ensured that the inhibitors had no effect on the cell viability during the observation period.

Results

ACMV are formed and loaded differentially with nuclear material during apoptosis

When we followed apoptosis in UV-B-irradiated H2B^{GFP} transfected HeLa cells, we observed the formation of early small (ACMV^S, < 1 μM), early intermediate (ACMV^M, 1 μM–3 μM) and late large (ACMV^L, > 3 μM) ACMV starting at approximately 180, 270 and 300 min after induction

of apoptosis, respectively (Fig. 1a–d); we analysed approximately 50 cells by fluorescence microscopy.

Whereas at time-point 270 min none of the ACMV contained detectable H2B^{GFP} (Fig. 1c), 30 min later few ACMV were loaded with chromatin fragments generated from the degraded nucleus (marked with arrows in Fig. 1c,d). Even after prolonged monitoring for up to 10 h, most of the ACMV did not contain visible amounts of H2B^{GFP} (Fig. 1e,f). Observations of late cell death phases showed that some of the ACMV^L which had been filled with H2B^{GFP} lost their content by disintegration (Fig. 1g,h).

Morphometric measurements of H2B^{GFP}-transfected HeLa cells revealed that the chromatin contracted significantly 6–7 h after induction of cell death, reflected by increased MFI values (Fig. 1i). Significant shrinkage of the nuclear area was to be observed after 9–10 h (Fig. 1j).

Cytoskeletal rearrangements are required for the morphological changes of apoptosing cells

Next we studied the morphological changes of apoptotic HeLa cells in the presence of inhibitors for cytoskeletal dynamics.

Jasplakinolide is a macrocyclic cyclodepsipeptide, extracted from the marine sponge *Jaspis johnstoni* [26]. It competes with phalloidin for the binding to F-actin. Induction of actin polymerization and stabilization of pre-existing actin filaments cause cytotoxic and anti-proliferative effects [27]. The effect of jasplakinolide on HeLa cell apoptosis is the almost immediate formation of motile ACMV^S 10 min after the addition of jasplakinolide (Fig. 2a). The cells lose volume and shrink. However, the loading of H2B^{GFP} material into late apoptotic ACMV^L remains unaffected (Fig. 2b). Compared with control cells and cells treated with alternative inhibitors of cytoskeletal dynamics [Y27632, 1-(5-chloronaphthalene-1-sulphonyl)-1H-hexahydro-1,4-diazepine (ML-9), latrunculin A, N-benzyl-p-toluene sulphonamide (BTS)], we detected a significantly greater amount of ACMV in cells incubated with jasplakinolide 90 min after apoptosis induction (Fig. 2c).

Latrunculin A is a macrolide toxin of the Red Sea sponge *Latrunculia magnifica*. It creates an equimolar complex with G-actin affecting the actin polymerization and interferes with microfilament organization. Latrunculin A has no impact on microtubules [28]. Within 10 min after addition of latrunculin A, HeLa cells generate ACMV^S and ACMV^M transiently and form membranous tubular extensions (Fig. 2d). After 90 min, HeLa cells treated with latrunculin A do not release newly generated ACMV^{S/M}, and therefore show similar amounts of released ACMV compared with the control cells (Fig. 2c). The nuclei of latrunculin A-treated cells appear plastic, whereas the cytoplasm of the cell is somewhat flat (Fig. 2e). This plasticity disappears during progression of apoptosis. Inhibition of G-actin has

no impact on nuclear shrinkage and on the redistribution of H2B^{GFP} into ACMV^L (Fig. 2f).

The pyridine derivate Y-27632 inhibits the functions of Rho-associated protein kinase (ROCK)-I and ROCK-II by binding to the catalytic site of these protein kinases. During the apoptotic process, ROCK-I is cleaved by caspase-3 at the carboxy-terminal region, thus becoming a constitutively activated kinase inducing membrane blebbing [29–31]. Treatment with Y-27632 inhibits uropod protrusion (Fig. 2g; arrow). Some cells (marked with '1' in Fig. 2g,h) still performed the usual morphological changes with cell shrinkage and the formation of ACMV^S. However, formation and loading of ACMV^L was not affected (Fig. 2h); 90 min after apoptosis induction HeLa cells treated with Y-27632 have released similar amounts of ACMV compared with the control cells (Fig. 2c).

BTS is an aryl sulphonamide, which specifically inhibits the Ca²⁺-stimulated myosin S1 ATPase and compromises gliding motility reversibly. BTS does not compete for the nucleotide-binding site of myosin II; rather, it handicaps myosin's interaction with F-actin. The primary target of BTS is Ca²⁺-stimulated S1 ATPase and the secondary target is actin-stimulated ATPase [32]. If HeLa cells are incubated with BTS, most of them contract and adopt a round shape. The cells are not 'paralyzed', as they still maintain their ability to move (Fig. 2i). ACMV^L were formed and loaded with H2B^{GFP} in the presence of BTS (Fig. 2j). BTS-treated HeLa cells show normal numbers of ACMV after 90 min (Fig. 2c).

Microtubules are required for the nuclear shrinkage of apoptosing cells

Paclitaxel is an anti-proliferative drug isolated from the bark of the pacific yew tree (*Taxus brevifolia*). It stabilizes microtubule formation by interacting with tubulin on its β -tubulin subunit [33] and prevents disassembly of the microtubule polymer. We observed that paclitaxel-treated H2B^{GFP}-transfected HeLa cells formed ACMV^S and ACMV^M within 30 min after irradiation (not shown); 60 min later the cells adopted a unique phenotype. They remained adherent, formed ACMV^L, lost their mobility and did not condense and degrade their chromatin and nuclei, respectively (Fig. 3a). This appearance lasted for at least 960 min. At this time-point, most nuclei displayed a viable morphology with no signs of condensation and shrinkage. Faint ACMV^L were visible; however, none of these were loaded with H2B^{GFP} (Fig. 3b). Morphometric measurements of the area of nuclei during a period of 10 h revealed that cells incubated with paclitaxel, an inhibitor of microtubules, stopped the shrinkage process at an early point of cell death (Fig. 4). The cells are not able to degrade and condense their nuclear material. Thus, they cannot translocate the histone H2B^{GFP} fusion protein into ACMV (Fig. 3b).

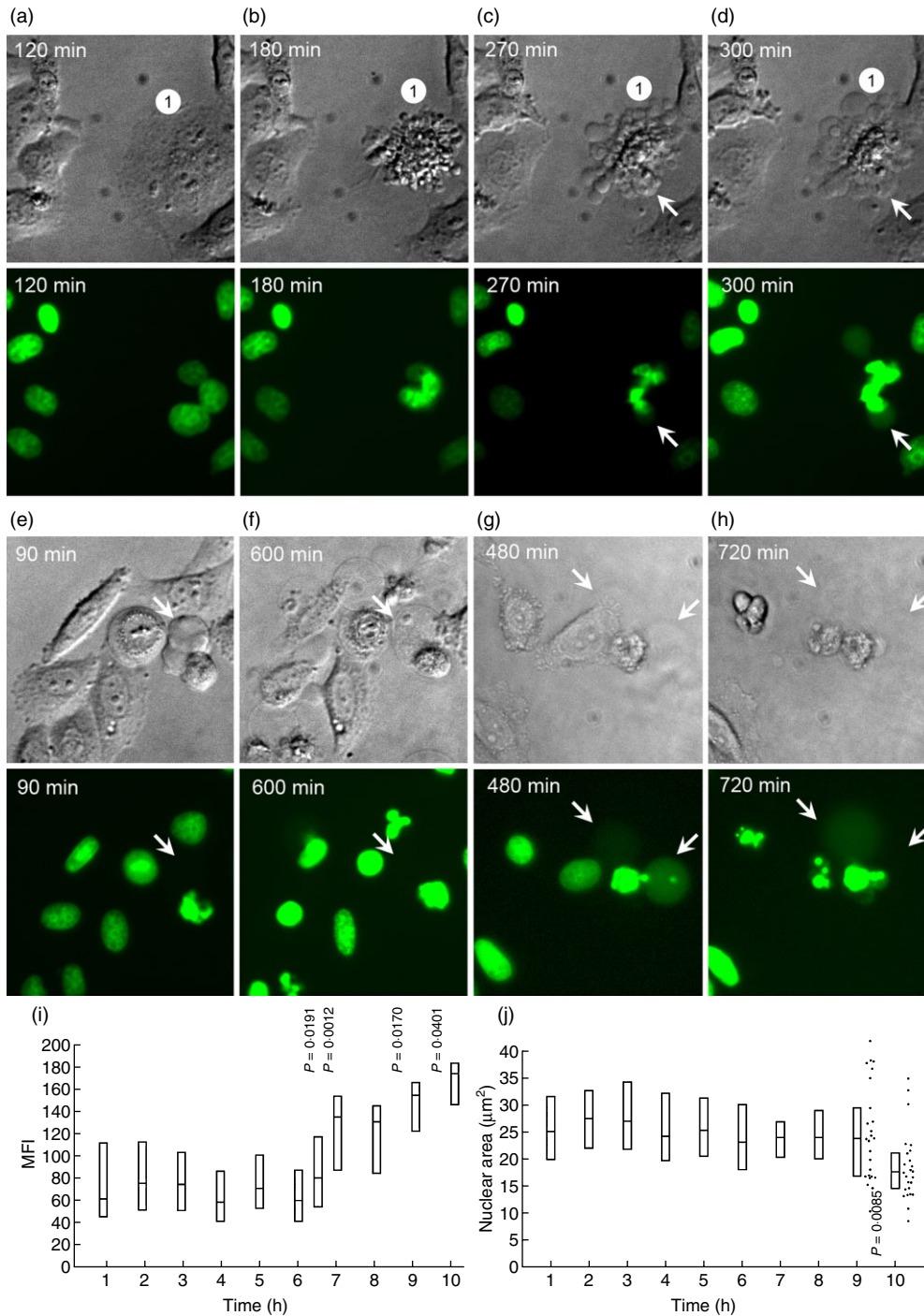


Fig. 1. Redistribution of histone 2B-green fluorescent protein (H2B^{GFP}) during ultraviolet (UV)-B-induced apoptosis in HeLa cells. Adherent HeLa cells expressing fluorescent H2B^{GFP} histone were cultured to a density of 3×10^4 cells/cm². After UV-B irradiation with 900 mJ/cm² the distribution of H2B^{GFP} was monitored by video-microscopy. (a–d) The characteristic nuclear changes that had been described for adherent cells undergoing apoptosis. After 270 min the cell marked with ‘1’ had undergone waves of formation of apoptotic cell-derived membranous vesicles (ACMV). None of these contained detectable amounts of H2B^{GFP}; 30 min later two of the vesicles were filled with substantial amounts of H2B^{GFP} (arrows). Note that (1) not all ACMV contain degraded chromatin (H2B^{GFP}); (2) ACMV are formed initially without nuclear material, and are loaded in a second step (arrows). (e,f) Some large ACMV^L remain unloaded even 10 h after their formation. (g,h) In very late phases of apoptosis some ACMV^L which had been loaded with H2B^{GFP} disintegrate and release their load into the culture supernatant, most probably during secondary necrosis. (i) mean fluorescence intensity (MFI) of the nuclei during the 10 h after irradiation. Note a significant increase of the MFI starting 6 h after irradiation (Student’s *t*-test of cellular MFI at $t = 0$ versus $t = \times$ hours after irradiation). (j) nuclear area during the 10 h after irradiation. Note a significant shrinkage of the nuclei 9 h after irradiation (Student’s *t*-test of cellular nuclear area at $t = 0$ versus $t = \times$ hours after irradiation).

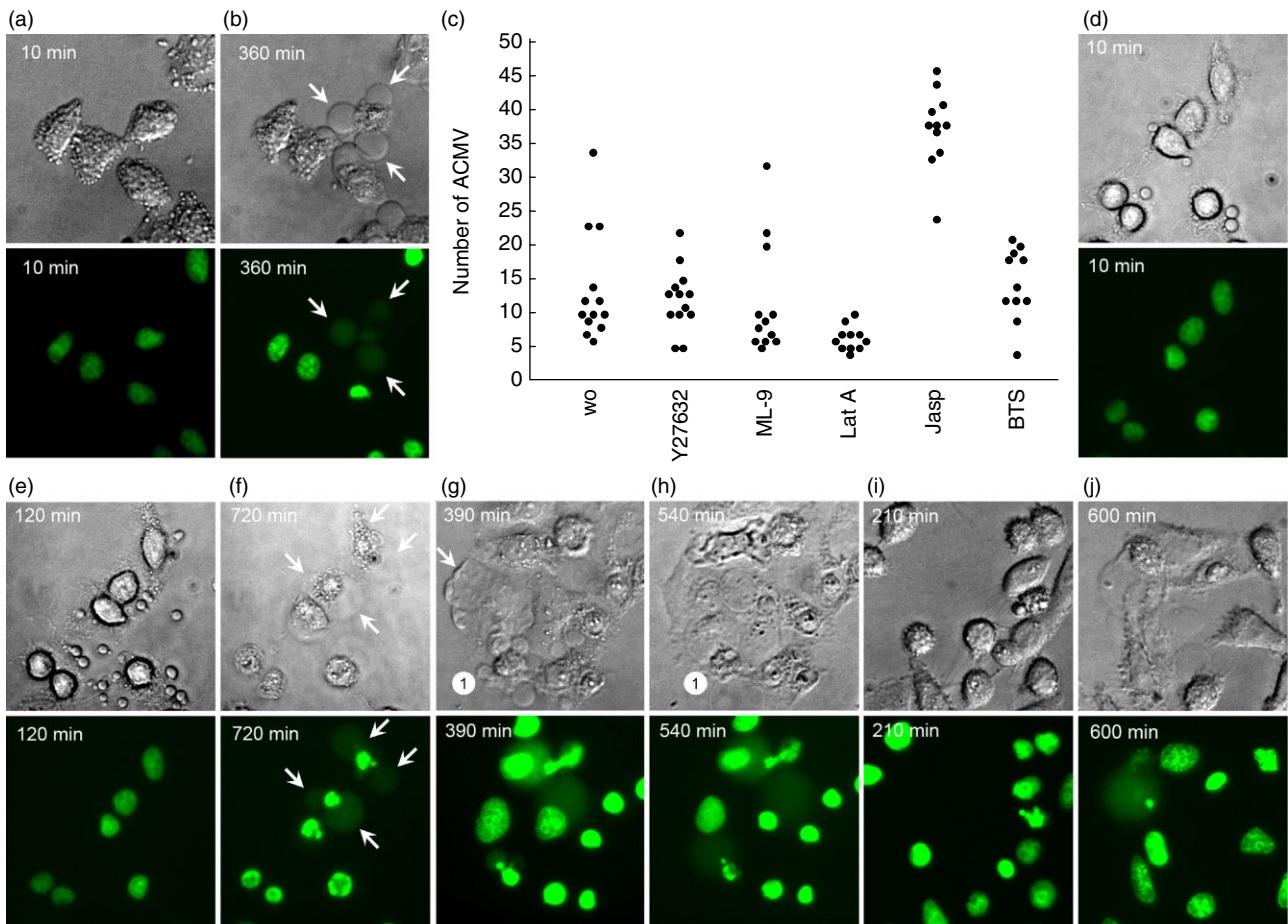


Fig. 2. F- and G-actin, p160^{ROCK} and myosin are not required for loading into apoptotic cell-derived membranous vesicles (ACMV)^L of histone 2B-green fluorescent protein (H2B^{GFP}). Culturing and induction of apoptosis in HeLa cells was performed as described in Fig. 1. (a,b) HeLa cells were incubated with the F-actin inhibitor jasplakinolide. (a) Ten min after addition of the inhibitor, the cells start to form large numbers of ACMV^{S/M}. (b) This leads to a loss of cell volume followed by shrinkage of the cell corpses. Whereas the inhibition of F-actin causes quick and dramatic changes in plasma membrane appearance, the creation and the loading of ACMV^L with H2B^{GFP} remains unaffected as to be observed 360 min after irradiation (arrows). (c) ACMV count 90 min after induction of apoptosis. HeLa cells incubated with jasplakinolide (Jasp) show a significant higher number of released ACMV^{S/M}. HeLa cells treated with latrunculin A (Lat A) show no significant change in the ACMV count (most ACMV were observed transiently during the first 10–60 min (see d)). (d,e,f) HeLa cells were incubated with the G-actin inhibitor latrunculin A. (d) Ten min after addition of the inhibitor, the cells start to form small ACMV^{S/M}. (e) After 120 min the nuclei appear plastically with a flat cytoplasmic rim. (f) Note that loading of ACMV^L with histone H2B^{GFP} fusion protein remains unaffected as to be observed 720 min after irradiation (arrows). (g,h) HeLa cells were incubated with the p160^{ROCK} inhibitor Y-27632; (g) 390 min after addition of the inhibitor, some cells display abnormalities in uropod protrusion (arrow). (g,h) Note that loading of ACMV^L with H2B^{GFP} remains unaffected (1). (i,j) HeLa cells were incubated with the myosin inhibitor N-benzyl-p-toluene sulphonamide (BTS); (i) 210 min after addition of BTS, most cells adopted a round shape but preserved their capabilities to move. (j) Note that ACMV^L were still loaded with H2B^{GFP}. Small, medium-sized and large apoptotic cell-derived membranous vesicles are marked ACMV^{S/M/L}, respectively.

MLCK activity is required for the loading of H2B into late ACMV^L

ML-9 is a synthetic compound that inhibits the catalytic site of MLCK with high selectivity. It competes with ATP and acts as an inhibitor of the phosphate acceptor in the catalytic centre [34]. Employing ML-9, we observed a somewhat normal execution of apoptosis in HeLa cells; 270 min after irradiation most cells harboured condensed chromatin and regularly formed ACMV^{M/L} (Fig. 3c). Up to 960 min after

induction of apoptosis the nuclei appeared shrunken and displayed condensed amounts of H2B^{GFP}. However, we never observed loading into ACMV^L of H2B^{GFP} (Fig. 3d). ML-9-treated cells showed normal numbers of ACMV compared with the apoptotic control cells 90 min after apoptosis induction (Fig. 2c).

Table 1 and Figs 3e and 4 summarize the results of the inhibitor study. Whereas in the apoptotic control cells and in the presence of the inhibitors jasplakinolide, latrunculin A, Y-27632 and BTS H2B^{GFP}-loaded ACMV^L are readily seen

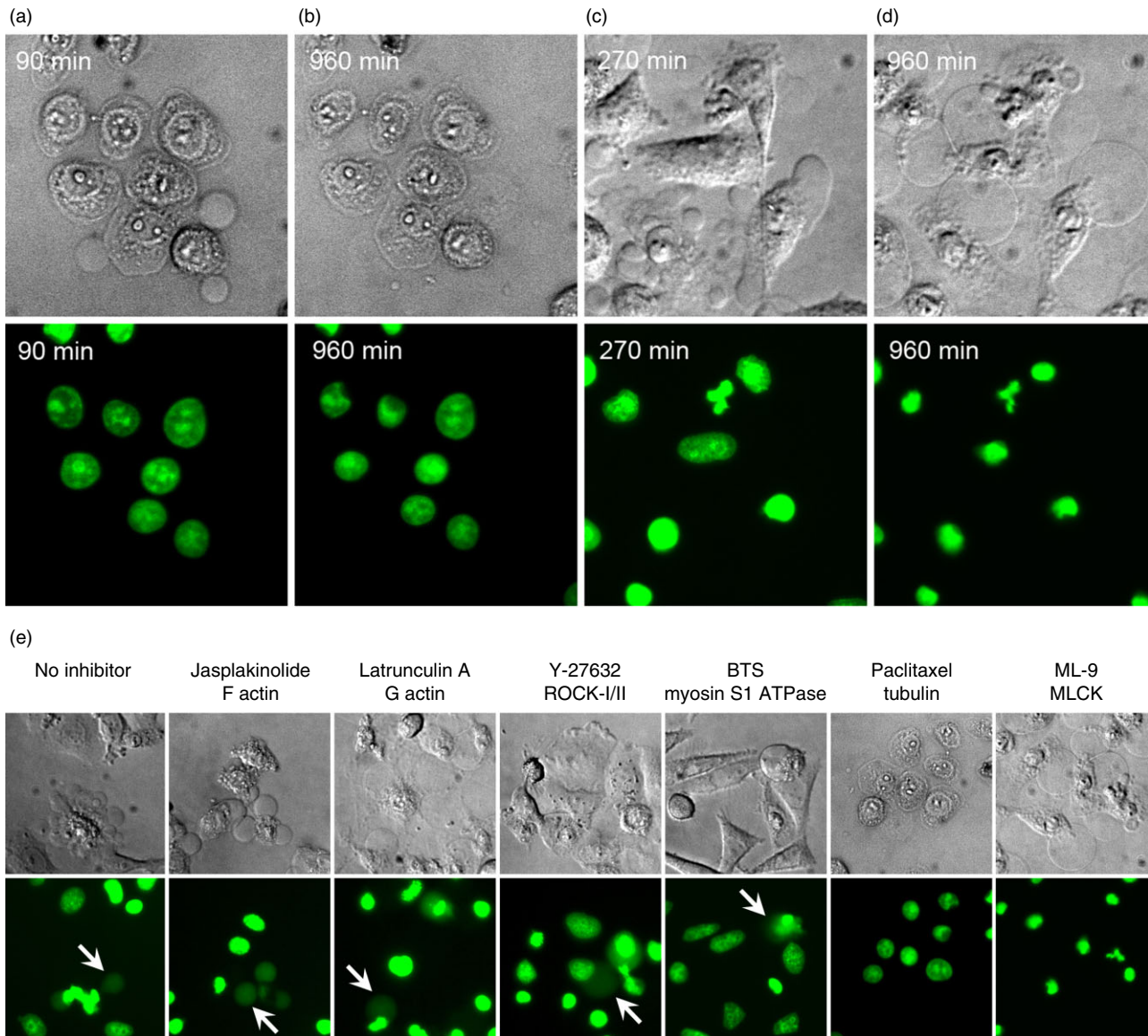


Fig. 3. Microtubules and myosin light chain kinase (MLCK) are required for nuclear shrinkage and loading of apoptotic cell-derived membranous vesicles (ACMV)^L with histone 2B-green fluorescent protein (H2B^{GFP}), respectively. (a,b) HeLa cells incubated with an agent stabilizing microtubules (paclitaxel) are arrested at an early stage of apoptosis. (b) Nuclear shrinkage is impaired as well as the sorting of nuclear autoantigens into late ACMV^L. (c,d) The formation of ACMV^L is not affected during apoptosis in the presence of 1-(5-chloronaphthalene-1-sulphonyl)-1H-hexahydro-1,4-diazepine (ML-9), an inhibitor of myosin light chain kinase (MLCK). (d) Nuclear shrinkage and chromatin condensation is performed regularly. However, sorting of nuclear autoantigens into late ACMV^L cannot be observed. (e) In the absence of inhibitors, and in the presence of jasplakinolide, latrunculin A, Y-27632 and N-benzyl-p-toluene sulphonamide (BTS) histone H2B^{GFP}-positive ACMV^L are readily observed (arrows). Paclitaxel conserves the cell at an early state of cell death. Nuclear shrinkage and chromatin condensation is arrested. Cells incubated with ML-9 regularly generate ACMV^L; translocation of nuclear autoantigens into these was not detected.

(arrows), paclitaxel and ML-9 inhibited the formation and loading, respectively, of ACMV^L (Figs 3e and 4).

Discussion

In this study, we analysed the fate of a prototypic nuclear autoantigen recognized by the humoral immune response in patients with SLE in the execution phase of apoptotic cell

death *in vitro*. We observed that the histone H2B^{GFP} fusion protein is loaded into ACMV^L that are generated in the late stages of apoptosis. Pharmacological inhibition studies revealed that microtubules and MLCK are required for nuclear shrinkage and for packing nuclear autoantigens into membranous apoptotic vesicles, respectively (Fig. 5).

During apoptosis, cells undergo a plethora of morphological changes, including cellular shrinkage, condensa-

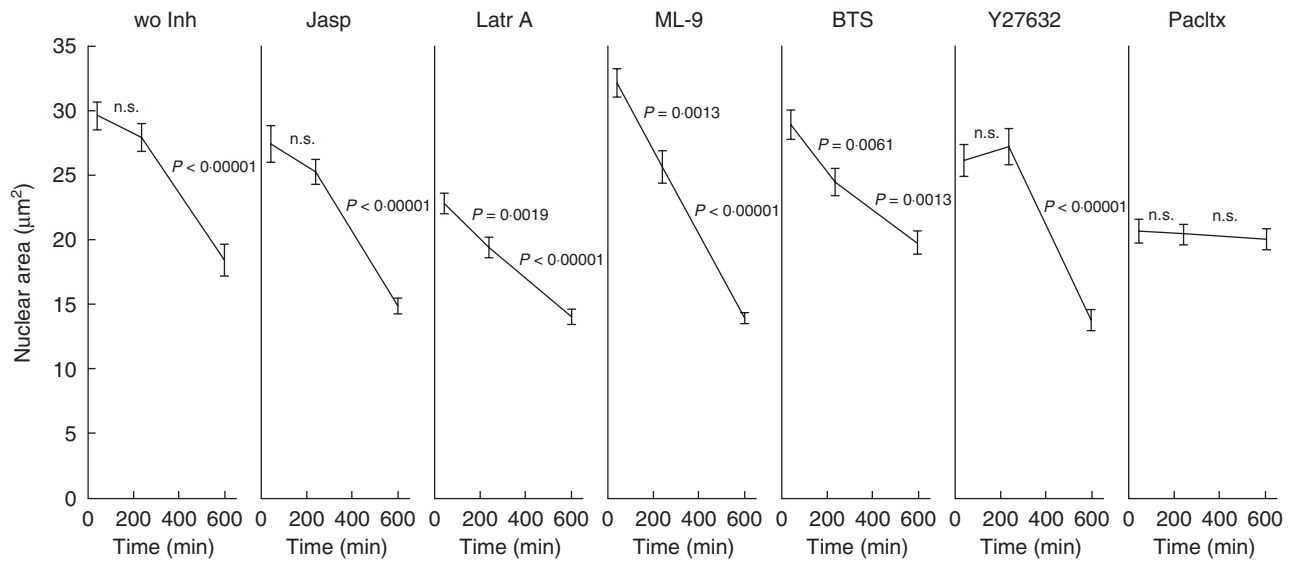


Fig. 4. Morphometric measurement of nuclear size 30, 240 and 600 min after irradiation. Y-27632 and jasplakinolide do not have any influence on nuclear shrinkage. 1-(5-chloronaphthalene-1-sulphonyl)-1H-hexahydro-1,4-diazepine (ML-9) and N-benzyl-p-toluene sulphonamide (BTS) significantly accelerate and decelerate nuclear shrinkage, respectively. Cells treated with latrunculin A or paclitaxel (Pacltx) already display a significantly smaller nuclear area 30 min after treatment. Whereas cells treated with latrunculin A continue shrinkage, the nuclei of paclitaxel-treated cells maintain their size for at least 10 h.

tion of chromatin (pyknosis), nuclear fragmentation (karyorrhexis) and development of bubble-like surface blebs (zeiosis), and break down into subcellular membranous vesicles (referred to in this paper as ACMV) (Fig. 5). Apoptotic cells usually release ‘find-me’ signals, expose ‘eat-me’ signals and orchestrate their swift and early clearance accompanied by the release of the tolerogenic cytokines interleukin (IL)-10 and transforming growth factor (TGF)- β [35,36].

Consequently, apoptotic cells are rarely seen in healthy tissues, and even sites of massive apoptosis do not show any signs of inflammation. Indeed, apoptotic cells have been reported to dampen inflammation [36]. For rare cases of apoptotic cells that had escaped this primary, phosphatidylserine-dependent clearance, back-up mechanisms exist that opsonize late apoptotic cells that expose immature glycoproteins [37,38].

A proper clearance, as found in most subjects, means that apoptotic cells are phagocytosed before they lose their membrane integrity without induction of inflammatory responses. In many patients with SLE the clearance process is impaired, as reflected by the occurrence in the affected tissues of post-apoptotic debris and inflammation. In the germinal centres of the lymph nodes, nuclear autoantigens released from secondary necrotic cells have been identified to decorate the surfaces of follicular dendritic cells and may there serve as selecting antigens for B cells that have accidentally gained autoreactivity during somatic diversification [39]. The molecular basis of this defect includes deficiencies for the complement components C1q [40], C2 [41] and C4 [42] and even a lupus-associated genetic variant in CR3 [43]. Furthermore, anti-dsDNA and other

autoantibodies have been identified to shift the clearance of apoptotic cells towards inflammation [8,44]. Clearance deficiency, humoral autoimmunity and inflammation induced by antibody-bound late apoptotic/secondary necrotic cells fuel a vicious circle that precipitates tissue inflammation in patients with SLE [7].

The accumulation of cellular remnants is prone to challenge self-tolerance due to access of the immune system to post-apoptotic material in an inflammatory environment. Therefore, we investigated the fate of the prototypic nuclear autoantigen histone H2B in the late phases of apoptosis, which occur predominantly in individuals with a deficient clearance. We observed that nuclear histone H2B^{GFP} is detectable in large membranous vesicles (ACMV^L) formed in the late stages of apoptosis *in vitro*. In the early-occurring ACMV^{S/M} we did not observe histone H2B^{GFP}-related fluorescence (Fig. 5). However, this does not exclude the presence of chromatin in these vesicles; it only shows that the amount of DNA has not reached the level of detection, which requires other methods [45,46]. We used the H2B^{GFP} distribution to follow the translocation of bulk chromatin for several hours and did not intend to detect minor traces of chromatin.

Coleman and colleagues described that ROCK I is necessary and sufficient for formation of membrane blebs and for relocalization of fragmented DNA into blebs and apoptotic bodies [29]. In our system, treatment with Y-27632 inhibits uropod protrusion and generation of ACMV^S. However, loading of ACMV^L was not prevented. In agreement with the above-mentioned paper, we observed that MLCK activity is required for loading with chromatin (reflected by H2B^{GFP} translocation) of ACMV^L [29].

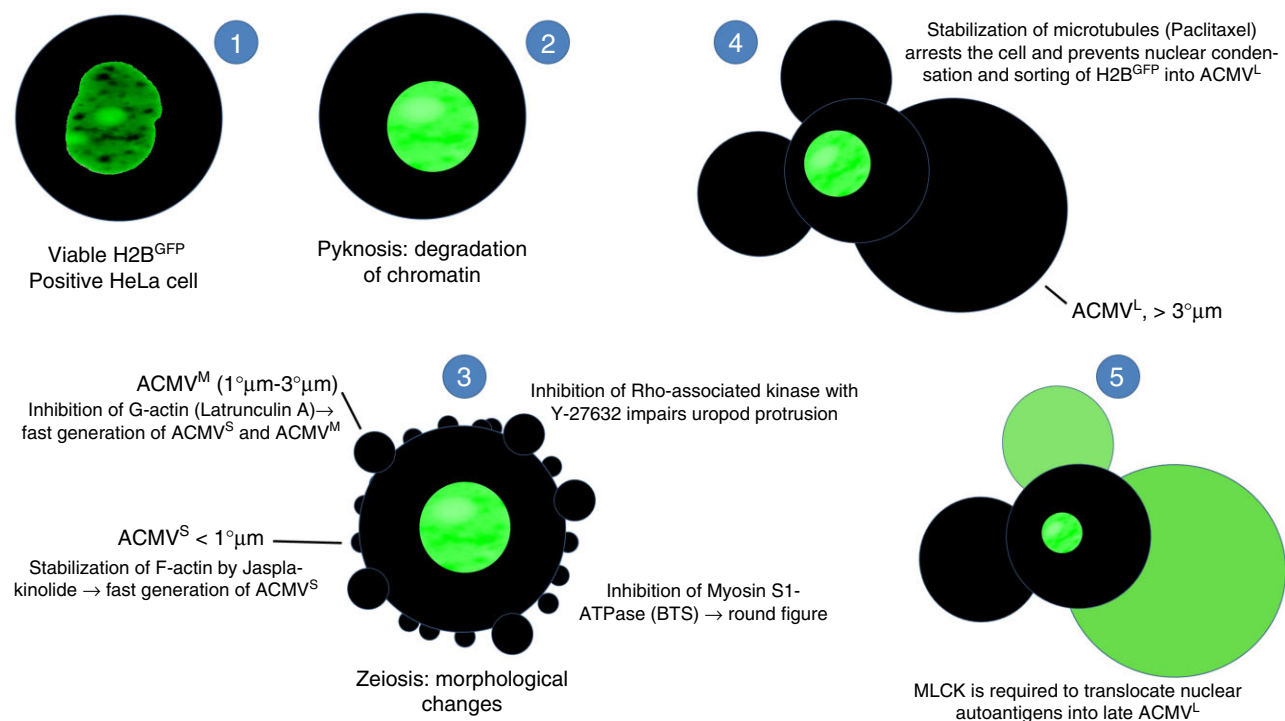


Fig. 5. Synopsis: schematic display of the translocation in apoptotic cells of nuclear autoantigens. HeLa cells were transfected with pBOS-histone 2B-green fluorescent protein (H2B^{GFP}) to label histone H2B as a representative of nuclear proteins serving as autoantigens in patients with systemic lupus erythematosus (SLE). After induction of apoptosis via irradiation with ultraviolet (UV)-B, processing and translocation of H2B was monitored employing fluorescence microscopy. The transfected cells were incubated with inhibitors of cell dynamics to identify the cytoskeletal proteins involved in the trafficking of H2B. (1) Viable H2B^{GFP} expressing cell with a regularly configured nucleus. (2) Apoptosis starts with chromatin condensation and degradation (*pyknosis*). (3) In early apoptosis, the cells undergo dramatic morphological changes. They shrink and generate apoptotic cell-derived membranous vesicles (ACMV), zeiosis. Initially small (< 1 µm) and intermediate (1–3 µm) ACMV^{S/M} are generated. These ACMV do not contain substantial amounts of H2B^{GFP}. Several inhibitors of cell dynamics have impact on the morphology of the dying cell. p160^{ROCK} (Y-27632) is required for uropod protrusion. Inhibition of F-actin (jasplakinolide) or G-actin (latrunculin A) leads to the early generation of ACMV^S and ACMV^M. Inhibition of myosin S1 ATPase [N-benzyl-p-toluene sulphonamide (BTS)] causes rounding of the cell. (4) However, all the above listed inhibitors do not have any impact on formation of late ACMV^L and the translocation of H2B into these. Inhibition of microtubules (paclitaxel) arrests the apoptotic process at an early time-point. It impairs nuclear shrinkage as well as formation of ACMV^L and translocation of H2B^{GFP}. (5) Inhibition of myosin light chain kinase (MLCK) by 1-(5-chloronaphthalene-1-sulphonyl)-1H-hexahydro-1,4-diazepine (ML-9) does not preclude the formation of ACMV^L, but impairs their loading with histone H2B^{GFP}.

In summary, we describe that translocation of the hallmark autoantigens addressed by the humoral autoimmune response in patients with SLE are loaded actively into large preformed membrane vesicles (ACMV^L) in the late stages of apoptosis (Fig. 5). Although these stages are somewhat rare, and seldom occur in healthy subjects, they may become prominent in individuals with deficiency of apoptotic cell clearance. It may be speculated that this translocation of cleaved chromatin is involved in the aetiopathogenesis of SLE. However, whether or not pharmacological interference with chromatin redistribution can be employed for the treatment of SLE needs further investigation.

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Author contributions

M. Z., C. J., L. M. and B. F. performed the experiments; R. V. provided the stably transfected HeLa cells. C. G., G. S. and M. H. designed the study and wrote the manuscript.

Disclosures

The authors declare no conflicts of interest.

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