

Lupus nephritis: The role of renal DNase I in the progression of the disease



Natalya Seredkina

A dissertation for the degree of Philosophiae Doctor
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2. List of papers

- I. Reduced fragmentation of apoptotic chromatin is associated with nephritis in lupus-prone (NZBxNZW)F1 mice. *Zykova S.N., Seredkina N., Benjaminsen J., Rekvig O.P. Arthritis Rheum. 2008 58: 813-825.*
- II. Progression of murine lupus nephritis is linked to acquired renal DNase I deficiency and not to up-regulated apoptosis. *Seredkina N., Zykova S.N., Rekvig O.P. Am. J. Pathol. 2009 175: 97-106.*
- III. Anti-dsDNA antibodies promote initiation, and acquired loss of renal DNase I promotes progression of lupus nephritis in autoimmune (NZBxNZW)F1 mice. *Fenton, K., S. Fismen, A. Hedberg, N. Seredkina, C. Fenton, E. S. Mortensen, O. P. Rekvig. PLoS. One. 2009 4: e8474.*
- IV. Acquired loss of renal nuclease activity is restricted to DNase I and is an organ-selective feature in murine lupus nephritis. *Seredkina N., Rekvig O.P. Manuscript submitted for publication.*

3. Abbreviations

ACR	American College of Rheumatology
Bid	B-cell lymphoma 2 family proteins Interacting Domain
bp	Base pairs
C1q	Subcomponent of complement 1
CAD	Caspase activated deoxyribonuclease
CPT	Camptothecin
DNase I	Deoxyribonuclease I
DNase III	Deoxyribonuclease I-like 1
dsDNA	Double stranded deoxyribonucleic acid
EBV	Epstein-Barr virus
EDS	Electron dense structure
EM	Electron microscopy
Endo G	Endonuclease G
GBM	Glomerular basement membrane
HMGB1	High-mobility group box 1
IC	Immune complexes
ICAD	Inhibitor of caspase activated deoxyribonuclease
IFN-β	Interferon-beta
Ig	Immunoglobulin
IL-10	Interleukin 10
ISR/RPS	International Society of Nephrology and Renal Pathology Society
LMW DNA	Low molecular weight deoxyribonucleic acid
MFG-E8	Milk fat globule epidermal growth factor-8
MMP	Metalloproteinase
MPs	Microparticles
mRNA	Messenger ribonucleic acid
PGE₂	Prostaglandin E ₂
SLE	Systemic lupus erythematosus
TGF-β	Transforming growth factor beta
TI	Tubulointerstitial inflammation
TNF-α	Tumor necrosis factor-alpha
TUNEL	Terminal transferase biotin-dUTP nick end-labeling

4. Introduction

Systemic lupus erythematosus (SLE) is a chronic autoimmune disease with a wide spectrum of clinical and immunological disorders. Prevalence of SLE is higher in females, while males have lower survival rates (1). The mostly involved tissues in SLE include skin, joints, kidneys, central nervous system, serous membranes and hematological systems while other organs can also be affected but with lower frequency. SLE is characterized by presence of a bewildering range of antibodies against self antigens. Clinical manifestations of the disease are imposed by the tissue damaging impact of circulating autoantibodies and deposition of immune complexes.

4.1 Epidemiology of SLE

Epidemiological data demonstrate marked variations in gender, age and race. According to recent studies, the overall age-adjusted prevalence of SLE varies from 20.6 to 78.5 per 100 000 persons (2,3) and is approximately 2 to 3 times higher in people of African or Asian background than in the white population (4). The incidence of the disease has increased approximately 3 times during the last 50 years, likely because of better diagnostics of mild SLE cases (5,6). The strongest risk factor of lupus is gender. In most studies, more than 90% of patients are women. The female-to-male ratio in general is 7:1, while in the childbearing years it increases to 11:1 (7). Known as a disease that develops mostly in women of reproductive age, in white population SLE however has the highest age-specific incidence rates after the age of 40 (8). Published data for Afro-Americans or Hispanic in USA and Latin America show that they develop lupus earlier in life (9-11).

4.2 Etiological factors

Classically, three main factors are considered in the etiology of SLE: genetic, hormones and environment.

Familial clustering, differences in the concordance rate between monozygotic (24-57%) and dizygotic (2-5%) twins, suggest a genetic basis in lupus (12,13). Currently, more than 20 loci of SLE susceptibility genes are known to contribute to risk of the disease, most of which are involved in immune complex processing; Toll-like receptor function and type I interferon production; and immune signal transduction in lymphocytes (reviewed in (14,15)). However, no single gene polymorphism was identified to cause lupus itself and SLE is considered as a genetically complex condition where 2 or more genetic risk factors need to occur in an individual to increase risk of the disease (14).

Predominantly development of SLE in females, implicates an important role of sex hormones. Estrogen and prolactin have been shown to have influence on the regulation of immune system including alteration of B-cell maturation and selection, proliferation of T-cells and promotion of a Th1 response (16-18). Several studies demonstrated increased risk of SLE in association with menstrual irregularity or with both short and long menstrual cycles (19-21). Protective effect of breastfeeding three or more babies compared with none was shown in the Carolina Lupus Study (20). Menopausal status, age at menopause and postmenopausal hormone therapy were also shown to be risk factors for SLE (20,21).

Historically, SLE was considered to be a viral disease. However, last decades of investigation did not confirm a viral etiology of lupus. The most promising finding is serological evidence of Epstein-Barr virus (EBV) infection in SLE patients. In one study almost 100% of patients with pediatric SLE were sero-positive to EBV (22). Retrospective analysis of serum samples collected from US military recruits showed markedly higher anti-

EBV antibody titer in people who later developed SLE compared to “non-lupus” individuals (23).

Environmental factors which also can likely be etiological for SLE are chemicals. Exposure to silica has been associated with increased risk of SLE (24,25). There are several reports about hair dye use as a risk factor for lupus (26,27), however this observation was not confirmed in a large prospective study (28).

4.3 Etiopathogenesis of SLE

The pathogenesis of SLE is composed of two pathological processes: i. break of self-tolerance that results in production of antibodies to self-antigens and ii. organ-damaging impact of circulating autoantibodies and deposition of immune complexes (IC).

The immune system normally defends our body from pathogens coming with bacteria, viruses or parasites. While the innate immune system acts fast, recognizes pathogens and responds in a generic non-specific way, the adaptive immune system has the an ability to recognize and remember specific pathogens with response getting stronger each time a pathogen is encountered. Aggression of immune system against the host organism is prevented through the mechanism of immunological tolerance where immature B- and T-cells which bind self antigens are eliminated in bone marrow and thymus (central tolerance) or mature autoreactive cells which enter the periphery are suppressed by T-regulatory cells and become anergic in the absence of co-stimulation by antigen presenting cells (peripheral tolerance) (29,30)

Several B- and T-cell abnormalities were observed in human and murine SLE including abnormal B-cell activation and differentiation to memory or plasma cells (31) and regulatory dysfunction of T-cells (32). However, defects in B- and T-cells can not explain the main phenomena in the pathogenesis of SLE – how self intracellular antigens become immunogenic and trigger a strong and prolonged autoantibody response (33,34).

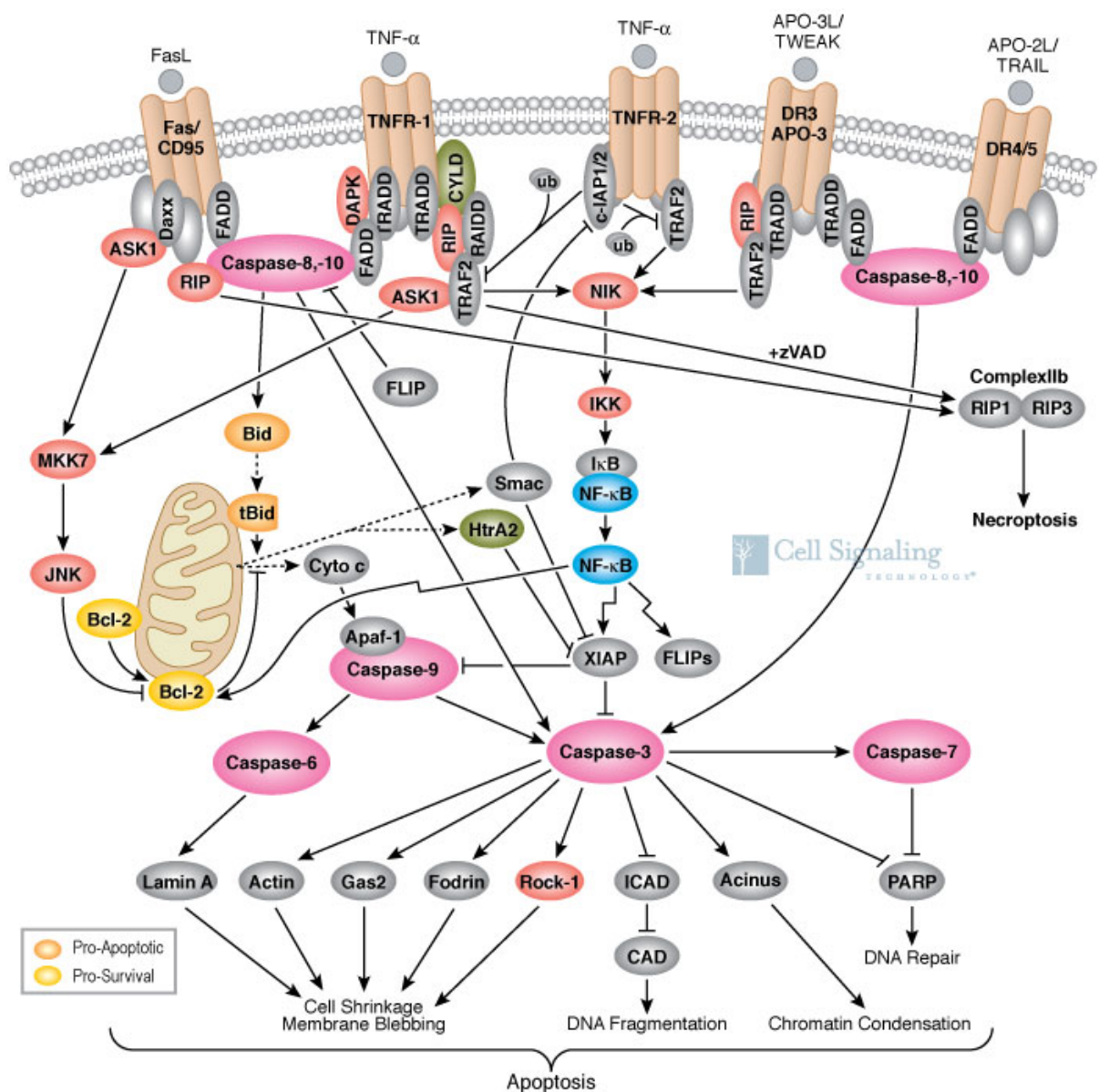
The central target for autoantibodies in SLE is nucleosomes. Nucleosomal antibodies have been shown to be highly specific for patients with SLE (35-37). Break of self tolerance to nucleosomes can similarly contribute to development of autoantibodies to dsDNA as well (37,38). Nucleosomes are normal products of apoptosis and generated in vivo only by endonuclease digestion of chromatin, therefore accelerated apoptosis, or defects in DNA fragmentation or impaired clearance of apoptotic cells can provide a potential mechanism for breaking self-tolerance and antigen-driven prolonged autoantibody response (39-41).

4.3.1 Apoptosis in pathogenesis of SLE

General characteristic of apoptosis

Apoptosis is a programmed genetically controlled cell death characterized by condensation of chromatin, DNA fragmentation, membrane blebbing and externalization of phosphatidylserine (42). It is initiated through the ligation of specific death receptors on the cell surface (extrinsic pathway) or from within the cell as response to DNA damage, defective cell cycle, hypoxia or other types of cell stress (intrinsic pathway). The initiation of apoptosis is followed by a cascade of enzymatic activations (Figure 1) and identifiable morphological changes in cells and in nuclei (43). In the last stage, apoptotic bodies, carrying cellular components, present “eat-me” signals and are engulfed by macrophages or dendritic cells (44,45). Clearance of intact dying cells prevents secondary necrosis of apoptotic cells and release of danger signals that may promote inflammatory process (46,47).

Figure 1. Death receptor signaling.



Pathway diagram reproduced courtesy of Cell Signaling Technology, Inc.

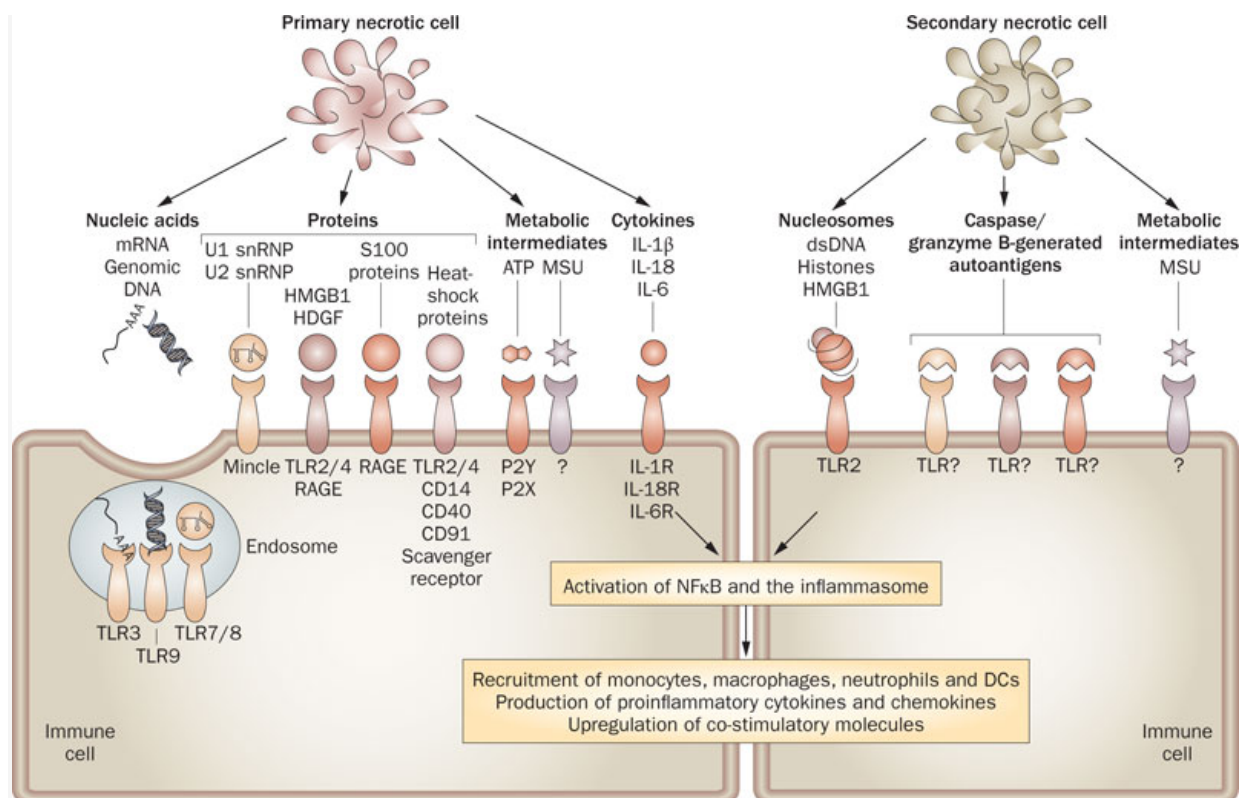
(www.cellsignal.com). Used with permission.

Apoptosis and autoimmunity

In contrast to apoptosis, primary necrosis is characterized by a rapid loss of the integrity of the cell membrane and exposure of intracellular components in the extracellular space, followed by activation of inflammasome (a large multimeric cytoplasmic protein complex that enables proteolytic processing of prointerleukin-1 β to its active form (48)) (49). Apoptotic cells

maintain their membrane integrity during the early stage of apoptosis, however at a late stage membrane integrity may be lost and cells become “secondary necrotic” (50). If apoptotic cells enter the stage of secondary necrosis, they start to release intracellular danger signals including high-mobility group box 1 (HMGB1) associated with nucleosomes (51,52), caspase-cleaved autoantigens (53) and uric acid (54). Immune cells respond to those signals with activation of inflammasomes and recruitment of more immune competent cells, production of cytokines and the up-regulation of co-stimulatory molecules, which finally cause immune system to be “alarmed” and to break tolerance to intracellular self-antigens (reviewed in (54,55)) as shown on Figure 2.

Figure 2. Danger signals from primary and secondary necrotic cells induce an alert immune system.



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Apoptosis and SLE

An increase in the apoptosis rate may exceed the local phagocytic clearance capacity. This may lead to accumulation of apoptotic cells and their transformation into secondary necrosis. Increased apoptotic activity among peripheral blood cells from SLE patients including lymphocytes (56), neutrophils (57) and monocytes (58) and its positive correlation with autoantibody production and disease activity (57) has been shown by many researches. Correlation between SLE activity and the increased level of apoptosis suggests that high apoptotic rate may lead to the production of autoantibodies. Induction of apoptosis of monocyte/macrophage in vivo by the administration of chlodronate liposomes to lupus-prone mice results in increase of anti-nucleosome and anti-dsDNA antibody production and worsening lupus nephritis, while injection of chlodronate in non-lupus-prone mice lead to development of anti-nucleosome antibodies but not lupus nephritis (59). Induction of apoptosis has also been shown to be the initial event in the pathogenesis of pristane-induced lupus in mice (60), which also is complicated by development of lupus-like nephritis.

In a contrast to increased apoptotic activity, reduction of apoptosis also leads to induction of autoimmunity. MRL-lpr/lpr mice which have no expression of a functional apoptosis-inducing ligand Fas, develop a spontaneous lupus-like syndrome including production of anti-dsDNA antibodies, lupus nephritis and skin lesion (61). Insufficient elimination of lymphocytes, observed in those mice, demonstrates that autoreactive T cells can survive and cause break of immunological tolerance leading to humoral autoimmunity to components of chromatin. In human SLE, the Fas-dependent apoptotic pathway was shown to be unaffected (62), however in some lupus patients anti-Fas ligand antibodies were found in circulation (63). In vitro, they inhibited Fas-mediated apoptosis in cell lines. This indicates the possibility of in vivo inhibition of Fas-mediated elimination of autoreactive lymphocytes and disturbance of peripheral tolerance (63).

Apoptotic bodies or microparticles?

It has been shown that not only apoptotic bodies but microparticles (MPs) can also be generated during apoptosis. They incorporate nuclear and cytoplasmic components of dying cells and can mediate intercellular communication (64). The diameter range of MPs is 0.1-1.0 μm . They contain RNA (including ribosomal, messenger and microRNA) and cleaved DNA (65). Nucleic acids are presented both on the surface and inside the particles. MPs are proposed to participate in regulation of thrombosis, vascular reactivity, angiogenesis and inflammation (reviewed in (66)). Because of RNA and DNA incorporation MPs are suggested to act as adjuvants during the establishing of central B-cell tolerance (reviewed in (67)). Beside apoptosis they can also be generated during cell activation (64).

The role of microparticles in pathogenesis of SLE is of high interest since they may participate in both central tolerance and peripheral activation of B cells (67). Nucleic acids located on the surface of microparticles can interact with B-cell receptors triggering their activation while translocation of nucleic acids from MPs into B cells will lead to their activation through toll-like receptors and non-toll like receptor sensors. In normal individuals this would cause central deletion of autoreactive B cells but in SLE patients this will rather contribute to promoting survival of autoreactive B cells due to demonstrated defects at checkpoints of negative selection of B cells (68,69). In the periphery, interaction of autoreactive B cells with MPs might further lead to their differentiation into autoantibody-producing plasma cells (67). Therewith, MPs have been demonstrated to be a source of extracellular DNA and serve as an autoantigen for anti-DNA antibodies (65,70) and increased level of circulating MPs was observed in SLE patients (71,72).

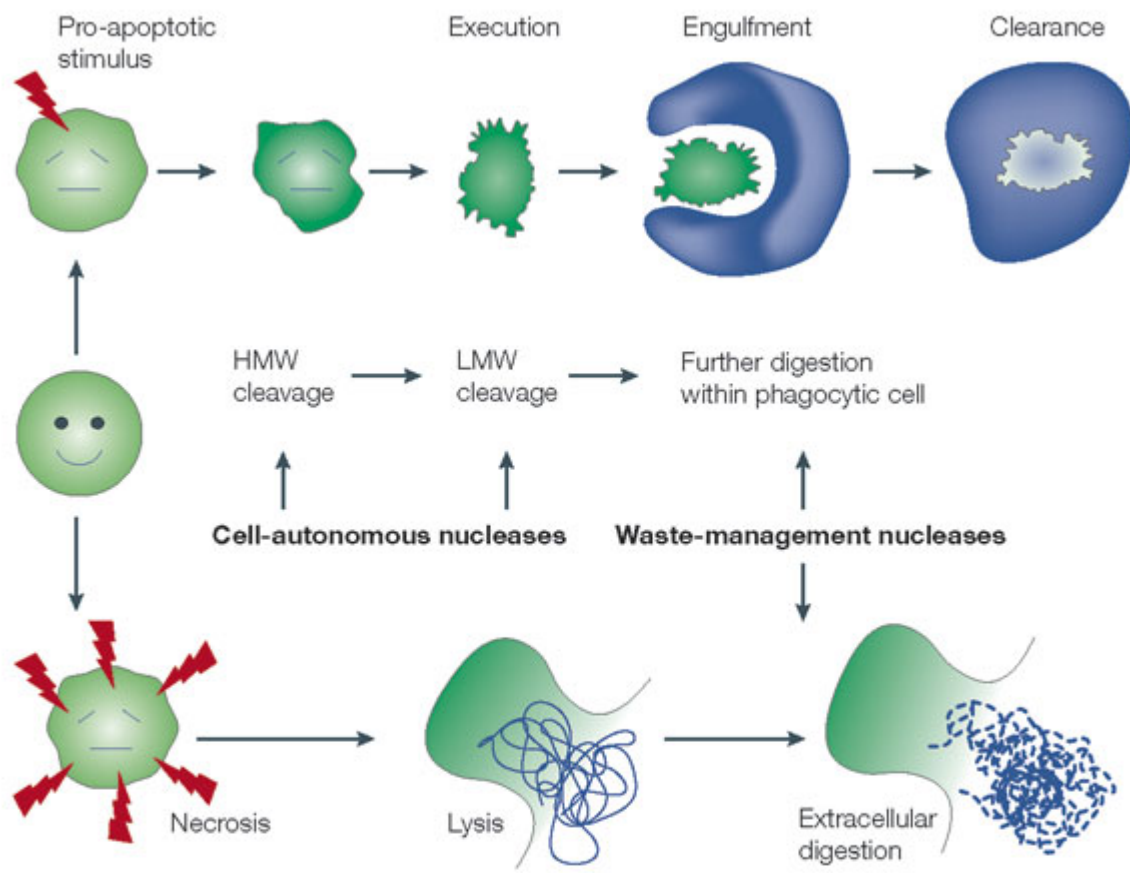
4.3.2 Nucleases in pathogenesis of SLE

In addition to dysregulated apoptosis or microparticles, impaired degradation of DNA during cell death is another process that may lead to extracellular chromatin exposure, break of self-tolerance and appearance of autoantibodies to chromatin components.

General characteristics of nucleases

In cells undergoing apoptosis, chromosomes are condensed and cleaved at internucleosomal regions to generate approximately 200-bp DNA ladders. Chromosome fragmentation is a complex biochemical mechanism that involves endonucleases with distinct nuclease activities and substrate specificities (73). Two classes of apoptotic nucleases participate in programmed cell death according to Samejima and Earnshaw (reviewed in (74), Figure 3). Cell-autonomous nucleases, which cleave the DNA within a cell, and waste-management nucleases, which digest chromatin originated from other cells, not from cells where those nucleases were produced. Cell-autonomous nucleases have direct access to the nuclei, while waste-management nucleases are enclosed in lysosomes or secreted into the extracellular space. The lysosomal nucleases participate in chromatin degradation during, for example, phagocytosis, and in case of insufficient chromatin fragmentation by cell-autonomous nucleases perform the final DNA digestion (75). The secreted nucleases exert their function in the blood stream and gastrointestinal tract to clean up DNA released from necrotic cells. Some nucleases can represent both classes, when secreted waste-management nucleases could under certain conditions be released into cytoplasm of a cell and function as cell-autonomous nucleases (76).

Figure 3. Cell-autonomous and waste-management nucleases in apoptosis and necrosis.



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There are two apoptotic nucleases clearly identified to degrade DNA within a cell – caspase activated deoxyribonuclease (CAD) and endonuclease G (Endo G).

CAD is the “professional” apoptotic nuclease. In cells it presents itself as inactive, in complex with the inhibitor of CAD (ICAD). When apoptotic stimuli activate the caspases, caspase 3 cleaves ICAD from the complex and active CAD digests double-stranded DNA at positions within internucleosomal linker DNA (77,78). Cleavage by CAD results exclusively in double-stranded breaks (79). In cells that are deficient in CAD or have a caspase-resistant form of ICAD, chromatin degradation is markedly reduced (75,80,81), suggesting that CAD

is the main cell-autonomous nuclease. At the same time, ICAD-deficient mice develop normally, lack of apoptotic DNA fragmentation does not lead to induction of autoimmunity in those mice and they still show residual DNA fragmentation (80,81), suggesting the existence of other apoptotic nucleases (74,82).

Endo G is identified as a mitochondrial endonuclease which can induce chromatin degradation in cells lacking CAD (82). It translocates to the nucleus after induction of apoptosis and proceeds DNA fragmentation (82). Endo G can be activated through caspase-independent apoptotic pathway (pro-apoptotic factors Bid and Bim) (82), or in order to release of cytochrom c and caspase 3 activation – caspase-dependent apoptotic pathway (83). It was shown that cleavage by Endo G results in single-stranded breaks between nucleosomes and its function is optimized in presence of DNase I (84). Interestingly, expression of Endo G via cisplatin-induced kidney injury was lower in DNase I knockout mice than in wild-type mice, demonstrating a potential link between those two nucleases (85). Results of studies on Endo G knockout mice remain controversial. The first study showed that Endo G-deficient mice died prenatally (86), but the second study reported they are viable (87). In any case, living mice without Endo G expression in cells did not demonstrate a compromised immune system (87).

DNase II is classified as a waste-management nuclease (74). It is packed in lysosomes and plays the main role in engulfment-mediated DNA degradation (88,89). DNase II has been shown to be essential for life since degradation of expelled nuclei from erythroid precursor cells proceeds by DNase II in bone marrow macrophages (88). DNase II-deficient mice die at birth, owing to severe anemia and defects in the diaphragm (88,89). Lack of DNase II expression in macrophages leads to accumulation of DNA fragments in those cells and hyperproduction of interferon- β (IFN- β) (75). DNase II knockout mice deficient in interferon type I receptor were born alive and normal. However, macrophages in 1-month-old mice

carrying undigested DNA started to produce TNF- α , and at 2-3 month of age mice developed polyarthritis resembling rheumatoid arthritis (88). Interestingly, the knockout of CAD gene in DNase II-deficient mice increases interferon- β production up to 10-fold (75). Thus lack of engulfment-mediated DNA degradation, especially in combination with reduced chromatin fragmentation via apoptosis, contributes to abnormal activation of the innate immune system (75).

DNase I is a secreted protein detected in serum, saliva, intestinal juice, urine, seminal fluid and lacrymal fluid (90). Primary regarded as an enzyme of gastrointestinal tract that digests DNA in food, it has been found to be required in chromatin breakdown during apoptosis and necrosis (91-93), and to function as cell-autonomous nuclease in certain circumstances (76). Knockout of *DNase I* gene in mice on SLE-predisposed background leads to induction of autoimmunity, appearance of anti-nucleosome antibodies and development of nephritis (94). Indeed, DNase I-deficient mice with a “non-autoimmune” background have reduced DNA fragmentation in the intestine (95), indicating physiological role of DNase I in the death of intestinal cells. The same mice have been shown to be protected against cisplatin-induced kidney injury (96) and gamma radiation (95) – two circumstances known to be associated with endonuclease-mediated DNA fragmentation damage.

There are three other nucleases that were reported to have 39-46% identity to DNase I – DNase I-like 1 (DNase IL1), DNase IL2 and DNase IL3. They can function as cell-autonomous nucleases and participate in chromatin degradation during apoptosis (97-99). General characteristics of the nucleases mentioned here are summarized in Table 2.

DNA degradation is an essential process for life and development. Therefore it is a well protected mechanism with complex nuclease interactions. Several cell-autonomous enzymes can cleave apoptotic chromatin, while the final digestion proceeds in lysosomes of macrophages by waste-management DNase II. DNase I is essential to degrade DNA in

extracellular space and body fluids, however it can also function as cell-autonomous nuclease (reviewed in (74)).

Table 2. Properties of the main apoptotic nucleases.

Nucleases	Cofactor	pH optimum	Inhibitor	TUNEL*	Location	Secreted	Ref.
CAD	Mg ²⁺	Neutral	Zn ²⁺	+	Nuclei, cytoplasm	Not	(78)
EndoG	Mg ²⁺ , Mn ²⁺	Neutral	Zn ²⁺	+	Mitochondria	Not	(84)
DNase II1	Ca ²⁺ , Mg ²⁺ , Mn ²⁺ , Co ²⁺	Neutral	G-actin, Ni ²⁺ , Zn ²⁺	+	Cytoplasm	Not	(98,100)
DNase II2	Ca ²⁺ , Mg ²⁺ , Mn ²⁺ , Co ²⁺	Acidic	Zn ²⁺	+	Cytoplasm	Yes	(100)
DNase II3	Ca ²⁺ , Mg ²⁺ , Mn ²⁺ , Co ²⁺	Neutral	Ni ²⁺ , Zn ²⁺	+	Nuclei	Yes	(100-102)
DNase I	Ca ²⁺ , Mg ²⁺ , Mn ²⁺ , Co ²⁺	Neutral	G-actin, Ni ²⁺ , Zn ²⁺	+	Cytoplasm	Yes	(93,100)
DNase II	None	Acidic	-	-	Lysosomes	Not	(103)

* the "+" indicates that the nuclease generates 5'-P and 3'-OH ends that can be detected by TUNEL reaction.

Nucleases and SLE

Only one nuclease has been shown to be involved in the pathogenesis of SLE so far. Reduced serum DNase I activity has been reported in lupus patients (104-107) and lupus-prone (NZBxNZW)F1 mice (108,109) and was proposed to cause accumulation of undigested DNA and induce production of autoantibodies against chromatin components (104). Therefore, a study with administration of DNase I in lupus-prone mice that develop nephritis was performed by Macanovic et al. (110). Published data suggested positive therapeutic effect of DNase I since progression and severity of the disease were decreased in mice injected intraperitoneally with murine DNase I (110). However those results were not reproduced in larger study on lupus-prone mice (111) and intravenous and subcutaneous administration of recombinant human DNase I to 17 patients with lupus nephritis did not show any effect on

disease activity (112). Moreover in an experimental mouse model with pristane-induced lupus, mice deficient in CAD did not produce antinuclear antibody (113). Thus, animals lacking chromatin fragmentation are impaired in ability to produce antibodies against nuclear components. Therewith, knockout of the *DNase I* gene in mice with “non-autoimmune” background did not lead to induction of autoantibodies (96). Taken together those data suggest that lacking or reduced chromatin fragmentation per se does not contribute to break of immunological tolerance to components of chromatin.

4.3.3 Impaired clearance of apoptotic cells in pathogenesis of SLE

Increased amount of apoptotic, secondary necrotic chromatin as a main antigen in SLE can also occur in the case of impaired clearance of apoptotic cells. Normally, cells undergoing apoptosis are removed immediately by non-inflammatory phagocytosis (114). The fast, efficient and silent removal of apoptotic cells protects them from transformation into secondary necrosis. If clearance is reduced, apoptotic cells reach a stage of secondary necrosis, expose danger signals (including HMGB1, heat shock proteins and uric acid) and trigger inflammation (reviewed in (55), Figure 2). Detection of nuclear remnants from apoptotic cells in germinal centers in association with the surfaces of follicular dendritic cells in SLE patients can explain the mechanism of termination of immunological tolerance to chromatin components in SLE (115). Several studies demonstrated functional defects in clearance of apoptotic cells in human and murine SLE (115-117). Mice deficient in C1q (C1q mediates immune complex and apoptotic cell opsonisation and phagocytosis) and MFG-E8 (MFG-E8 recognizes and binds apoptotic cells that enhances the engulfment of apoptotic cells by macrophages) develop anti-nuclear antibodies and immune-complex mediated lupus-like nephritis (118,119). This indicates an important role of effective clearance of apoptotic cells as a defensive mechanism to maintain tolerance for e.g. chromatin autoimmunity. Only C1q deficiency so far was found to be strongly associated with SLE in humans (120,121). Other

genetic defects causing impaired clearance of apoptotic cells in SLE patients remain unknown.

Thus, several pathological processes can contribute to termination of tolerance to self chromatin components in SLE and induce production of anti-dsDNA/anti-nucleosome antibodies. Interaction between the autoantibodies and antigens leads to formation of immune complexes (IC) that deposit in organs, trigger cascades of inflammation causing tissue injury and manifestation of clinical symptoms of the disease. Deposition of IC in patients with SLE has been identified in several sites including glomeruli, blood vessels and skin. IC presence may be explained by the deposition of circulating IC or by local formation of autoantibody-antigen complexes in case when target antigen is present within the site. Circulating IC can effectively and quickly be cleared by the reticulo-endothelial system in liver and spleen (122-124). Several studies have reported abnormal processing of IC in SLE patients (125-127) including reduced splenic uptake. This may likely be due to complement deficiency (125-127). But at the same time uptake of IC by liver was found to be increased (125) and final clearance of IC was faster in lupus patients (127). On another side, several constitutively expressed components of glomeruli have been shown to be recognized by anti-chromatin antibodies (including laminin (128,129) and α -actinin (130,131)) while two main components of GBM - collagen IV and heparan sulphate, could serve nucleosome-mediated binding of anti-nuclear antibodies to glomerular membrane (reviewed in (132,133)). However, there is no international consensus about the mechanism of IC deposition in SLE and future investigations are required. Nevertheless autoantibodies can by themselves cause cell damage by Fc receptor mediated inflammation (134) and/or by direct cytotoxicity. Some hematological disorders in SLE as hemolytic anemia and thrombocytopenia are most caused by direct lytic effect of the autoantibodies (135,136).

4.4 Classification and diagnosis of SLE

Since most organs can be affected by the disease, SLE often presents a diagnostic challenge. The main serological marker of SLE is presence of antinuclear antibodies including antibodies against dsDNA and nucleosomes. They are present in approximately 80% of lupus patients and correlate with disease activity (137,138). Prognosis of SLE is based on disease severity and known to be the most unfavorable in case of kidney and nervous system involvement. American College of Rheumatology (ACR) developed classification criteria for lupus, consisting of the most common clinical and laboratory manifestations, to classify SLE for clinical studies. Those criteria however are also provisionally used for the disease diagnosis. The 11 ACR criteria for SLE are presented in Table 1. Combination of 4 or more of them simultaneously or accumulated over time permits to classify lupus with 96% specificity and sensitivity between other autoimmune illnesses (139); nonetheless those criteria were never tested on non-autoimmune diseases (140).

Table 1. Criteria for classification of Systemic Lupus Erythematosus (SLE) modified from Tan E.M. et al. (139).

Criterion	Definition
1. Malar rash	Fixed erythema, flat or raised, over the malar eminences
2. Discoid rash	Erythematous circular raised patches with adherent keratotic scaling and follicular plugging; atrophic scarring may occur
3. Photosensitivity	Exposure to ultraviolet light causes rash
4. Oral ulcers	Includes oral and nasopharyngeal ulcers, observed by physician
5. Arthritis	Nonerosive arthritis of two or more peripheral joints, with tenderness, swelling or effusion
6. Serositis	Pleuritis or pericarditis documented by ECG or rub or evidence of effusion
7. Renal disorder	Proteinuria >0.5 g/d or +3, or cellular casts
8. Neurologic disorder	Seizures or psychosis without other causes
9. Hematologic disorder	Hemolytic anemia or leukopenia (<4000/mm ³) or lymphopenia (<1500/mm ³) or thrombocytopenia (<100000/mm ³) in the absence of offending drugs
10. Immunologic disorder	Anti-dsDNA, anti-Sm, and/or anti-phospholipid antibody
11. Antinuclear antibodies	An abnormal titer of ANA by immunofluorescence or an equivalent assay at any point in time in the absence of drugs known to induce ANAs

4.5 Lupus nephritis

The classical immune-complex mediated complication in SLE is lupus nephritis.

4.5.1 General characteristics of lupus nephritis

Lupus nephritis is potentially the most severe clinical manifestation of SLE that affects 30-60% of lupus patients (141,142). Before 1970, 5-year survival rate of SLE patients with lupus nephritis was low and reached only 25-40% (143). The situation has changed during the last decades and survival rate increased to 80-95% (144) as a result of using broad-spectrum immunosuppressive therapy, introduction of dialysis and renal transplantation. Despite intervention, the results of integrated therapy are still insufficient since complete remission rates of lupus nephritis are around 50% (145) with relapse rates of up to 30% over 2 years (146). Therewith the incidence of end-stage renal disease in SLE has tended to increase in US especially among African-Americans and Hispanic (147).

The diagnosis of lupus nephritis also invokes challenges. The initial clinical symptoms of the disease are persistent proteinuria and/or appearance of cellular casts in urine, however it can manifest from full-blown nephrotic syndrome with fast progression into end-stage renal failure. Laboratory findings in urine as well as a monitoring of anti-dsDNA or other autoantibodies in serum do not indicate disease severity. The level of circulating autoantibodies is associated with overall disease activity but does not correspond to renal pathomorphological changes and degree of kidney damage (148). Correct evaluation of lupus nephritis therefore has to be performed by examination of renal biopsy. Histopathological evidence of inflammation and affection of different glomerular patterns has been shown to represent the extent of kidney injury and well predict the risk of development severe renal disease (149). At the same time, renal biopsy is an invasive procedure and has considerable procedural risk, and it needs to be repeated during disease progression to diagnose the possible transformation of one morphological pattern to another, and to provide correction in

therapy (150,151). Investigation of new less-invasive markers of the disease severity is of high interest in lupus nephritis. Several candidates were reported to provide sufficient prognostic value of the disease but future studies are needed to be performed to conclude their significance in practice (152-154).

4.5.2 Classification of lupus nephritis

There are six classes of lupus nephritis according to classification criteria developed by International Society of Nephrology and Renal Pathology Society (ISR/RPS) working group (155). The last revised form of classification from 2003 is presented in Table 3. This classification is based on light microscopy, immunofluorescent and electron microscopy (EM) analyses of renal biopsies and focuses exclusively on glomerular pathology. Histological findings in different glomerular patterns (mesangial, epithelial and endothelial) including active or chronic inflammation, necrosis, crescents and IC deposition allow to discriminate severity of the disease and renal outcome. Thus Class I lupus nephritis (characterized by mesangial IC deposits detectable only by EM and/or immunofluorescence) and Class II (appearance of mesangial hypercellularity and extended deposition of IC in mesangium) are silent disease, therefore usually non-symptomatic and rarely progress to renal failure (155,156). Class III lupus nephritis is characterized by segmental endocapillary proliferative lesions and immune deposits in subendothelial glomerular pattern that affect less than 50% of all glomeruli, whereas in Class IV more than 50% of glomeruli have to be affected (155). Clinically, Class III and IV lupus nephritis present with mild or severe proteinuria (including nephrotic syndrome) and are associated with rapid progression to end-stage kidney disease (156). Lupus nephritis patients with subepithelial immune deposition in glomeruli (Class V) have low rate of progression, typically present with nephrotic syndrome and have high risk of thromboembolic events (155,157). Class VI lupus nephritis is the final-stage when chronic glomerular inflammation transforms into global glomerulosclerosis (155).

ISR/RPS classification primarily focuses on glomerular damage. However several studies have reported on the importance of tubulointerstitial inflammation (TI) and its predictive role for progression to renal failure (158-160). Since TI can occur independently of glomerular injury (161) and does not correlate with titers of anti-dsDNA antibodies it may indicate different pathogenic mechanisms for glomerular and interstitial tissue damages during development of lupus nephritis (160).

Table 3. Abbreviated International Society of Nephrology and Renal Pathology Society classification of lupus nephritis (2003), modified from Weening et al. (155).

Class I	Minimal mesangial lupus nephritis
Class II	Mesangial proliferative lupus nephritis
Class III	Focal lupus nephritis (indicate the proportion of glomeruli with active and with sclerotic lesions)
Class IV	Diffuse segmental or global lupus nephritis (indicate the proportion of glomeruli with fibrinoid necrosis and cellular crescents)
Class V	Membranous lupus nephritis (may occur in combination with class III or IV in which case both will be diagnosed)
Class VI	Advanced sclerosis lupus nephritis

4.5.3 Pathogenesis of lupus nephritis

Since lupus nephritis is an IC-mediated disease (162,163) three main questions have to be addressed: i. which autoantibodies are nephritogenic, ii. what is the central renal target antigen and iii. where and how are IC formed.

A wide spectrum of autoantibodies has been shown to be associated with lupus nephritis including anti-C1q, anti-Ro, antiribosomal antibodies, antibodies to laminin, fibrinogen and collagen (reviewed in (133,164)). However, the central role in the pathogenesis of lupus nephritis has been attributed to anti-dsDNA and anti-nucleosome antibodies (132,165-167). A direct nephritogenic role of anti-dsDNA and anti-nucleosome antibodies is suggested by the correlation of serum antibody levels with nephritis (168,169)

and the presence of anti-DNA antibodies in glomerular immune deposits (170,171). Concentration of anti-dsDNA antibodies in eluates from glomeruli exceeds their concentration in serum (172) and administration of DNA to autoimmune mice with circulating anti-dsDNA antibodies accelerates the progression of nephritis (173). In addition injection of anti-DNA antibodies to non-immune mice can induce nephritis (174-176). However immunization with dsDNA failed to induce anti-dsDNA antibodies with lupus specific characteristics, while the positive results came after administration of dsDNA complexed with histone-like DNA-binding proteins (177-179). It has been also shown that T cells directed against histones or nucleosomes were able to provide help for the production of anti-dsDNA antibodies (36,180). Those observations lead to the conclusion that nucleosomes are the driving autoantigen in SLE and lupus nephritis.

At the same time there exists evidence that nucleosomes are the central renal targets for nephritogenic autoantibodies since they are found in electron dense structure (EDS) in murine and human lupus nephritis (165,166). However, many researches have demonstrated cross-reaction of anti-nucleosomes antibodies with glomerular membrane components such as laminin, α -actinin and collagen (128,131,181-184). Ultra structural analysis of glomeruli by various electron microscopy techniques did not show presence of those components within the EDS (185,186), and the *in vivo*-bound antibodies were not observed in regular membranes or matrices. Again the main SLE enigma – how can intracellular antigen (nucleosome) become able to impose an immune response and at the same time serve as an antigen for the induced autoantibodies? The possible mechanisms such as disregulation of apoptosis, defective DNA degradation by endonucleases and impaired clearance of apoptotic cells are discussed in chapter 4.3. With regard to lupus nephritis, abnormal levels of apoptotic activity were observed in diseased kidneys. However published results remain controversial. Several studies demonstrated an increase of apoptotic cells in lupus nephritis (165,187,188) while

other reported decreased renal apoptosis (189,190). In all of these studies conclusions are based on histological determination of amount of apoptotic cells on kidney sections. While investigation of renal apoptotic activity with integrated assessment of apoptotic triggers, executioners and effectors and detection of final apoptotic chromatin fragmentation were not performed.

Decreased serum DNase I was found associated with active phase of class III/IV lupus nephritis (191) in respect to possible defect in endonuclease mediated DNA degradation. But involvement of serum DNase I in the pathogenesis of lupus nephritis remained elusive since administration of DNase I to patients with lupus nephritis did not have impact on the disease activity (112). Even the origin of serum DNase I is not well established. Just one recent set of data suggested liver as an organ secreting DNase I (192). At the same time little is known about DNase I expression and regulation in tissue and particularly in kidneys.

The mechanism by which autoantibodies form immune complexes in glomeruli has been discussed over decades, and the international consensus is still absent. In general there are two main theories to consider: 1) pre-formed circulating IC are passively trapped into glomeruli and 2) autoantibodies bind directly to endogenous renal antigens. The first theory is challenged by evidence that circulating IC should be rapidly cleared by the liver and spleen. In addition, administration of DNA/anti-DNA complexes to lupus prone mice resulted in decreased disease activity by reduction of autoantibody production (193). The second theory has many versions of antibody-binding mechanism including cross-reactions with constitutively expressed components of glomeruli (discussed above), nucleosome-mediated binding (132) and binding to exposed undigested chromatin fragments (194). None of those mechanisms have been ultimately proved and still is a matter of debate.

It is also not excluded that different stages of lupus nephritis relate to different pathogenetic antigens. Data from repeated renal biopsies of lupus patients suggest that 15-

40% of them switch one class of lupus nephritis to another during continuous follow-up (150,151). Some patients experience progression from the mild mesangial form to full-blown membrano-proliferative nephritis, while others remain with a benign mesangial pattern throughout life.

One of described mechanisms of lupus nephritis development suggests that deposited IC trigger a cascade of inflammatory events including activation of Fc receptors and complement (reviewed in (195)). Those events lead to recruitment of inflammatory cells. Infiltrating macrophages can be responsible for increased expression of metalloproteinase 2 (MMP-2) and MMP-9 (196,197). Alteration in the composition or integrity of glomerular membranes possibly caused by increased MMP activities can facilitate subepithelial deposition of IC or chromatin fragments (198). However this mechanism is not well established and future investigation are required.

4.5.4 Animal models for the study of lupus nephritis

Studies of lupus nephritis on human tissue samples are limited for practical reasons. These include problem with amount of renal biopsy material, timing difficulties to follow all stages of lupus nephritis development in one individual and lack of renal histology information at the time of initiation of nephritis. Use of animal models helps to solve many of those problems but retains additional question about relevance of research in animals to the human counterpart of the disease.

The (NZBxNZW)F1 mouse is known as an animal model that spontaneously develops SLE-like disease. They are generated by the F1 crossbreed progeny of New Zealand Black (NZB) and New Zealand White mice. (NZBxNZW)F1 mice develop typical lupus nephritis including production of anti-dsDNA antibodies, development of IC mediated glomerulonephritis and death from end-stage renal failure or cardiovascular disease (199-201). Studies in this thesis were performed on (NZBxNZW)F1 lupus-prone mice. The

relevance of research findings in the animal model is currently tested in our group on human renal biopsies.

5. Aims

- To investigate the mechanism of appearance and origin of chromatin fragments in glomerular EDS in kidneys of (NZBxNZW)F1 mice. (Paper I and II)
- To characterize the apoptotic rate in kidneys of (NZBxNZW)F1 mice at different stages of lupus nephritis. (Paper II)
- To determine the impact of anti-dsDNA antibodies, renal DNase I and MMPs on initiation and progression of lupus nephritis. (Paper III)
- To analyze the organ specificity of acquired renal DNase I reduction and its selectivity among endonucleases in (NZBxNZW)F1 mice during development of lupus nephritis. (Paper IV)

6. Summary of the papers

Paper I.

Reduced fragmentation of apoptotic chromatin is associated with nephritis in lupus-prone (NZBxNZW)F1 mice.

Electron dense structures (EDS) containing IgG and chromatin fragments are observed in glomerular basement membranes (GBM) of human and murine lupus nephritis. However the size of chromatin structures found in EDS was never analyzed. Thus demonstration of nucleosomal DNA fragments within the kidneys may be consistent with increased production of apoptotic DNA or its impaired clearance since nucleosomes are products of apoptosis and generated in vivo exclusively by endonuclease-mediated cleavage of DNA. While the absence of low molecular DNA, may be due to reduced fragmentation of chromatin in SLE nephritis. We performed analyses of DNA isolated from kidneys of female (NZBxNZW)F1 mice at different stages of lupus nephritis and did not find nucleosomal DNA fragmentation even in proteinuric animals where TUNEL-positive chromatin particles were detected in glomerular membrane by co-localization TUNEL immune electron microscopy. Induction of apoptosis ex-vivo by camptothecin in isolated kidneys from young, autoantibody negative (NZBxNZW)F1 mice and in kidneys from proteinuric mice with severe nephritis demonstrated markedly reduced DNA fragmentation in comparison to age- and sex-matched controls. Analysis of mRNA level of central renal nucleases CAD, EndoG and DNase I demonstrated a dramatic loss of renal DNase I transcription in mice with nephritis, while mRNA levels of CAD and EndoG in all groups of lupus-prone mice remained unchanged compared to healthy controls. Thus TUNEL-positive chromatin particles deposited in the glomerular membranes of nephritic mice are likely large chromatin fragments accumulated in glomeruli due reduced chromatin fragmentation and clearance of chromatin in nephritic (NZBxNZW)F1 mice.

Paper II.

Progression of murine lupus nephritis is linked to acquired renal DNase I deficiency and not to up-regulated apoptosis.

Antibodies to dsDNA and nucleosomes are strongly associated with lupus nephritis. Accumulation of apoptotic DNA has been suggested as a possible mechanism of nucleosome conversion into self-antigens that may initiate autoimmune response and participate in glomerular immune complex deposition. Normally, apoptotic cells are rapidly cleared by macrophages. In case of increased apoptotic activity the local phagocytic clearance capacity may be exceeded and accumulation of apoptotic chromatin fragments may occur for example in glomeruli. Therefore we analyzed mRNA level of apoptosis-related genes and presence of activated apoptotic factors within kidneys in (NZBxNZW)F1 mice during development of lupus nephritis. We did not find changes in apoptotic activity in kidneys at the time of appearance of anti-dsDNA antibodies as well as at the time of formation of mesangial immune deposits. In contrast, in kidneys with membrano-proliferative lupus nephritis, characterized by deposition of large chromatin fragments in glomerular basement membranes, we found increased amount of activated caspase 3-positive cells in kidney sections, unchanged mRNA levels of the apoptosis-related genes and a dramatic decrease in renal *DNase I* gene expression. Accumulation of activated caspase 3-positive cells was also observed in isolated nephritic kidneys where apoptosis was induced ex-vivo by camptothecin and lack of apoptotic chromatin fragmentation was observed. Taken together, our data suggest that apoptotic activity in kidneys of (NZBxNZW)F1 mice is not accelerated. Down-regulation of DNase I leading to accumulation of undigested apoptotic cells may be responsible for the transformation of mild mesangial lupus nephritis into severe membrano-proliferative end-stage organ disease.

Paper III.

Anti-dsDNA antibodies promote initiation, and acquired loss of renal DNase I promotes progression of lupus nephritis in autoimmune (NZBxNZW)F1 mice.

There is an international consensus that appearance of anti-chromatin antibodies is an initial event in the pathogenesis of lupus nephritis. The mechanism of the renal disease progression is elusive. We demonstrated that EDS in glomeruli of lupus-prone (NZBxNZW)F1 mice contain chromatin fragments and that there is a defect in apoptotic DNA fragmentation in nephritic kidneys associated with reduced mRNA level of renal DNase I. We have also reported that activity of metalloproteinases (MMPs) is increased during progression of nephritis in (NZBxNZW)F1 mice. To analyze the factors that contribute to lupus nephritis development we performed a study where mRNA levels and activities of DNase I, MMP2 and MMP9 were correlated with each other and with anti-dsDNA antibody production, with successive deposition of IC in the mesangial matrixes and in glomerular basement membranes (GBM), and with progressive proteinuria.

We observed that lupus nephritis in female (NZBxNZW)F1 mice is principally two-stepped organ disease. The early phase correlated with deposition of complexes of chromatin fragments and IgG in the mesangial matrix. Progression of the disease, which was characterized by deposition of large chromatin fragments in GBM and severe proteinuria, correlated with an acquired loss of renal DNase I mRNA level and enzyme activity. We proposed that loss of DNase I, as a dominant renal nuclease, leads to reduced chromatin degradation during regular apoptosis in the kidneys. In case of impaired clearance of apoptotic cells, this may explain exposure of chromatin fragments in membranes and matrices of affected glomeruli. Increased MMP2 and MMP9 expression, observed in nephritic kidneys, may contribute to alterations in the composition or integrity of GBM that facilitate binding of chromatin fragments to glomerular membranes.

Paper IV.

Acquired loss of renal nuclease activity is restricted to DNase I and is an organ-selective feature in murine lupus nephritis.

Reduced DNase I was observed in nephritic kidneys of (NZBxNZW)F1 mice at the stage of IC deposition in GBM. An acquired loss of renal DNase I has been suggested as a promoter of transformation of mild mesangial lupus nephritis into membrano-proliferative end-stage organ disease. However, DNase I expression in other organs of (NZBxNZW)F1 mice as well as expression profiles of other endonucleases in (NZBxNZW)F1 mice has never been examined. Only reduced serum DNase I activity in nephritic animals was reported but its importance in kidney pathology during development of lupus nephritis seems unclear. We analyzed mRNA level of DNase I, DNase IL1-3, caspase activated deoxyribonuclease (CAD), Dnase2a, and endonuclease G (Endo G) in kidneys, spleens and livers isolated from lupus-prone mice at different stages of lupus nephritis. DNase I activity and total nuclease activity were measured in kidneys, livers, spleens and sera of (NZBxNZW)F1 mice during progressive kidney disease. Our results demonstrate no reduction of DNase I mRNA level and enzyme activity in liver, spleen and serum samples of (NZBxNZW)F1 mice throughout all stages of lupus nephritis. DNase I was dramatically and selectively reduced only in kidneys of mice with severe nephritis and was the only nuclease that was down-regulated, while 6 other nucleases (Dnase1L1-3, CAD, Dnase2a, and Endo G) in kidneys were normally expressed. Loss of renal DNase I was not accompanied by changes in serum DNase I activity, suggesting an independent mechanism of the nuclease regulation in circulation and in kidneys and absence of compensatory up-regulation of serum DNase I activity in the case of renal DNase I deficiency. Thus, silencing of renal DNase I is a unique renal feature in membrano-proliferative lupus nephritis.

7. Discussion

7.1 Origin of chromatin fragments in glomerular EDS – accelerated renal apoptosis or defect in renal DNA degradation?

Antibodies to dsDNA and nucleosomes are strongly associated with lupus nephritis. Nucleosomes were suggested to play a dual role in the pathogenesis of the disease; they may initiate an autoimmune response and participate in glomerular immune complex deposition (34,132). Electron-dense structures containing TUNEL-positive autoantibody-binding chromatin are associated with glomerular capillary membranes and mesangial matrix in nephritic lupus-prone (NZBxNZW)F1 mice (165,166,202). Presence of TUNEL-positive extracellular chromatin indicates DNA fragments with 5'-P and 3'-OH ends that are generated by endonucleases during apoptosis. Accessibility of such fragments for autoantibody binding at physiological circumstances is prevented by formation of apoptotic bodies containing degraded DNA and their rapid clearance by macrophages. Thus appearance of chromatin fragments in EDS and manifestation of lupus nephritis likely occur due to disturbances in apoptotic DNA cleavage and/or impaired clearance of apoptotic cells.

The origin of IC accumulated in glomerular membranes is not clear. They can come from circulation and deposit due to filtration through the capillary walls or they can be formed locally with glomerular antigens. Studies with administration of pre-formed IC demonstrated their deposition mainly in mesangial matrix and subendothelial spaces, but not subepithelially (reviewed in (203,204)). In situ deposit formation was shown to occur in all glomerular patterns: mesangial, subendothelial and subepithelial (204). Different structures that can serve as a renal antigen in lupus nephritis were matter of discussions during the last decades. Cross-reaction of anti-dsDNA antibodies with inherent glomerular components like laminin (129) or α -actinin (182), or with mesangial cells components (205) was shown by many researches

and proposed as a central mechanism of antibody-binding in glomeruli. However those structures were not indentified in EDS by EM analysis (185,186). In respect to presence of DNA fragments in EDS and inability of circulating IC to deposit in subepithelial glomerular spaces as demonstrated experimentally (204) we proposed that pathological process leading to accumulation of chromatin within glomeruli can rather have a renal origin at least at the stage of membrano-proliferative nephritis. One simple explanation could be an increase in glomerular cell apoptosis as was already reported by different research groups (165,187,188). However they based the conclusion only on increased amount of apoptotic cells in kidney sections that can also occur due to impaired clearance of apoptotic material by macrophages. Moreover some studies demonstrated reduced apoptosis in lupus nephritis (189,190). To thoroughly determine the renal apoptotic rate in lupus nephritis we performed an integrated assessment of apoptotic triggers, executors and effectors in kidneys of (NZBxNZW)F1 mice at different stages of the disease (206). Our results demonstrate no evidence of accelerated renal apoptosis before or at the time of anti-dsDNA antibody production as well as at the time of clinical manifestation of kidney disease (mesangial nephritis). Only slightly increased amount of activated caspase 3-positive cells was found in kidney sections from mice with membrano-proliferative lupus nephritis mostly in tubular cells and in the interstitium (206). This increase was not accompanied by changes in mRNA level of apoptotic regulators and executors. These data suggested that rate of apoptosis in kidneys of (NZBxNZW)F1 mice is not increased by activity, and accumulation of extracellular chromatin in glomerular membranes at the stage of nephritis most likely occur due to other pathological processes. This conclusion was also consistent with our previous observation, that presence of TUNEL-positive chromatin particles in glomerular membranes in kidneys of (NZBxNZW)F1 mice correlated with lack of detection of LMW DNA fragments in DNA isolated from nephritic kidneys (202). Demonstration of oligonucleosomes in kidneys with glomerular EDS would

suggest increased ongoing apoptosis and/or impaired clearance of apoptotic cells. Absence of low molecular weight DNA in kidneys with TUNEL-positive EDS in glomerular membranes could indicate presence of large chromatin fragments that most likely appear due to defects in apoptotic chromatin fragmentation.

We determined LMW DNA in kidneys of (NZBxNZW)F1 mice using the Agilent bioanalyzer that is a sensitive electrophoretic assay. Clear LMW DNA ladders were observed in isolated BALB/c kidneys exposed to apoptosis inducer camptothecin (CPT), whereas only few active caspase 3-positive cells were present. Surprisingly, when we induced apoptosis *ex vivo* by CPT in isolated kidneys from (NZBxNZW)F1 mice we found markedly reduced DNA fragmentation compared to age- and sex-matched healthy controls (202). The amount of active caspase 3-positive cells observed in those kidneys was significantly higher than in controls (206) demonstrating that apoptosis was induced in (NZBxNZW)F1 mice but was not completed with final DNA degradation. These results together indicate that TUNEL-positive extracellular chromatin particles found in EDS can represent large DNA fragments accumulated in glomeruli due to defective chromatin fragmentation in kidneys of (NZBxNZW)F1 mice. Defects in DNA cleavage may be explained by lack of DNase I since dramatically reduced mRNA level of this nuclease was found to be associated with nephritis in lupus-prone animals (202,206).

7.2 Acquired loss of renal DNase I in development of lupus nephritis

DNase I in kidneys has been shown to represent approximately 80% of the total nuclease activity and mostly expressed in tubular epithelial cells (96,202). Role of the enzyme is not well established but it has been reported that mice deficient in DNase I expression are protected from kidney injury mediated by cisplatin (96). This chemotherapeutic drug induces apoptosis and necrosis in cells in a dose-dependent manner (207,208). Moreover DNase I was

up-regulated during kidney injury induced by ischemia-reperfusion in in vivo experiment (209). Those data indicate an importance of DNase I to clear death-associated chromatin.

The mechanism of chromatin fragmentation and breaking down during necrosis by serum DNase I was experimentally demonstrated. Results showed that the nuclease can penetrate necrotic cell, accumulate in the cytoplasm and nucleus and proceed DNA cleavage (92). How DNase I can participate in apoptotic chromatin fragmentation is not clear since this is a secreted protein and does not have direct access to the cell nucleus. Data from experimental induction of apoptosis in cells deficient to DNase I are also controversial. While one paper demonstrate resistance to apoptotic stimuli in cells after disruption of the *DNase I* gene (76), another paper report that no difference in the sensitivity towards the induction of apoptosis was observed in tissues and cells from DNase I knockout mice with lupus predisposed background in comparison to wild-type animals (210). On the other side, reduced chromatin fragmentation and cell death was found in the intestine of DNase I deficient mice with “non-lupus” background (95). In spite of contradicting results, the fact that DNase I activity in kidneys is dominant compared to other nucleases and importance of DNase I in renal cells death implicates its essential role for DNA digestion during the cell life cycle.

Abnormal levels of DNase I activity was observed in association with a variety of diseases. A high serum DNase I was measured in patients with advanced liver diseases (211), acute hemorrhagic pancreatitis (212), several cancers (213-215) and renal failure (216), whereas low level was found in patients with xeroderma pigmentosum (108). Involvement of DNase I in the pathogenesis of lupus nephritis was discussed back in 1981 when decreased serum DNase I activity was observed in lupus patients (107) and later on in lupus-prone mice (108). Detection of DNA in circulation together with low DNase I activity in serum could indicate insufficient DNA fragmentation in blood and was proposed as a promoter of

autoimmunity to self chromatin in SLE (107). Interest to this idea was cooled down when intravenous administration of DNase I to patients with lupus nephritis did not lead to suppression of disease activity (112).

Discovery of a defect in apoptotic DNA fragmentation of ex-vivo camptothecin induced apoptosis in kidneys of (NZBxNZW)F1 mice stimulated us to analyze renal expression of endonucleases during development of lupus nephritis (202). The mRNA levels of CAD, EndoG and DNase I were measured in kidneys of (NZBxNZW)F1 mice at different age groups. Dramatic reduction of the transcription level of DNase I was observed when mesangial nephritis progressed into end-stage organ disease, whereas CAD and EndoG remained unchanged in all groups of animals. To investigate the relevance of DNase I renal gene expression to development of lupus nephritis we performed a longitudinal study where we analyzed mRNA level and enzyme activity of DNase I in kidneys of (NZBxNZW)F1 mice and correlated the data with appearance of circulating anti-dsDNA antibodies, deposition of IC in the mesangial matrix and/or glomerular membranes, and with proteinuria (217). Our data demonstrate that initiation of lupus nephritis was associated with anti-dsDNA antibody production and correlated with appearance of EDS in the mesangial matrix. End-stage nephritis on the other hand, was characterized by severe proteinuria and IC deposition in GBM and an acquired loss of renal DNase I (217). Based on these results we proposed that lupus nephritis in female (NZBxNZW)F1 mice is a two-stepped organ disease with possibly two different pathogenetical mechanisms of forming deposition of IC in glomeruli. Initiation of lupus nephritis can be due to deposition of IC possibly coming from circulation. It can explain the systemic character of tissue damage in SLE. Comparative studies of components of IC and their localization in skin and glomeruli demonstrated that they have similar structure and are consistently observed in capillary lumina in both skin and kidney (218,219). However, no other associations between skin and glomerular deposits were found (218,219).

Importantly, deposition of IC in glomeruli did not predict deposition in skin. Examination of DNase I and MMP expression in skin in MRL-lpr/lpr mice demonstrated stable low activity of DNase I and an increase in MMP-2 and MMP-9 enzyme activities during disease progression (219). Those results confirm that circulating IC can be an initial event of SLE manifestation in different organs, but mechanism of tissue damage progression might be organ restricted. Our data suggest that progression of lupus nephritis is caused by accumulation of large chromatin fragments in glomeruli due to insufficient DNA fragmentation in kidneys deficient of renal DNase I expression. Access of such fragments in GBM can be facilitated by increased MMP activity since increased expression and activities of MMP-2 and MMP-9 were observed at the time when severe nephritis developed in (NZBxNZW)F1 mice (217).

7.3 Loss of renal DNase I – a systemic error or an organ-selective feature?

Distribution of the DNase I activity in different tissues and cells has been published for humans and other mammals (90,220,221). According to the expression pattern within the gut they can be grouped into three types: pancreas type (pig and human), parotid type (rat and mouse) and mixed pancreas-parotid type (bovine and rabbits) (221). Difference in DNase I expression is most probably reflecting variation in the eating habits (221). Furthermore beside the intestinal juice, DNase I is secreted into urine (kidney), seminal fluid (prostate), lacrimal fluid (lacrimal gland) and serum (90). The origin of serum DNase I is not known. Recent data indicated liver as a source of DNase I in serum (192). The dominant function of serum DNase I is digestion of chromatin material released in circulation. If serum DNase I contribute to intracellular DNA fragmentation has never been shown.

Reduced serum DNase I activity was demonstrated in human and murine SLE. We found lack of DNase I in kidneys at the time of severe lupus nephritis in (NZBxNZW)F1 mice. Therefore, it became important to verify if loss of the DNase I expression is an organ-

specific feature or if it reflects a systemic error in BW mice. In Paper IV we performed analysis of DNase I expression and enzyme activity in spleen and liver tissues in comparison to pathological changes in kidneys during progressive lupus nephritis. Our data demonstrate that loss of renal DNase I is an organ-selective feature in lupus-prone mice with membrano-proliferative nephritis. Moreover analysis of mRNA levels of 6 other known endonucleases (DNase I1-3, CAD, Dnase2a, and EndoG) demonstrates absence of compensatory up-regulation in kidneys or livers, neither at the time of mild nephritis nor after its transformation into severe disease with renal DNase I deficiency.

Interestingly we did not observe reduction in serum DNaseI activity in mice with full-blown nephritis in contradiction to previously published data (109). Nuclease activity in serum of (NZBxNZW)F1 mice with membrano-proliferative nephritis measured by a DNase radial diffusion assay and serum DNase I activity measured by denaturing SDS-PAGE zymography were comparable to activities detected in pre-nephritic animals. Notably, the degree of serum DNase I activity in the different groups of (NZBxNZW)F1 mice correlated with DNase I activity in the liver and not in the kidney, in accordance with results provided by Ludwig et al. (192). Those findings refer to the important question - is serum DNase I a required pool for the kidneys in the situation where renal DNase I is lost? We do not have data to provide an answer to this question. Oppositely, failure of an effect on lupus nephritis after DNase I administration in lupus patients (192) or in (NZBxNZW)F1 mice (111) indicates low-relevance of serum DNase I in renal environment. This leaves us with the perception that renal, intra-cellular DNase I is required for safe degradation and elimination of chromatin from dying renal cells. Without this enzyme, chromatin degradation is impaired, which results in deposition of large chromatin fragments in situ, where they are released from dying cells.

7.4 Clearance deficiencies in lupus nephritis

A large amount of cells undergo apoptosis every day but to detect them in situ is difficult, because they are quickly engulfed by macrophages and immature dendritic cells (44). An accumulation of apoptotic cells in the bone marrow and in the skin of lupus patients has been reported (222,223) as well as decreased clearance of apoptotic cells by macrophages in human and murine SLE (115,117,224). The exact mechanism leading to impaired clearance of apoptotic cells in lupus is not known. Deficiency in the components of the classical complement cascade including C1q, C2 and C4 are associated with high risk of SLE (225). Mice deficient in C1q develop lupus-like disease (226). Acquired and transient deficiency of C1q commonly found among SLE patients may be due to consumption by large amounts of IC or binding to anti-C1q antibodies rather than genetic defect (226).

Professional phagocytes detect apoptotic cells due to recognition of “eat-me” signals on their surfaces (including phosphatidylserine, intercellular adhesion molecule-3, Annexin I and cardiolipin) (227-229). Absence of such signals or defects in phagocyte recognition may lead to impaired clearance of apoptotic material (119,230). The uptake of apoptotic cells by phagocytes induces the expression of “tolerate me” signals including interleukin 10 (IL-10), transforming growth factor β (TGF- β) and prostaglandin E₂ (PGE₂) (55,231). If apoptotic cells reach a stage of secondary necrosis due to for example impaired clearance, their uptake is followed by production of pro-inflammatory cytokines and up-regulation of co-stimulatory molecules. In other words, impaired clearance of apoptotic cells may lead to central pathological processes in the pathogenesis of SLE: i. it may break self-tolerance to chromatin and ii. it may enable exposure of extracellular chromatin (self antigen) in tissue (reviewed in (55,194)).

Generation of large chromatin fragments due to lack of DNase I likely can be connected to defective “eat-me” signal presentation. In this case loss of renal DNase I may

contribute to clearance deficiency and accumulation of extracellular DNA in nephritic kidneys (reviewed in (232)).

7.5 Why is renal DNase I shutting down?

Based on our data, the question “why is renal DNase I shutting down? “, becomes the most important to answer since factors regulating renal DNase I can be future therapeutic targets in lupus nephritis. So far the mechanisms leading to silencing of renal DNase I are not clear but are currently analyzed in our laboratory. Contemporarily we follow three lines of analyses: transcriptional interference with convergent encoded genes, regulation by microRNAs, or by DNA methylation. The initial event, accounting for DNase I shut-down may, however, represent a response to inflammatory signals provided by early mesangial nephritis - the deposition of IC in the mesangial matrix.

8. Concluding remarks

Our investigations illuminated important mechanisms in the pathogenesis of lupus nephritis. We proposed the origin of chromatin fragments in glomerular EDS and processes leading to their formation. We have demonstrated defects in the process of apoptotic chromatin in kidneys of lupus-prone mice and have renewed an interest to the dominant renal nuclease DNase I. Our data allow us to generate new idea to describe kidney disease progression in SLE. We demonstrated that lupus nephritis in lupus-prone mice is initiated through deposition of anti-DNA antibody-nucleosome complexes in glomerular mesangial matrixes, a process that largely is sub-clinical. Transformation of mild mesangial nephritis into membrano-proliferative end-stage kidney disease is accompanied by severe proteinuria and is associated with a dramatically reduced renal DNase I. We have shown that shut-down of renal DNase I contribute to accumulation of large chromatin fragments observed as EDS in glomerular membranes. Deposition of such IC promotes transformation of mild mesangial nephritis into membrano-proliferative nephritis, induction of severe proteinuria and end-stage kidney disease. The observed defect in renal DNase I expression is demonstrated to be organ specific and shut-down of renal DNase I is shown to be selective among 7 nucleases analyzed in this study. Future investigation of mechanism leading to DNase I down-regulation may provide a new therapeutic target in lupus nephritis.

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Paper I

Paper II

Paper III

Paper IV



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