

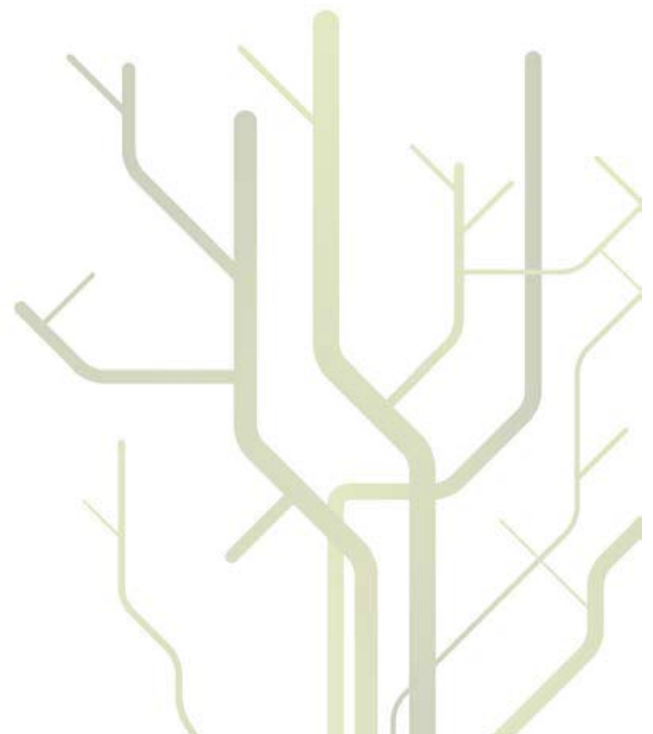
Progressive lupus nephritis: Biological consequences of renal DNaseI gene silencing



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A dissertation for the degree of Philosophiae Doctor

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By

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Abbreviations

ACR	American College of Rheumatology
ANA	Antinuclear antibodies
CAD	Caspase activated deoxyribonucleases
Clec4e	C-type lectin 4e
DNA	Deoxyribonucleic acid
DNaseI	Deoxyribonuclease I
DNaseIII	Deoxyribonuclease I like 1
dsDNA	double stranded deoxyribonucleic acid
EDS	Electron dense structure
Endo G	Endonuclease G
FC γ R	Fc gamma receptor
GBM	Glomerular basement membrane
HLA	Human leukocyte antigen
IgG	Immunoglobulin G
IL	Interleukins
IL 1 β	Interleukin 1 beta
INF γ	Interferon gamma
MMP	Matrix metalloproteinase
RNA	Ribonucleic acid
RNAP	RNA polymerase

RPTEC	Renal proximal tubule epithelial cell
SAP130A	spliceosome associated protein 130A
SLE	Systemic lupus erythematosus
SLICC	Systemic Lupus International Collaborating Clinics
TLR	Toll like receptors
TNF α	Tumor necrosis factor alpha
TRAP1	Tumor necrosis factor receptor-associated protein-1
UTR	Untranslated region
WHO	World health organization

List of Papers

1. Silencing of Renal DNaseI in Murine Lupus Nephritis causes Exposure of Large Chromatin Fragments and Activation of Toll Like Receptors and the Clec4e. **Dhivya Thiyagarajan***, Silje Fismen*, Natalya Seredkina, Søren Jacobsen, Thomas Elung-Jensen, Anne-Lise Kamper, Chris Fenton, Ole Petter Rekvig, and Elin Synnøve Mortensen. PLoS One. 2012 7(3): e34080.
2. Impact of the tumor necrosis factor receptor-associated protein 1 (Trap1) on renal DNaseI shutdown and on progression of murine and human lupus nephritis. Fismen S*, **Thiyagarajan D***, Seredkina N, Nielsen H, Jacobsen S, Elung-Jensen T, Kamper AL, Johansen SD, Mortensen ES, Rekvig OP. Am J Pathol. 2013 Mar;182(3):688-700
3. Stimulation of human renal proximal tubule epithelial cells with TNF α and IL-1 β increases mRNA and protein expression of DNaseI and translocate DNaseI from cytoplasm into the nucleus. **Dhivya Thiyagarajan**, Ole Petter Rekvig, Natalya Seredkina. Manuscript 2013

*Both have contributed equally and share 1st authorship

Research motivation

Certain diseases cause a heavy burden for affected patients and their families, for the society and for the social economy. Substantial effort is needed in order to develop a better insight into disease mechanisms, diagnostic procedures and biomarkers for progression of the disease and therapy response. One still enigmatic disease represents the focus for this project: the autoimmune syndrome Systemic lupus erythematosus (SLE), and its most serious and life threatening organ complication - lupus nephritis. SLE is a poorly defined autoimmune syndrome, and the frequency of SLE varies by race and ethnicity. Internationally, the research on SLE is intense (since 1946, 55301 papers have been published (search term: Systemic lupus erythematosus), and since 1959, 33279 papers have been published on the origin and role of anti-DNA antibodies (search term: Anti-DNA antibodies, PubMed 2013). This reflects that we still do not know how to provide aim-directed therapeutic interventions – simply because there is still no international consensus that explains disease mechanisms. Thus, despite such tremendous international efforts to understand the nature of SLE and its pathogenic mechanisms, still, our insight is fragmental and elusive. In a translational scientific approach we aim to reach fundamental insight into the pathogenesis of human and murine lupus nephritis.

SLE – an overview

Systemic lupus erythematosus (SLE) is an autoimmune disease which may affect any part of the body. It was in 1948, Dr. Hargraves made the first observation with SLE specific “LE” cells (1). These phagocytic cells have ingested nuclei of dead cells. Later LE cells were regarded as a diagnostic tool for SLE (2). Soon thereafter, it was found that there was an association between anti-nuclear antibodies (ANA) and clinical subsets of SLE (3-5). SLE is more common among women than among men, with female to male ratio of 9:1. SLE occurs at any age but most often between the ages 10-50 years (6). The most commonly affected organs are skin, joints, heart, kidney, brain in addition to other organs. The main pathological effects are caused by immune complex deposits with subsequent development of inflammation in different organs.

Deposition of chromatin-anti-dsDNA antibody immune complexes is the core factor that imposes renal inflammation in SLE. The origin of the components of these complexes is, however, unknown. Antibodies to dsDNA were discovered in 1957 by four independent research groups (2,3,7,8). Since then, a strong international effort has been made in order to understand how and why these antibodies have been produced, although only fragmental insight into these processes has been obtained (4,9-14). Nevertheless, internationally, it is a consensus that antibodies to dsDNA (and to nucleosomes) are the result of cognate interaction of autoimmune B cells and immune or autoimmune T cells (15-17). Likewise, the role of chromatin as a central target structure in early and late lupus nephritis has been debated, although there is no international consensus for this idea (for review, see e.g.(18-21)).

One of the major hallmarks of SLE is sustained production of autoantibodies. Although these autoantibodies are specific for a broad range of cellular auto-antigens deriving from nuclei, cytoplasm or membranes, many of them may serve as markers for SLE. Others may be associated with SLE but not specific for the disease. The mechanism of autoantibody production is still unknown, but it could be a result of B and T cells abnormalities, and/or impaired clearance mechanisms of dead cells (reviewed in (22)). Since all key components of the immune system are involved in SLE, the syndrome is regarded as basically autoimmune. The clinical symptoms are diverse and include arthritis, nephritis, seizures, psychosis, serositis and others (23). Approximately 30-60% of SLE patients acquires lupus nephritis which is a most challenging problem due to its severity (24). The most characteristic feature of lupus nephritis is end stage

organ disease (renal failure) which is associated with immune complex deposits in glomerular basement membranes (GBM) (25). In the next section, different aspects of SLE and lupus nephritis will be introduced.

Classification of SLE

The classification of SLE is important and therefore need to be sensitive and specific (26). According to the American College of Rheumatology (ACR) classification system the patients must satisfy at least four of the criteria listed in Table 1 (26,27), and according to The Systemic Lupus International Collaborating Clinics (SLICC) criteria, at least one clinical and one immunological criteria must be present (28). SLICC criteria for SLE are intended to be used in clinical and scientific studies (29).

Table.1 The 11 ACR classification criteria for SLE, last revised in 2002 modified from Petri M .et al.(26)

1. Malar Rash
2. Discoid Rash [Also subacute cutaneous lesions]
3. Photosensitivity
4. Oral Ulcers
5. Arthritis
6. Serositis (pleuritis or pericarditis)
7. Renal disorder
8. Neurological disorder
9. Hematologic disorder
10. Immunologic disorder
11. An abnormal titer of Antinuclear antibody

Epidemiology

SLE demonstrates a high degree of variation between gender, race and age. Prevalence of SLE varies among populations primarily based on ethnicity. Approximately 85% of SLE develops before the age of 50 and approximately 20 % of the SLE patients are children below 16 (30).

Most of the SLE patients are female and the ratio between male to female is 1:9, whereas in childbearing years it increases to 1:11 (31). The disease manifestations differ between males and females (32). Onset of disease is earlier among females than among males and the prevalence of the disease is 2 to 3 times higher in Africans or Asians compared to Caucasians. In addition Afro-Americans or Hispanics in USA and Latin American develop SLE in early life and experience a more severe disease than Caucasians (32). Incidence of disease has increased 3 times during the last 50 years, possibly due to better diagnostic tools and procedures (33).

Etiological factors

Classically, three main factors are considered to be central in the etiology of SLE. These factors encompass genetics, hormones and environmental factors.

The concordance rate of SLE is 25% in monozygotic twins and 2% in dizygotic twins (23,34) which indicate the genetic contribution to SLE. Like other autoimmune diseases, HLA (Human Leukocyte Antigen) regions are associated with the risk of SLE and production of specific autoantibodies, although the basis for this association is not clear (35). Also deficiency of Fc gamma receptor II (FC γ RII) has been implicated with the pathogenesis of SLE and lupus nephritis (36,37). Many other genes have been identified by whole genome scanning from families (38) with SLE. Genes that include early complement complex and genes that cause immunological tolerance are also associated with SLE. In addition, Toll like receptors (TLR), and type I interferon production are reported to be involved in SLE (39,40). Except for a genetic deficiency of C1q of the first complement factor, none of the other genes alone cause SLE (41). It is estimated that at least a combination of four genes is necessary for the development of disease (42). In mice, genetic loci which promote SLE are designated as Sle1, Sle2 and Sle3. These deal with loss of immunological tolerance, T cell dysregulation and B cell hyperactivity (38).

Ultraviolet radiation is another environmental factor linked to SLE (43). Exposure to silica (44), tobacco (45), infectious agent (Epstein–Bar virus) has also been associated with the risk of SLE (46). Use of hair dyes have been assumed to be a risk factor for SLE, but no confirmed evidence was obtained in large prospective studies (47).

Since SLE is predominantly a female disease, female hormones may play a major role in the disease. Hormones like estrogen and prolactin can induce SLE in mouse models (48,49). Use of

contraceptive pills and hormone replacement therapy is associated with increased risk of disease. Flares of SLE are known to occur during sudden hormonal change like pregnancy, ovulation stimulation during *in vitro* fertilization and exogenous estrogen administration. These estrogens have multiple actions on various immune cells that can lead to increased B cell proliferation and antibody production (50-53). Estrogen also hinders the T cell response, which indicates T cells are sensitive to estrogens, and estrogen affect the cytokine profile of Th2 with impact on autoantibody production (22).

Pathogenesis of SLE

As the etiology of SLE is multifaceted with no unambiguously identified main factor, the pathogenesis of SLE is also composed of diverse processes that are not necessarily linked to each other. This unclear picture of the etiology, predisposing factors and pathogenic processes may indeed point at SLE as a poorly defined syndrome. Nevertheless, two pathological processes have been identified: i. termination of self-tolerance that imposes sustained production of organ-specific and organ-unspecific autoantibodies and ii. the inflammatory consequence of *in vivo* binding of the induced autoantibodies to membranes or to soluble or particulate antigens that may deposit in tissue as immune complexes.

The immune system is normally tolerant to autologous tissue. The main obligation for the immune system is therefore to defend our body from pathogens coming with bacteria, viruses or parasites. Aggression of the immune system against autologous structures is prevented by tolerizing the immune system, a process initiated when immature B- and T-cells bind self-antigens followed by elimination in bone marrow and thymus (central tolerance) or mature auto-reactive cells enter the periphery and become anergic in the absence of co-stimulation by antigen presenting cells (peripheral tolerance) (54,55).

B- and T-cell abnormalities have been observed in human and murine SLE (56-58) . However, defects in B- and T-cells cannot explain the main phenomena in the pathogenesis of SLE – how intracellular self-antigens become immunogenic and trigger a strong and prolonged autoantibody response (59,60).

One of the central targets for autoantibodies in SLE is nucleosomes. Anti-nucleosomal antibodies have been shown to have a strong impact on pathogenic processes in patients with SLE (61-63),

and encompass a large array of individual autoantibodies, of which anti-dsDNA antibodies are regarded the most central pathogenic factor in overt SLE (63,64). 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 potential mechanisms accounting for breaking self-tolerance and induction of antigen-driven prolonged autoantibody responses (65-67). In this sense, defects in apoptotic processes may be linked to pathogenesis of manifestations linked to SLE.

Impaired clearance of apoptotic cells *in vivo*

Apoptosis is a programmed genetically controlled cell death characterized by condensation of chromatin, DNA fragmentation, membrane blebbing and externalization of phosphatidylserine (68). It is initiated through binding of death receptors on the cell surface (determining the extrinsic pathway of apoptosis), or from within the cell as response to DNA damage, defective cell cycle, hypoxia or other types of cell stress. This determines the intrinsic pathway to execute apoptosis. Apoptosis is fulfilled in an orderly way by interaction of cascades of apoptosis-associated proteases and endonucleases. These enzymatic activities end up with morphological changes in the cell, typical for apoptosis (69). In the last stage, apoptotic bodies present “eat-me” signals and are engulfed by macrophages or dendritic cells (70,71). Clearance of intact apoptotic cells are important, as their silent removal prevents secondary transformation of the cells into necrotic debris with the potential to promote inflammation (72,73).

In general, apoptotic cells are silently and efficiently cleared with the help of various receptors

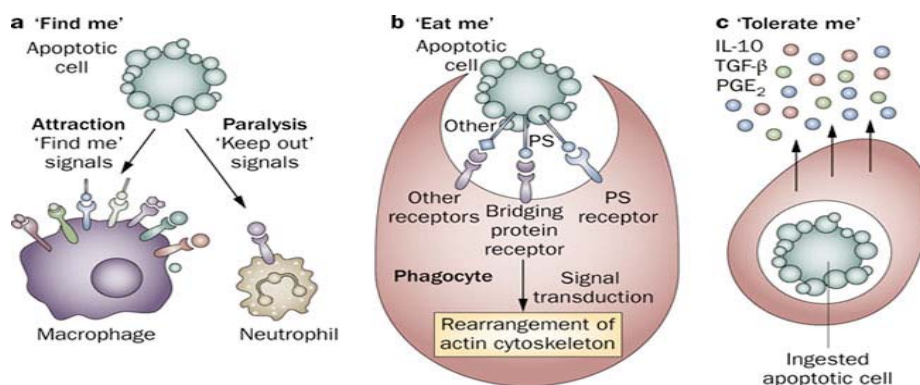


Figure 1: Clearance of apoptotic cells by professional phagocytes. Reprinted with permission from Review article “The role of defective clearance of apoptotic cells in systemic autoimmunity” by Munoz LE et al., 2010 (83).

and ligands, as illustrated in Figure 1. Dying apoptotic cells release or expose molecules such as phosphatidylserine, ATP (adenosine tri-phosphate), UTP (uridine di-phosphate) and S19 to attract macrophage by “Find me” signal (74-76). In addition, the dying cells protect themselves from neutrophils by “Keep out” signal molecules such as lactoferrin (77). Thus, initiation of phagocytosis in apoptotic cells depends on detection of “find me/eat me” signaling molecules for the engulfment. This maintains the tolerance specific for structures contained within the apoptotic cells.

However, it has been shown that in SLE, impaired clearance of apoptotic cells or continuous overload of apoptotic cells leads to accumulation of apoptotic debris (78,79). This apoptotic debris may, if not removed, release danger signals provided by secondary necrotic structures unmasked from apoptotic blebs (80). Danger signals induce the dendritic cells to up-regulate co-stimulation, present auto-antigens, and secrete inflammatory cytokines. This leads to inflammation, breakdown of self-tolerance and in the end cause autoimmunity that may include autoimmunity to e.g. nucleosomes, typical for SLE (81-83). The distinct balance between silent removal of apoptotic cells and exposure of secondary necrotic debris is illustrated in Figure 2.

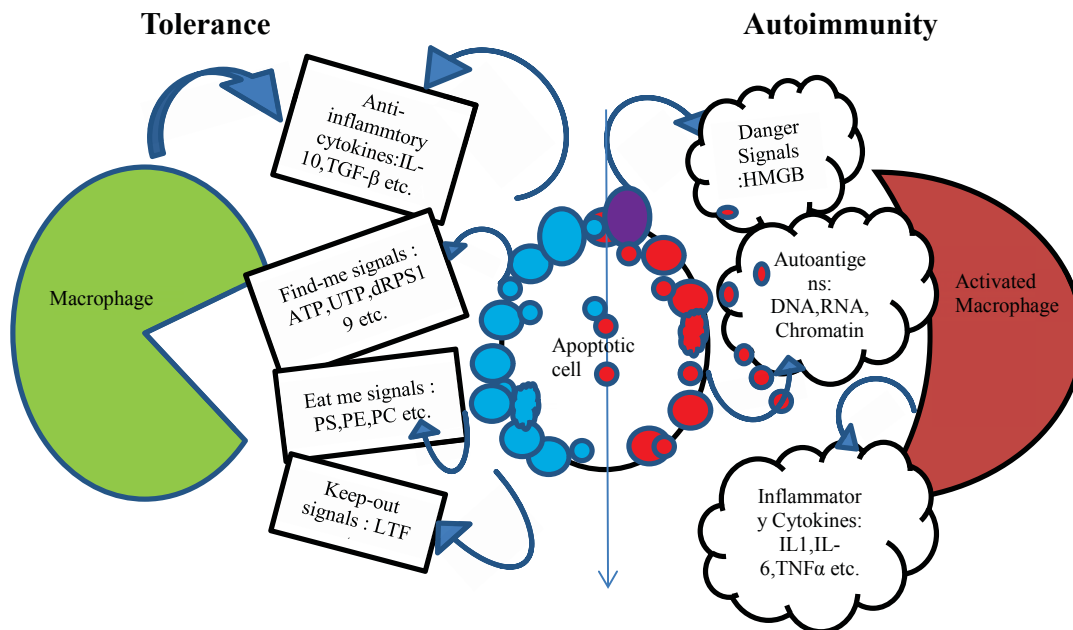


Figure 2: Distinct balance between the apoptotic cell clearances. Modified from Shao WH and Cohen PL, with permission from Biomed Central, Arthritis Research & Therapy 2011, 13:202 (81).

Lupus Nephritis

Lupus nephritis is still the most severe clinical manifestation of SLE. 30-60% of the SLE patients may be affected during lifetime (24,84). Before 1970, 5-year survival rate of SLE patients with lupus nephritis was about 25-40% (85) which by itself indicates how serious lupus nephritis in fact is. However, during the last 2 decades, the survival rate increased to 80-95% (86) due to better drugs and renal transplantation regimes. The results of modern and integrated therapy are still insufficient since complete remission rates of lupus nephritis are around 50% (87) with frequent relapses (88).

Proteinuria and biochemical changes in the urine are early clinical symptoms of the disease. The level of circulating autoantibodies is associated with overall disease activity but does not correspond to changes in glomeruli or degree of kidney damage (89). Therefore, the only approach to study degree of renal disease is to analyse biopsies by light microscopy, immunofluorescence microscopy and by electron microscopy. Histopathological changes and the extent of kidney injury indicate the risk of severe renal disease (90).

Epidemiology

The epidemiology of lupus nephritis is variable, and the incidence and prevalence of disease varies in different populations. In a study from northwest England it was observed that the incidence of disease was $0.4/10^5$ with a prevalence of $4.4/10^5$ (91). In another study, incidence of lupus nephritis in SLE was higher in Asia (55%), Africa (51%) and in Hispanic (43%) compared with Caucasians (14%) (92). When lupus nephritis develops, it develops early in the progression of SLE, which turns out to be a poor predictor of the outcome of the disease. On the other hand, 5% of SLE patients develop lupus nephritis several years after onset of the disease (93). In some cohort studies renal involvement is more common in males than in females, whereas in other groups this was not seen (94-96).

Etiology

The actual reason for the development of lupus nephritis is still under debate, and there is no international consensus related to its pathogenesis. Indeed the progression of lupus nephritis is believed to be reliant on production of autoantibodies and deposition of immune complexes. The major hallmark of the disease is anti-nuclear antibodies, which can be differentiated into

antibodies against specific nuclear antigens (97). The immune complexes will deposit in the kidney which influence the disease progression. Human studies and mouse model analysis reveal that various factors in the host are involved in the pathogenesis of lupus nephritis (98). Not all patients with lupus nephritis produce anti-dsDNA antibodies. This may indicate that lupus nephritis is not always linked to the presence of these antibodies. This has been experimentally demonstrated in murine models of lupus nephritis, where T cells accounted for the kidney injury (99,100). Consistent with the latter observations, the production of anti-chromatin autoantibodies is not absolutely required for the development of lupus nephritis (101). Thus, although antibodies to dsDNA and chromatin are important in lupus nephritis, its pathogenesis includes elements of antibody-independent processes.

Classification of lupus nephritis

The first classification of lupus nephritis was made in 1974 by the World Health Organization (WHO) who divided the organ disease into five classes. In 1982 the criteria was modified after the identification of new patterns of immuno-staining of kidney sections. Thus, the classification criteria is based on various staining patterns on renal biopsies. In 2003, The International Society of Nephrology and Renal Pathology Society (ISR/RPS) Working Group on the classification of lupus nephritis, revised and published the latest version in 2004 (Table 2). This classification system is based on light microscopy, immunofluorescence microscopy and by electron microscopy analyses of renal biopsies and focuses exclusively on glomerular pathology, and separate lupus nephritis into 6 classes (28).

Class I lupus nephritis is characterized by mesangial immune complex deposits detectable only by EM and/or immunofluorescence. Class II is characterized by appearance of mesangial hypercellularity and extended deposition of immune complexes in mesangium. Class I and II are clinically silent processes, and rarely progress into renal failure. Class III lupus nephritis present segmental endocapillary proliferative lesions and immune deposits sub-endothelially and affect less than 50% of all glomeruli. In Class IV, more than 50% of glomeruli are affected. Clinically, Class III and IV lupus nephritis are associated with progression into end-stage kidney disease. Lupus nephritis patients with subepithelial immune deposition (Class V) have low progression, typically presented with nephrotic syndrome and have high risk of thromboembolic events. Class

VI lupus nephritis is the final-stage when chronic glomerular inflammation transforms into global glomerulosclerosis.

Table.2 Classification of lupus nephritis by ISR/RPS working group, modified from Weening et al. (28).

Classes	Description of injury
Class I	Minimal mesengial nephritis
Class II	Mesangial nephritis
Class III	Focal lupus nephritis
Class IV	Gobal lupus nephritis
Class V	Membranoproliferative lupus nephritis
Class VI	Advanced sclerosing lupus nephritis

Pathophysiology of lupus nephritis

The course of SLE is hampered with the constant production of autoantibodies against components of chromatin. Predominant autoantibodies are anti-dsDNA antibodies, which are directly associated with the pathogenesis of lupus nephritis (14,19-21) . However other autoantibodies which are associated with lupus nephritis includes among others anti-C1q, anti-Ro, antibodies to laminin, collagen, fibrinogen etc. (reviewed in (21),(102)).

The exact mechanism for deposition of immune complexes in the kidney is still unknown, but

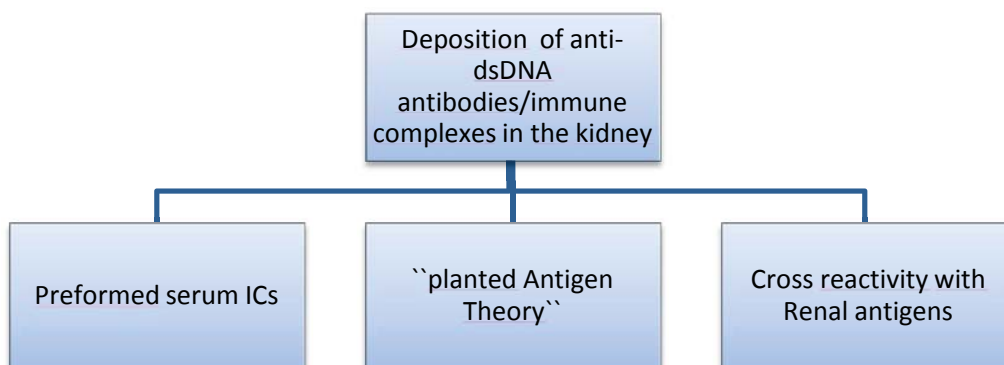


Figure 3: Three proposed theory for the immune complexes deposits in the kidney. Adopted from the Review article by Alberto de Zubiria Salgado et. al, Autoimmune diseases, 2012 (25).

there are some proposed mechanisms (illustrated in Figure 3). These include: 1) deposition of preformed serum immune complexes in the kidney, 2) anti-dsDNA antibodies binds to chromatin fragments that are exposed in glomerular matrix and membranes (103), and 3) direct binding of antibodies to the renal antigen by cross reactivity.

A wide spectrum of autoantibodies has been shown to be involved in the genesis of lupus nephritis. Many of these bind components of nucleosomes, while other bind components of glomerular membranes (reviewed in (21), (102)).

There is today a consensus that anti-dsDNA and anti-nucleosome antibodies play a central role in both early and late stages of lupus nephritis (14,19-21,104). But there is no international consensus how they bind in glomeruli. Our group has provided evidences that chromatin fragments represent the central renal targets for nephritogenic autoantibodies. This has been demonstrated by immune electron microscopy and by variants of this technique (14,18-21). There is, however, today not an international consensus on this point. Several groups have demonstrated that nephritogenic autoantibodies cross-react with glomerular structures such as laminin, α -actinin and collagen (105-110) and lately also with entactin (111). By immune electron microscopy assays we were not able to show presence of such components in areas where autoantibodies bound *in vivo* (112,113). On the other hand, the *in vivo* bound antibodies were not observed bound in regular membranes or matrices.

The cross-reaction model that was developed to explain the nephritogenic potential of anti-dsDNA antibodies is problematic. Dual specificity of an antibody does not allow us to identify which one of the cross-reactive renal ligands that indeed bound these antibodies *in vivo*. Even proving that antibodies eluted from nephritic kidneys cross-react with dsDNA and α -actinin (109), or with laminin (110,114) or entactin (111) does not reveal which of the target structures these antibodies actual bind *in vivo*. It is important to solve this apparent paradox how antibodies are involved in lupus nephritis in order to design new and concise therapy regimes.

In our opinion, there is firm support for a model of lupus nephritis in which extra-cellular chromatin plays the role as target structure for anti-dsDNA/anti-chromatin antibodies. Thus, it is reasonable to think that chromatin fragments exposed in glomeruli are released from dying renal cells. In context of lupus nephritis, these fragments are not appropriately degraded during the

apoptotic process because of an acquired loss of the dominant renal endonuclease DNaseI (see below). In this situation, chromatin fragments may be targeted by potentially nephritogenic anti-chromatin antibodies. Therefore, in both murine and human lupus nephritis exposure of chromatin in glomeruli and its complex formation with IgG are central events in disease pathogenesis and disease progression. In this picture, there is no place for cross-reactive anti-dsDNA autoantibodies as a major cause for lupus nephritis.

Apoptosis, autoimmunity and lupus nephritis

Apoptosis is a programmed cell death. Apoptosis is executed through an intrinsic and an extrinsic pathway. It leads to chromatin condensation, DNA fragmentation and cell shrinkage (115) (see above). These apoptotic bodies then send “eat me” signals to phagocytes/macrophages to digest the bodies. Apoptosis is regulated by sets of genes and clearance of dying cells prevents the formation of secondary necrotic cells and release of pro-inflammatory response (72,73). In contrast, necrosis (not genetically programmed), a process where the cell membrane loses its integrity, leads to exposure of intracellular components. This is followed by the activation of inflammasomes. The inflammasome is a large multimeric cytoplasmic protein complex that facilitates proteolytic processing of prointerleukin-1 β to its active form (116,117). Thus in the late stage of apoptosis, if the cell membrane loses its integrity, this is called secondary necrosis (118). If the apoptotic process transforms into secondary necrosis the necrotic cell debris releases “danger signals” which contribute to breakdown of immunological tolerance (119,120).

In vitro studies have shown that there is an impaired phagocytosis of apoptotic cells by monocyte-derived macrophages in SLE patients compared to healthy individuals (121). Increased apoptotic activity among peripheral blood cells from SLE patients including lymphocytes (122), neutrophils (123) and monocytes (124) and its positive correlation with autoantibody production and disease activity (123) has been demonstrated. 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 monocytes/macrophages *in vivo* by the administration of chlodronate liposomes to lupus-prone mice resulted in increased production of anti-nucleosome and anti-dsDNA antibodies and worsening of lupus nephritis. On the other hand, injection of chlodronate in non-lupus-prone mice leads to development of anti-nucleosome antibodies but not lupus nephritis (125). Induction of apoptosis has also been shown to be the

initial event in the pathogenesis of pristane-induced SLE in mice (126), which also is complicated by development of lupus-like nephritis (125).

In addition, detection of increased number of apoptotic cells was seen in *in vivo* studies of patients with SLE compared with non-SLE individuals (127-129). Furthermore there was a typical morphological difference in macrophages (tingible body) observed in the sections of lymph nodes from SLE patients. But in non-SLE patients these tingible bodies were normal and were not reduced in number (130). Furthermore, there are evidences that defects in any apoptotic molecules in murine models were associated with development of autoimmunity (131-133). Thus, impaired clearance of apoptotic cells, in addition to a yet unknown genetic background, may push the development of autoimmunity in the SLE patients (82).

Interestingly, and in contrast to increased apoptotic activity, reduced apoptosis is also phenomenologically linked to autoimmunity. MRL/lpr-lpr mice, due to a mutation in the Fas receptor gene, have a non-functional apoptosis-inducing Fas system, and a consequent reduced extrinsic pathway of apoptosis. These Fas deficient mice develop a spontaneous lupus-like syndrome including production of anti-dsDNA antibodies, lupus nephritis and skin lesion (lupus dermatitis) (134). Insufficient elimination of lymphocytes in these mice may explain why auto-reactive T cells can survive and participate in autoimmune responses. Such autoimmune responses may encompass humoral autoimmunity to components of chromatin. This brings us to the role of endonucleases in SLE and lupus nephritis.

Nucleases

In 1980, Andrew Wyllie found that during the process of apoptosis, digestion of chromatin was carried out by special endogenous endonucleases. He observed that these endonucleases targeted the inter-nucleosomal linker DNA (135,136). The endonucleases are involved in the chromosome fragmentation and have complex biochemical mechanisms and substrate specificities (137). These nucleases were classified into cell autonomous and waste management endonucleases (138). Functional nucleases involved in apoptosis and necrosis are demonstrated in Figure 4. Cell autonomous nucleases implicate the cleavage of DNA within cells when they undergo apoptosis. The waste management nucleases digest extra-cellular DNA/chromatin when released from necrotic cells. Thus, these waste management nucleases are enclosed in lysosomes or secreted

into the extracellular space. After secretion, these nucleases clean up the DNA in blood stream and in the gastrointestinal tract.

The two most common identified nucleases, which cleave chromatin within the cells, are CAD (caspase activated deoxyribonuclease) and endonuclease G (endo G).

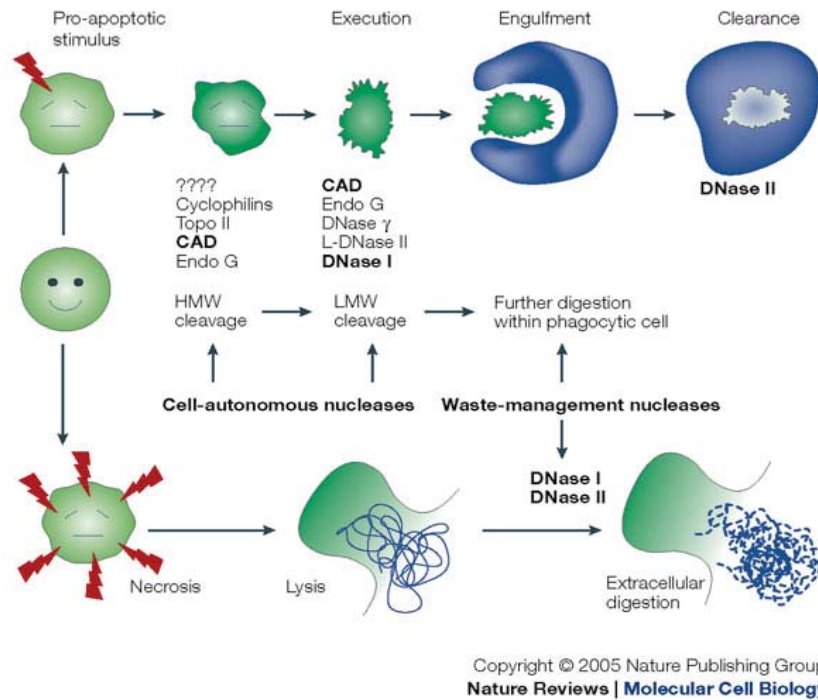


Figure 4: Cell-autonomous and waste management nucleases in apoptosis and necrosis. Reprinted with the permission from Macmillan Publishers Ltd: Nature Review copyrights 2005(138).

CAD, which is referred to as a professional apoptotic nuclease, plays a role in condensation of chromatin and fragmentation of DNA (139). CAD forms a strong complex with ICAD (inhibitor of CAD) and remains inactive in the nucleus (140). The interaction of CAD/ICAD is cleaved by the induction of apoptosis through caspase 3, caspase 7/granzyme B (141-143).

Then activated CAD digests the dsDNA at positions within inter-nucleosomal linker DNA (144). CAD is the main cell autonomous nuclease since the chromatin degradation is significantly reduced when the mice are deficient in CAD (145-147). At the same time ICAD deficient mice

do not show any lack in the apoptotic DNA fragmentation, thus signifying that other apoptotic nucleases are present in the cells.

Endo G, an endonuclease which is shown to be located in mitochondrial intermembrane space, has a dominant specificity for ssDNA and RNA (139,148). Endo G induces DNA cleavage during apoptosis in the cells that lack functional CAD (149). During apoptosis, Endo G translocates into the nucleus from mitochondria and induces low molecular weight DNA fragmentation. Unlike CAD, Endo G does not require caspase activation prior to its action (150). Nevertheless, in some cases, Endo G and apoptosis-inducing factors require caspase activation for their release from mitochondria (151). Studies in Endo G knockout mice remain controversial. One study showed that Endo G deficient mice died prenatally (152) whereas the second study showed that Endo G deficient mice are viable (153).

DNaseII is classified under waste management nucleases that are packed in lysosomes. The main role of DNaseII is DNA degradation through an engulfment-mediated process which requires acidic environment (154). The DNaseII deficiency leads to disturbance in the macrophage function, which causes an impaired cellular immunity. DNaseII deficient mice die at birth due to asphyxiation (155). The mice that lack both CAD and DNaseII have defective thymus and kidney development (147). This leads to the accumulation of undigested DNA in the macrophages that activates the innate immunity. This results in high production of interferon β (156).

DNaseI is a secreted protein detected in saliva, urine, intestine and other secretory organs and is primarily considered as digestive enzyme for the digestion of DNA and chromatin in the food (157). Later it was found that DNaseI is a candidate endonuclease that facilitate chromatin breakdown during apoptosis and necrosis (158,159). DNaseI acts in certain situation as a cell autonomous endonuclease (160). DNaseI knockout mice show SLE like symptoms with production of anti-nuclear antibodies and development of lupus-like nephritis (161). This mouse model has, however, not been further studied, leaving the validity of this model with a certain doubt. The same knockout mice, however, showed, protection against cisplatin-mediated kidney injury (162) and gamma radiation (163). DNaseI serum level and activity has been shown to be reduced in SLE patients, which correlated with SLE specific cutaneous lesions (164).

In addition to DNaseI, three other DNaseI homologous endonucleases were identified. They include DNaseI like 1 (DNaseIL1), DNaseIL2, DNaseIL3 (165). These nucleases are also involved in degradation of chromatin during apoptosis, and are therefore regarded as cell autonomus endonucleases (166).

DNaseI and its role in autoimmunity

It has been shown that among other endonucleases, DNaseI plays a major role in the pathogenesis of lupus nephritis. Both DNaseI knockout mice and a mutation in DNaseI gene in humans leads to the induction of autoimmunity, and development of SLE with the production of high anti-dsDNA antibody titers (161,167). Also reduced levels of DNaseI is shown both in human SLE patients (168-171) and in lupus prone (NZBxNZW)F1 mice (172,173). In addition to lupus, it has been shown that reduced DNaseI expression plays a role in autoimmunity in other diseases as well (174). Studies from the Macanovic group showed that administration of recombinant murine DNaseI intraperitoneally in lupus prone mice reduced the severity of the disease progression (175). But this was not reproduced when lupus nephritis patients were treated with human recombinant DNaseI (176,177). (NZBxNZW)F1 mice with reduced renal DNaseI expression levels suffered from progressive lupus nephritis (178,179). This is nicely associated with the fact that DNaseI is selectively reduced among all nucleases in kidneys and is reduced only in the kidneys during progression of lupus nephritis (180).

Biological and pathophysiological consequences of silencing of the renal DNaseI gene

One of the main topics for this study was to analyse the pathophysiological consequences of silencing of the renal DNaseI gene. If chromatin is not fragmented and cleared, they may activate the innate immune system through interaction with Toll-like receptors (TLR) 7-9 (181). The chromatin-mediated stimulation of TLR may also up-regulate certain matrix metalloproteases (MMPs) (182,183). For example, engagement of TLRs can up-regulate pro-inflammatory cytokines (TNF α , IFN γ) (184,185), and Interleukins in cells of the innate immune system, and in resident cells like mesangial cells (186). Up regulation of these cytokines is linked to the MAPK, ERK kinase or REL through NF κ B gene activation (187), and this in turn can directly up-regulate MMPs. On the other hand, incomplete clearance of apoptotic cells may transform them into secondary necrotic cell debris (83,188-190). Such debris contains SAP130 (spliceosome associated protein 130), which serves as a ligand for the inflammation-related receptor C-type

lectin 4e (Clec4e) (191). Downstream signaling induced by SAP130-Clec4e interaction also promotes production of pro-inflammatory cytokines (192,193) and a consequent up-regulation of MMPs. Thus, the mechanisms that lead to inflammation in lupus nephritis may therefore involve TLRs (181,194) and the Clec4e receptor (195). Several studies suggest that TLR signaling is important in the pathogenesis of lupus nephritis (196-199), while the role of Clec4e in this context remains undetermined.

So what would the role of secreted MMPs be? MMP2 and MMP9 have the potential to disintegrate and remodel membranes and matrices by enzymatic degradation (200). This biological event may facilitate deposition of chromatin fragment-IgG complexes. MMP2/MMP9 activities are increased within kidneys of nephritic, but not pre-nephritic (NZBxNZW)F1 mice (173,178,201). Reduced expression of renal DNaseI and increased expression of renal MMPs via the TLR system make a reasonable explanation as to how large chromatin fragments generated within the kidneys get access to membranes and matrices. This is a focus for detailed murine studies in this thesis.

How is DNaseI regulated in lupus nephritis?

Despite the fact that DNaseI has been known for more than 50 years, mechanisms that regulate expression and also secretion of this enzyme are not clear. This is also uncertain for renal DNaseI. Silencing of DNaseI gene expression in kidneys during progression of lupus nephritis may theoretically be caused by several regulatory pathways. One possibility is a direct effect of early inflammation and pro-inflammatory cytokines, although no data supporting this exist. By inspection of the DNaseI gene organization in the UCSC genome browser (<http://genome.ucsc.edu/>) (202) we found an overlap in the annotated transcript with a transcript from the convergently transcribed tumor necrosis factor receptor-associated protein 1 (Trap 1) gene in their 3' un-translated regions (UTR) Figure 5 (203).

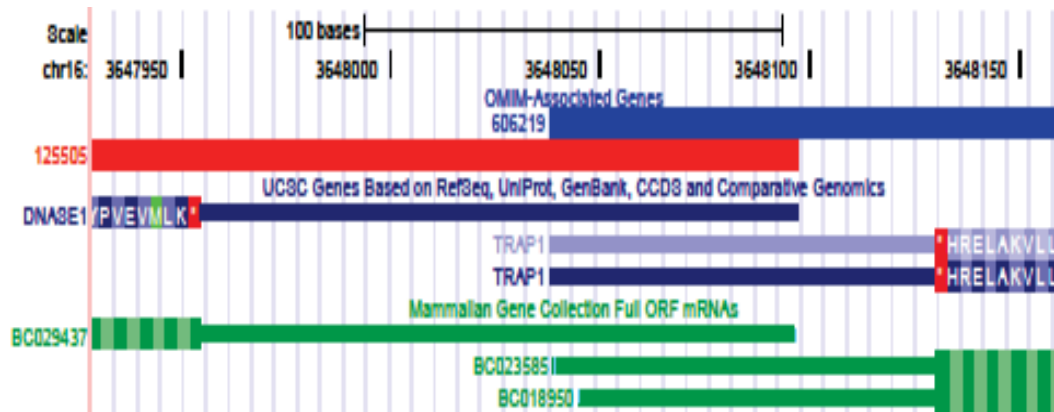


Figure 5: DNaseI/Trap1 overlap by 59 nucleotides in their UTR regions. The figure taken from public encode database (<http://genome.ucsc.edu/>)

This gene organization is peculiar and found in several human transcription units (H. Nielsen, unpublished observation) but is likely to preclude co-expression of the two genes. Given that transcription proceeds well beyond the 3' end of the mature transcript (204), the overlap between the transcripts of the two genes is substantial and it is unlikely that they are transcribed simultaneously. First, recent evidence suggest that two RNA polymerase II (RNAPII) molecules elongating in opposite directions cannot bypass each other, and second, elongating RNAPII suppresses downstream antisense transcription by chromatin modification through the Set2/Rpd3S pathway (205). Based on this, we consider the DNaseI and Trap 1 genes as a coupled gene pair and use the term transcriptional interference (in a broad sense) to describe the concept that the expression of one gene is affected by the expression of the other at the transcriptional level. This is another focus for this thesis.

Transcriptional interference as a gene regulation principle in biology and disease

The term "transcriptional interference" is widely used but poorly defined in the literature (206). Transcriptional interference usually refers to the direct negative impact of transcription of one gene on transcription of another gene provided the genes are transcribed in opposite directions and that the two genes overlap with each other. Transcriptional interference is potentially widespread throughout biology; therefore, it is timely to assess exactly its nature, significance and operative mechanisms especially in clinical medicine.

Convergent gene pairs are prevalent among eukaryotes. The existence of such transcription units

raises the question of what happens when RNA polymerase II (RNAPII) molecules collide head-

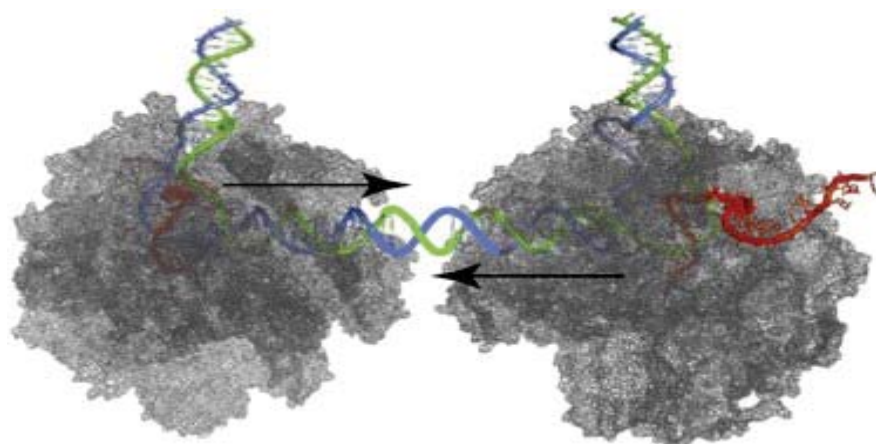


Figure 6: Convergently transcribing RNAPIIs cannot transcribe and pass one another *in vivo*: Reprinted with permission from Mol Cell by Hobson DJ et al., 2012 November 9; 48(3): 365–374(207).

to-head (207). It is demonstrated that polymerases transcribing opposite DNA strands cannot bypass each other. Upon head-to-head collision *in vitro*, the RNAPII molecules stop on the DNA strands, but they do not dissociate from the DNA strands (Figure 6). This suggests that opposing polymerases represent obstructions for each other. In this situation, transcription of both genes is therefore blocked.

This model also explains what happens if transcription of one of the gene pairs is initiated. In that situation, transcription of the convergent gene is blocked by the transcriptional activity of the first gene. This perception provides insight into fundamental mechanisms of gene traffic control and point to an unexplored effect of antisense transcription on gene regulation via RNA polymerase collision. This model is also valid if the genes overlap in their UTR since the primary transcripts is elongated far beyond UTR (208,209).

Thus, the present study focuses on whether transcriptional interference through Trap 1 has any role in regulation of DNaseI expression.

Role of cytokines in lupus nephritis

Cytokines are essential molecules involved in the differentiation, maturation and activation of cells. They also play a significant role in immuno-inflammatory responses (see Table 3 for a simple overview). Cytokines are involved in tissue destruction at the site of inflammation by

recruiting innate and adaptive immune cells. Overproduction of cytokines leads to severe inflammation and to damage in the organ, as in lupus nephritis (210). To understand the disease process that occurs locally in glomerulus, it is important to know the immune signals mediated by cytokines and their role in inflammation/tissue damage. So far several cytokines have been shown to play a major role in the kidney destruction in lupus nephritis. These includes $TNF\alpha$, interleukins, $INF\alpha$, $INF\beta$, $INF\gamma$, transforming growth factor β and others (reviewed in (211)). Effect of several cytokines and their relevance in lupus nephritic kidneys are listed in Table 3 (adopted from reference (212)). This information derives from studies on human SLE nephritis and relevant mouse models of lupus diseases.

In lupus nephritis the immune complex deposits in the GBM lead to an end-stage organ disease. Cytokines plays a pivotal role during the progression of lupus nephritis (213). The resident renal cells like mesangial cells and endothelial cells are activated by immune complexes that induce the production of pro-inflammatory cytokines. These cytokines on the other hand activate monocytes and enhance the inflammation which in the end leads to tissue fibrosis (212).

We see a tendency for up-regulated DNaseI in mesangial nephritis (178). Since this early up-regulation of DNaseI appears during mesangial nephritis, it became obvious to assume that increased production of pro-inflammatory cytokines may account for the increased DNaseI expression in early lupus nephritis. The cytokines may contribute to the pathogenesis of lupus nephritis from early initiation to the progression of disease (214). Why up-regulation of DNaseI is followed by loss of DNaseI expression and progression of lupus nephritis remains to be determined. For example, it has been shown that reduction in proteinuria can be achieved through cytokine blockade therapy (213) and contribute to improved renal function. Thus, it is essential to understand the role of pro-inflammatory cytokines as regulators of DNaseI expression.

Table.3 Cytokines of probable local relevance in the lupus kidney. Modified from Cytokine expression in lupus kidneys. Aringer M et.al from Lupus 2005, 14:13 (212).

Cytokines	Main negative local effects
TNF α	Tissue damage propagation
Interferon gamma	Inflammation,tissue damage
Interleukin-1	Tissue damage
Interleukin-4	Tissue fibrosis
Interleukin-6	Hypercellularity
Interleukin-8	Leukocyte recruitment
Interleukin-10	Increase in apoptosis
Interleukin-18	Interferon gamma stimulation
Transforming growth factor β	Tissue fibrosis

In the next sections, the information given above will be translated into research hypotheses described as aims of the study, and a general discussion of the results and data obtained in this study.

Experimental strategies and tissue applied to this study – some general comments

Animals and tissue materials

Breeding the animals and the animal experiments have been approved by the Norwegian ethical and welfare board for research animals. Female BALB/c and (NZBxNZW)F1 mice were used in the studies, and the animals were euthanized by CO₂ suffocation regularly from the age of 4 up to 40 weeks. Proteinuria was checked using urinary dipstick analyses. This assay was accurate enough for the present analyses. Blood samples were collected by standard and accepted methods on regular basis. Kidneys were immediately extirpated, sliced and preserved in RNA later™ for qPCR, fixed in 4% paraformaldehyde for immune electron microscopy, embedded in paraffin for immunohistochemistry, or snap frozen in liquid nitrogen, or embedded in optimal cutting temperature compound (OCT) and kept at -80°C. In (NZBxNZW)F1 mice, we analysed the progressive elements of murine lupus nephritis in unmanipulated mice. Gene expression profiles were subsequently interpreted and translated into strategies for cell culture experiments. For all these experiments and analyses of responses and gene expression profiles it is crucial to ensure that the results are consistent when analyzing mRNA and protein levels. In some of the results, it was clear that expression of mRNA indicated a response, while that could not be observed for the translated protein level.

Cell culture experiments

Human renal proximal tubule epithelial cells (RPTEC) were grown in Clontec REGM™ BulletKit (CC-3190) containing Renal Epithelial Cell Basal Medium with the following growth supplements: hEGF, Hydrocortisone, Epinephrine, Insulin, Triiodothyronine, Transferrin, GA-1000, and fetal bovine serum at 37° C in 95% humidified air and 5% CO₂. Cells were grown to 80% confluent and used for stimulation experiments. Stimulations of the cells were done with a spectrum of pro-inflammatory cytokines to analyse their impact on DNaseI and Trap 1 expression profiles. In each of these biological experiments, gene expression responses known to occur for each stimulus was included in the analyses to ensure that the stimulation experiments were technically valid. Only then, the results were interpreted

Gene expression analyses at mRNA level, protein expression level and protein functional level

Specific mRNA expression levels do not always reflect the levels of protein expression in a given tissue or cell. Therefore, in these studies, we analysed gene expression at different levels, starting with qPCR to determine mRNA levels. These were compared to protein expression levels by semi-quantitative western blots, by in situ protein expression using immune electron microscopy, immunofluorescence, immunohistochemistry and/or confocal microscopy. By the in situ protein expression analyses, loci and translocation of proteins were determined.

DNaseI Zymography

DNaseI activity in the kidneys or cell cultures was determined after protein separation in a 10% SDS–polyacrylamide gel containing 40µg/mL of heat-denatured salmon sperm DNA (Invitrogen, Carlsbad, CA). The gel was then incubated in 0.2% triton solution to remove the SDS and incubated at 37°C for 16-24hrs in a DNaseI reaction buffer. The gel was then stained with Nucleic acid stain (Gel-Red) and the enzyme activity detected under UV illuminator. Cleared areas in the gel demonstrated DNaseI endonuclease activity. The band that appeared as generated by DNaseI was compared with western blot bands from the same samples. By this approach, we can determine if proteins of interest are enzymatically active.

Aims of the study

Lupus nephritis is a two-stepped organ disease (see introduction, (178)). The early phase, mesangial nephritis, correlates in time with production of anti-dsDNA antibodies, and with immune complex deposits in the mesangial matrix. Progression into membrano-proliferative nephritis (end stage nephritis) correlates in time with silencing of the renal DNaseI gene, with deposits of immune complexes in the GBM and in the mesangial matrix, and development of severe proteinuria (178). The progression of lupus nephritis is linked to an acquired loss of renal DNaseI (179). Since loss of DNaseI in the kidney promotes end stage disease, one can assume that impaired clearance of DNA is one of the main etiological factors in pathogenesis of lupus nephritis. It is therefore crucial to know why renal DNaseI is lost during the development of the disorder. How and why the renal DNaseI is down-regulated and why renal DNaseI is selectively silenced in the kidney and not in any other organ (180) is still an unresolved matter.

In accordance with these unresolved problems, the aims of this study were to investigate the following:

1. What is the biological consequence of silencing of the renal DNaseI gene expression? (Paper I)
2. What is the role of TRAP 1 in the regulation of DNaseI in kidneys through transcriptional interference? (Paper II)
3. Is DNaseI (down-) regulation linked to the effect on tubular cells of certain pro-inflammatory cytokines? (Paper III)

Summary of the papers

Paper I

Silencing of Renal DNaseI in Murine Lupus Nephritis causes Exposure of Large Chromatin Fragments and Activation of Toll Like Receptors and the Clec4e

Recent studies have demonstrated that progression of mild lupus nephritis into end-stage organ disease is imposed by a sudden silencing of renal DNaseI gene expression in the lupus-prone (NZBxNZW)F1 mice. Down-regulation of DNaseI results in reduced chromatin fragmentation and in deposition of extracellular chromatin in complex with IgG in glomerular basement membranes provided that the individuals produce IgG anti-chromatin antibodies. The main focus of the present study is to describe the biological consequences within the kidneys of renal DNaseI shut-down. The study particularly had a focus on whether reduced renal chromatin fragmentation led to exposure of large chromatin fragments and activation of Toll like receptors and the necrosis-related Clec4e receptor in murine and human lupus nephritis. Furthermore, analyses were performed to determine if matrix metalloproteases are up-regulated as a consequence of chromatin-mediated Toll like receptors/Clec4e stimulation. Mouse and human mRNA expression levels of DNaseI, Toll like receptors 7-9, Clec4e, pro-inflammatory cytokines and matrix metalloproteases 2 and 9 were determined and compared with in situ protein expression profiles and clinical data. We demonstrate in this study that exposure of chromatin indeed significantly up-regulated Toll like receptors and Clec4e in mice, and also, although less pronounced, in patients with lupus nephritis that were treated with immunosuppressive drugs. The conclusion of this study is that silencing of renal DNaseI gene expression initiates a cascade of inflammatory signals leading to progression of both murine and human lupus nephritis. Principal component analyses biplots of data from murine and human lupus nephritis demonstrate the importance of silencing of the DNaseI gene for progression of the organ disease.

Paper II

Impact of the tumor necrosis factor receptor-associated protein 1 (Trap 1) on renal DNaseI shutdown and on progression of murine and human lupus nephritis

Recent findings in our laboratory demonstrate that transformation of mild glomerulonephritis into end-stage disease coincides with shutdown of renal DNaseI expression in (NZBxNZW)F1 mice. Down-regulation of DNaseI results in reduced chromatin fragmentation and deposition of extracellular chromatin fragments in glomerular basement membranes where they appear in complex with IgG antibodies. In this study, we implicate the anti-apoptotic and pro-survival protein, tumor necrosis factor receptor-associated protein 1 (Trap 1) in the disease process, based on the observation that annotated transcripts from this gene overlap with transcripts from the DNaseI gene. Furthermore, we translate these observations into human lupus nephritis. In this study, mouse and human DNaseI and Trap 1 mRNA levels were determined by qPCR and compared with protein expression levels and clinical data. Cellular localization was analyzed by immune electron microscopy, immunohistochemistry, and by in situ hybridization. Data indicate that silencing of DNaseI gene expression correlates inversely with expression of the Trap 1 gene. Our observations suggest that the mouse model is relevant for aspects of disease progression in human lupus nephritis. Acquired silencing of the renal DNaseI gene has been shown to be important for progression of disease in both murine and human forms of lupus nephritis. Early mesangial nephritis initiates a cascade of inflammatory signals that we assume will lead to up-regulation of Trap 1 and a consequent down-regulation of renal DNaseI by transcriptional interference.

Paper III

Stimulation of human renal proximal tubule epithelial cells with TNF α and IL-1 β increases DNaseI mRNA and protein expression and translocates DNaseI from cytoplasm into the nucleus

Cytokines play a major role in inflammation and contribute to the pathogenesis and progression of lupus nephritis. One factor that may account for this progression may be loss of renal DNaseI expression that correlates with up-regulation of Trap 1 through transcriptional interference. Combining this information we assumed that it would be important to determine if pro-inflammatory cytokines have any impact on regulation of DNaseI and on Trap 1 genes. We have recently demonstrated that the major renal endonuclease DNaseI shows a tendency to be up-regulated in early mesangial nephritis, and severely down-regulated timely linked to transformation of mild mesangial nephritis into end-stage kidney disease. In order to understand these inverse expression profiles we focus in this study on processes that account for an early increase in renal DNaseI expression. The hypothesis was that the mesangial inflammation induced secretion of pro-inflammatory cytokines which directly increased DNaseI expression in tubular cells. The present study was performed to analyse transcriptional interference between the DNaseI and Trap 1 genes in early mesangial nephritis by stimulation of human proximal tubule epithelial cells with pro-inflammatory cytokines. DNaseI and Trap 1 gene expression in the cells was determined by qPCR, confocal microscopy, immune electron microscopy, and by western blots. Furthermore, since DNaseI may act as a transcription factor for the Fas receptor gene, we also analysed if increased expression of DNaseI also implies that DNaseI translocates from the cytoplasm to the nucleus where it may indeed act as a transcription factor for the Fas receptor gene. In the present study, we obtained data that indicate that transcriptional interference is involved in gene regulation of DNaseI and Trap 1, and that a 55 KD protein recognized by three different anti-DNaseI antibodies was increasingly expressed in nuclei of tubular cells in response to stimulation of the cells with TNF α and with IL-1 β . The translocation of the 55 KD variant of DNaseI is shown to correlate with increased expression of the Fas receptor gene.

General Discussion

Lupus nephritis is a serious complication of SLE and represents one of the SLICC and ACR classification criteria for SLE (28,29). It can develop in the early course of SLE and therefore becomes a major indication of poor prognosis. A striking finding in lupus nephritis is the fact that anti-dsDNA antibodies bind to chromatin fragments in the mesangial nephritis in early phases of the disease and in GBM during progression into end-stage organ disease (see Introduction for details). The chromatin fragments are believed to be released from necrotic or apoptotic renal cells and retained locally or systemic due to impaired clearance mechanisms. This defect renders large quantities of chromatin fragments available for anti-chromatin antibodies including those reactive with dsDNA (14,18,19,215).

Impaired clearance of apoptotic cells may play a major role in the pathogenesis of lupus nephritis. Furthermore, progression of lupus nephritis from silent mesangial nephritis to end-stage membrano-proliferative nephritis is associated with impairment of one of such clearance mechanisms, namely loss of the renal DNaseI endonuclease as determined in both murine and human lupus nephritis (179). Down-regulation of DNaseI mRNA and enzyme activity is selective for kidneys in (NZBxNZW)F1 mice and no other organ demonstrates a similar loss of this enzyme (180). Furthermore, in the kidneys DNaseI is the only one among a series of genes analysed so far that is silenced in lupus nephritis (180). This silencing may therefore be a key event in lupus nephritis, since the selective loss of the dominant renal endonuclease coincides with diminished elimination of chromatin in the kidneys (138,216). The main challenge for this study originated from the fact that silencing of renal DNaseI resulted in loss of DNaseI mRNA, protein and enzyme activity (173,178,179). The main consequence is exposure of large un-fragmented chromatin in the kidneys, and particularly in glomeruli.

This study was therefore generated to elucidate the following problems. Why is the renal DNaseI gene silenced, and what are the biological consequences of renal DNaseI shut down during the progression of the disease? Therefore we have analyzed consequences of, and mechanisms that may account for, the DNaseI gene silencing by different possible mechanisms with the following central foci:

- a. Biological consequences of loss of DNaseI
- b. Regulation of renal DNaseI by transcriptional interference
- c. Impact of pro-inflammatory cytokines on renal DNaseI expression

Activation of the immune system by exposed chromatin through signaling receptors in the kidney

To understand the biological consequences of DNaseI gene silencing in the kidneys, we have to consider the role of extra-cellular chromatin fragments and anti-dsDNA antibodies. Central in this context is that retention of chromatin may be due to reduced fragmentation and clearance during apoptosis or necrosis (79,83,188). Secondary to this, chromatin is exposed and may therefore activate dendritic cells through interaction with particularly TLR2, 7-9 and the Clec4e receptor (191,196,217-219). These cells present chromatin-derived peptides in the context of co-stimulatory molecules, and MHC class II molecules to peptide specific CD4+ T cells. If adequately primed, the peptide-specific T cells recirculate and bind the same chromatin-derived peptides presented in the context of MHC class II by chromatin-specific B-cells (here recognizing e.g. dsDNA in chromatin). As a consequence of these interactions dsDNA-specific B cells may transform into plasma cells that secrete IgG anti-dsDNA antibodies. Immune complexes that consist of IgG antibodies and chromatin fragments may subsequently bind in the glomerular mesangial matrix and initiate mesangial lupus nephritis. These interactions place exposed chromatin in the center of the events that end up with mesangial nephritis (215,220). Likewise, these interactions may be closely linked to, and caused by, silencing of renal DNaseI.

Early mesangial nephritis initiates several inflammatory events. Without linking them mechanistically to each other yet, production of pro-inflammatory cytokines, infiltration of immune cells including macrophages (221), and complement activation may be factors that are interdependent in mesangial nephritis, and that may promote silencing of the renal DNaseI gene in tubular and glomerular cells and subsequent accumulation of undigested chromatin fragments. The mechanistic link between the factors listed above may be explained as follows from data in Paper I. Chromatin fragments bind TLR and promote their up-regulation. Stimulation of TLR leads to up-regulation of pro-inflammatory cytokines and MMP, particularly MMP2 and to a lesser extent MMP9 (178). MMP may play a crucial role in further progression of lupus nephritis

due to their function as modulators of membranes and matrices, and to the fact that they have the potential to disintegrate membranes like GBM (200,222,223). This may in fact be the event that promotes deposition of large chromatin fragments within GBM. Since this does not take place during mesangial nephritis, the idea that mesangial nephritis promotes deposition in GBM through chromatin-TLR-induced up-regulation of MMPs may be true. Indeed, data obtained in Paper I of the present study demonstrate that there is a strong negative association between loss of DNaseI, increased expression of TLR, and increased expression of MMPs (Paper I). Finally, IgG autoantibodies recognize and bind chromatin fragments and these immune complexes deposit in the (assumably MMPs-mediated) disintegrated GBMs and aggravate renal inflammation. In this sense, chromatin fragments and anti-chromatin (here: anti-dsDNA) antibodies may be the partners that impose the classical murine and human lupus nephritis. Thus, anti-chromatin antibodies are pathogenic only when chromatin fragments are exposed in glomeruli. This model does, however, not exclude other processes that can initiate and maintain lupus nephritis.

The data discussed so far do also relate to the ongoing discussion on how anti-chromatin antibodies exert their nephritogenic potential. Our data demonstrate the impact of binding of the antibodies to exposed chromatin in glomeruli. However, several sets of data from other laboratories indicate that the antibodies bind directly to glomerular antigens, i.e. the model of cross-reactions (109-111,114,224,225). In our assays, we have never obtained data that support the cross-reactive model to explain lupus nephritis (226). For example, by high-resolution analytical approaches, such as IEM, co-localization IEM and co-localization terminal deoxynucleotidyl transferase dUTP nick end-labeling (TUNEL) IEM, IgG were never observed to bind *in vivo* directly to the GBM or to the mesangial matrix. But IgG did bind to TUNEL-positive EDS associated with glomerular matrices and membranes. We therefore think that previous and present data argue for a model to explain lupus nephritis that implies anti-chromatin antibody binding to chromatin exposed in the mesangium and in GBM. The model envisaged in Figure 7 harmonizes with these data.

So far there exists no data on the involvement and function of the Clec4e receptor in context of lupus nephritis. Exposed chromatin fragments in the nephritic kidneys can induce a possible activation of signaling pathways through the interaction of Clec4e receptor with SAP130A. This

is a molecule exposed in necrosis, but not in apoptosis (191). Our analyses of Clec4e in (NZBxNZW)F1 mice informed about a significant up-regulation of this receptor in both mesangial- and membrano-proliferative lupus nephritis (Paper I). Mesangial nephritis, although it is a clinically silent condition, was associated with significant activation of the surface macrophage receptor Clec4e. The activation of Clec4e receptor is strong in membrano-nephritic kidneys most probably due to the presence of large un-fragmented chromatin in the kidneys. Thus, activation of Clec4e, like of TLR, induces a cascade of signaling pathways that may be implicated in both innate and adaptive immune responses (191,193). Up-regulation of MMPs by Clec4e signaling pathway is linked to activation of the NFkB gene either by the canonical pathway, through serine tyrosine kinase (SYK) pathway or through engagement of the FCγR (187,227,228). In harmony with the murine data, human data revealed a marked down-regulation of DNaseI and up-regulation of TLR 7/8. However, the up-regulation was not significant. Furthermore, in contrast to mice, we did not see an up-regulation of TLR 9 or Clec4e in human nephritic kidneys. One possible reason for this may be that these patients were treated with immunosuppressive drugs while mice were not. Activation of Clec4e up-regulates pro-inflammatory cytokines, and like the result of TLR-stimulation, these stimulatory events may up-regulate MMP 2/9. Recent analyses reveal an association of mannose receptors that belong to C-type lectin family in crescentic glomerulonephritis (229). Interestingly, a study has demonstrated that C-type lectin receptors are connected to the development of glomerulonephritis (230). These and present data may give a new clue about the role of C-type lectin family in inflammation and pathogenesis of lupus nephritis. Thus, the up-regulation of TLR 7-9 and the Clec4e receptor may be indirectly involved in processes that facilitate exposure of chromatin in the kidneys. Therefore, loss of renal DNaseI may indirectly lead to exposure of un-fragmented chromatin in the kidney. TLR then impose up-regulation of MMPs and promote inflammatory responses by matrix degradation and disruption of glomerular membranes that lead to deposition of immune complexes in GBM and end-stage renal failure (231). This scenario is envisaged in Figure 7.

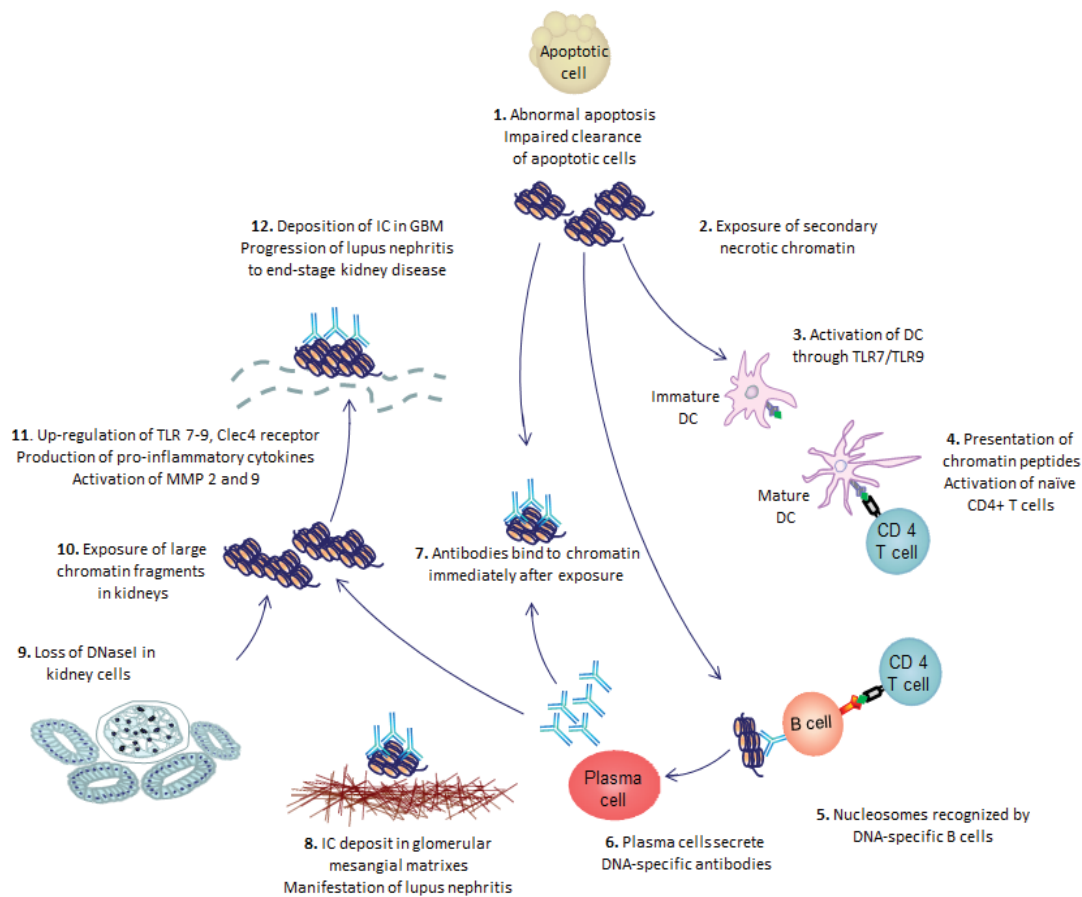


Figure 7: Biological consequences of renal DNaseI shutdown and exposure of chromatin in the pathogenesis of lupus nephritis. From Seredkina N et al. Mol Med. 2013 Jun 6. [Epub ahead of print]

Transcriptional interference as a possible mechanism that regulates renal DNaseI gene silencing during progression of lupus nephritis

Next, the aim was to understand how the renal DNaseI gene silencing was controlled during the progression of lupus nephritis. Understanding the reason behind the loss of renal DNaseI may be important to help us to develop new treatment strategies that e.g. may inhibit the process that silence the renal DNaseI gene. By inspection of the UCSC genome browser ([see http://genome.ucsc.edu/](http://genome.ucsc.edu/)) (202) a possible clue to analyse this problem was observed. There is a 59 nucleotides overlap between DNaseI in the annotated transcript with the transcripts from the

convergently transcribed Trap 1 in their 3' un-translated regions (UTR). The impact of this type of gene organization remains poorly analysed in humans. We now know that this gene organization prohibits co-expression of the two genes. The transcription of one gene will suppress the transcription of the anti-sense gene by chromatin modification through Set2/Rpd3S (204,205). Furthermore, in a head-to-head collision between two RNAPII, the transcription of both genes stops, but the two RNAPIIs do not dissociate from the DNA strands (232). In a situation where one gene is transcribed, this will suppress the transcription of the other gene in the gene pair. Thus, we consider that transcriptional interference could cause Trap 1 transcription to silence the DNaseI gene.

Interestingly, DNaseI and Trap 1 have contrasting functions. DNaseI is a death-associated protein involved in apoptosis and necrosis (138,233) whereas Trap 1 is an anti-apoptotic survival protein that is up-regulated in stress and cancer (234-237). Analysis of the expression of Trap 1 in (NZBxNZW)F1 mice over the first 40 weeks of life, revealed a tendency for reciprocal expression of Trap 1 and DNaseI (Paper II). In pre-nephritic mice, there was a stable expression of both DNaseI and Trap1 mRNA and protein levels. This may mean that in a normal situation, the two genes are alternatively transcribed to ensure a stored reservoir of the two proteins. In mice with mesangial nephritis, higher expression of Trap 1 compared to DNaseI was observed. In nephritic mice with immune complex deposits in GBM and with severe proteinuria, we observed low levels of both DNaseI and Trap 1 mRNA levels but the ratio of Trap1/DNaseI mRNA levels was still high to very high. We thus observed also in these mice with end-stage disease a reciprocal expression pattern of the two genes, with a relatively higher expression of Trap 1 compared to expression of DNaseI. However, in few nephritic mice both DNaseI and Trap 1 expression levels are simultaneously down-regulated. Thus in end stage, nephritic mice is characterized by two different expression patterns. One pattern demonstrated relatively high Trap1 and low DNaseI mRNA levels. The other pattern was characterized by a silencing of both genes. Both patterns can be explained by the effect of transcriptional interference. In a situation with sustained high Trap 1 expression, transcription of the DNaseI gene may be constantly suppressed. In stressed cells, like in severe inflammation, both genes may receive transcriptional stimuli. In that situation, RNAPII on both strands may collide head-to-head, a situation where transcription of both genes are blocked. This may be the explanation for silencing of both genes in end-stage disease, and is consistent with the collision-model described by Hobson et al. (207) .

Impact of pro-inflammatory cytokines in DNaseI regulation

In light of the reciprocal tubular cell expression of DNaseI and Trap 1 in different phases of progressive lupus nephritis, we decided to analyse this phenomenon in tubular cell cultures stimulated with various pro-inflammatory cytokines to mimic the inflammatory milieu in mesangial nephritis. We performed analysis of a possible transcriptional interference between DNaseI and Trap 1 genes by stimulation of human RPTEC with pro-inflammatory cytokines. DNaseI and Trap 1 gene expression in the cells was determined by qPCR (for mRNA), confocal microscopy (for in situ protein expression) and by semi-quantitative western blots for protein expression in cell lysates. Furthermore, DNaseI has been reported to have the potential to be a transcription factor for the Fas receptor gene (238) and thereby it may play a significant role in the regulation of the apoptotic machinery. Therefore, we analysed if an eventual increased expression of DNaseI in response to the stimuli also implied that DNaseI translocates from the cytoplasm into the nucleus and subsequently induces up-regulation of the Fas receptor gene (Paper III). Data that were obtained, demonstrated that 3 independent anti-DNaseI antibodies stained cell compartments differently as shown by confocal microscopy, as 2 antibodies preferentially stained cytoplasm, while 1 mainly stained the nucleus. Western blot analyses clearly demonstrated that two proteins were stained by all of the 3 antibodies, one at 40 KD, another at 55 KD. The antibody that stained the 55 KD protein most strongly also stained the nucleus, while the antibodies that preferentially stained the 40 KD protein stained cytoplasm as demonstrated by confocal microscopy.

Thus, we proposed that DNaseI gained function as a transcriptional factor for the Fas receptor gene when we stimulated the RPTEC with TNF α and IL-1 β . The observed translocation of the DNaseI protein from cytoplasm to nuclei supports this assumption. In addition, it is known that TNF α and IL-1 β can induce apoptosis in renal tubular cells through the Fas mediated apoptosis pathway (239,240). Interestingly staining of DNaseI with the different antibodies resulted in different staining patterns. It is known that DNaseI localize in both nucleus and cytoplasm (138,241-243). Thus, from staining patterns by confocal microscopy and strength of binding of the different anti-DNaseI antibodies to the 40 KD and 55 KD bands in western blots, we assume that the 55 KD band corresponds to the nuclear staining whereas the 40 KD band corresponds to the cytoplasmic staining. Although the SC anti-DNaseI antibody distinctly stained the nucleus even in the resting cells, we assume that it probably recognizes an inactive form of DNaseI.

Analysis of DNaseI enzyme activity by DNaseI gel zymography revealed a 40 KD band but no enzymatically active 55 KD band was observed. This made us to assume the presence of two variants of DNaseI, one inactive variant (trans-) located in nucleus whereas the other was located in the cytoplasm and had endonucleolytic activity.

When tubular cells were stimulated with $\text{TNF}\alpha$ and $\text{IL-1}\beta$ we observed that the staining intensity of the 55 KD band increased, and at the same time, staining of DNaseI by the AB DNaseI antibody in nuclei became evident (Paper III). At the same time, the Fas receptor gene was up-regulated. Concomitant with up-regulation of the DNaseI protein(s) and DNaseI mRNA, expression of Trap 1 mRNA and the protein was reduced, indicating that the 2 genes had a mutual negative influence on each other. The responses to these two stimuli ($\text{TNF}\alpha$ or $\text{IL-1}\beta$) were specific, since stimulation of the cells with e.g $\text{INF}\gamma$, IL-6 or IL-10 did not affect expression of DNaseI, Trap 1, or nuclear translocation of DNaseI, or expression of the Fas receptor gene. These results demonstrate that pro-inflammatory cytokines have impact on DNaseI expression, and that DNaseI and Trap 1 expression profiles are reciprocal, indicating that gene regulation by transcriptional interference is a real phenomenon *in vivo*, as well as *in vitro* cell culture experiments.

Concluding remark

At a certain time point in the life of (NZBxNZW)F1 mice, the expression of renal DNaseI endonuclease is lost. The actual cause for the loss renal DNaseI is still after the present studies elusive and only fragmental. Our findings in this study revealed, however, a new direction to analyse the regulation of DNaseI through transcriptional interference with the Trap 1 gene, and through possible actions of pro-inflammatory cytokines. In the present study, we analyzed the biological consequence of renal DNaseI shut-down. Reduced chromatin fragmentation and exposure of chromatin *in situ* lead to activation of TLRs and Clec4e signaling pathways. Signaling molecules representing responses to activation of these receptors are shown to have a direct role on activation of the innate and adaptive immune system. One central effect of these processes may be activation of chromatin-specific T and B cells, and a consequent production of antibodies reactive with components of chromatin. Activation of Clec4e in the context of lupus nephritis is a promising observation due to its link to secretion of MMPs, but also since this

receptor has been shown to play a role in pathogenesis of glomerulonephritis. In the end, the observations presented in this study open for new possible pathways to describe lupus nephritis, and for new regulatory mechanisms for renal DNaseI. These processes must be further studied in detail, to analyse if they may provide insight into new possible therapies of lupus nephritis.

Future perspectives in analyzing lupus nephritis

The renal DNaseI gene is silenced in the kidneys during the progression of lupus nephritis from silent mesangial nephritis into membrano-proliferative nephritis. We have examined the biological consequences of loss of the renal DNaseI enzyme in mice and to some extent in humans. Loss of this endonuclease was associated with exposure of large un-fragmented chromatin in the glomeruli. These chromatin fragments may induce a cascade of signals that lead to overt inflammation in the kidneys. Basic in these processes may be engagement of the TLRs and Clec4e signaling pathways. This may be important information and may give a clue how to suppress the renal inflammation in SLE. In the context of regulation of DNaseI, it is important to analyse the potential role of miRNA. Such analyses by Illumina miRNA profiling is currently analysed in the (NZBxNZW)F1 mouse model. In addition, it is important to understand the detailed role of Trap 1 in the processes that regulate expression of the DNaseI gene during development of lupus nephritis, since no previous data on this topic is available. Understanding the mechanism(s) behind the renal DNaseI shut-down will be useful for future therapeutic targets to treat and prevent lupus nephritis.

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Paper I

Paper II

Paper III

