UNIVERSITY OF TROMSØ UIT

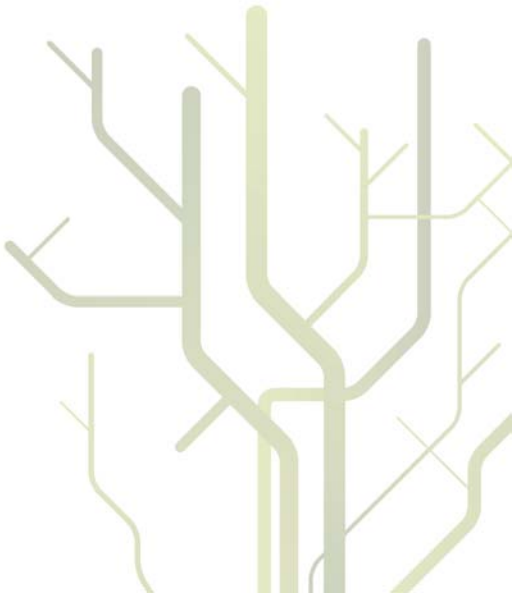
FACULTY OF MEDICINE
INSTITUTE OF MEDICAL BIOLOGY

Impact of chromatin in lupus nephritis and lupus dermatitis

– **mechanisms of induction and progression of disease**

Silje Fismen

A dissertation for the degree of Philosophiae doctor
June 2011



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Contents

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2 List of papers

I Circulating chromatin-anti-chromatin antibody complexes bind with high affinity to dermo-epidermal structures in murine and human lupus nephritis.

Fismen S, Hedberg A, Fenton KA, Jacobsen S, Krarup E, Kamper AL, Rekvig OP, Mortensen ES. Lupus. 2009 Jun;18(7):597-607.

II Deposition of chromatin-IgG complexes in skin of nephritic MRL-lpr/lpr mice is associated with increased local matrix metalloprotease activities.

Hedberg A, Fismen S, Fenton KA, Mortensen ES, Rekvig OP. Exp Dermatol. 2010. Aug 19 (8), e265-74.

III Anti-dsDNA Antibodies Promote Initiation, and Acquired Loss of Renal DNaseI Promotes Progression of Lupus Nephritis in Autoimmune (NZBxNZW)F1 Mice

Fenton KA[#], Fismen S[#], Hedberg A, Seredkina N, Fenton C, Mortensen ES, Rekvig OP. PLoS One. 2009 Dec 29;4(12):e8474

# These authors contributed equally to this work.

IV Renal upregulation of Trap1 and p62/SQSTM1 is associated with DNaseI downregulation during progression of murine and human lupus nephritis.

Fismen S, Thiyagarajan D, Jacobsen S, Fenton C, Krarup E, Kamper AL, Rekvig OP, and Mortensen ES. Manuscript submitted.

Appendix

V Pathogenesis of SLE Dermatitis – A Reflection of the Process in SLE Nephritis?
Fismen S, Rekvig OP and Mortensen ES
CurrentRheumatologyReviews, Volume 3, Number 2, May 2007. **Review**

VI Nuclease defiencies promote end-stage lupus nephritis but not nephritogenic autoimmunity in (NZB x NZW) F1 mice.
Fismen S, Mortensen ES, Rekvig OP.
Immunol Cell Biol. 2011; Jan: 89(1), 90-9. **Review**

3 Abbreviations

ANAs	Antinuclear antibodies
APC	Antigen presenting cell
BK virus	A polyomavirus. Named after the initials of a renal transplant patient.
CLE	Cutaneous lupus erythematosus
ss/dsDNA	Single stranded/double stranded DNA
DNaseI	Deoxyribonuclease 1
DC/pDC	Dendritic cell/plasmocytoid dendritic cell
DIF	Direct immunofluorescence
ECM	Extracellular matrix
EDS	Electron dense structures
ELISA	Enzyme-linked immunosorbent assay
GBM	Glomerular basement membrane
H1	Histone 1
HMGB1	High mobility group box protein 1
HSPG	Heparan sulphate proteoglycans
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
ISN/RPS	International Society of Nephrology/Renal Pathology Society
LN	Lupus nephritis
MHC	Major histocompatibility complex
MFG-E8	Milk fat globule-EGF factor 8 protein
MMP	Matrix metalloproteinases
(NZB/NZW)F1	F1 hybrids of New Zealand Black/New Zealand White mice
qRT- PCR	Quantitative Real Time Polymerase Chain Reaction
mRNA	Messenger ribonucleic acid
SDS	Sodium dodecyl sulphate
SLE	Systemic lupus erythematosus
SPR	Surface plasmon resonance
TBP	TATA box binding protein
TdT	Terminal deoxynucleotidyl transferase
TLR	Toll like receptor
TGF	Transforming growth factor
TNF	Tumor necrosis factor
TRAP1	TNF receptor-associated protein 1
TUNEL	Terminal deoxynucleotidyl transferase dUTP nick end labelling

4 Summary

Different concepts have been discussed to describe the pathogenesis linked to early lupus nephritis and its progression from mild into end-stage disease. There is a general consensus that anti-dsDNA and anti-chromatin antibodies are central in the initiation and maintenance of lupus nephritis, but there is yet no agreement as to how they interact with glomerular structures. One theory is that this is due to cross-reactions of anti-chromatin antibodies with glomerular structures like laminin, α-actinin or membrane components of mesangial cells. Another theory favours the binding of anti-chromatin antibodies to chromatin fragments exposed in affected glomeruli. Data from our group support the latter model, as several publications have demonstrated that chromatin fragments possess high affinity for glomerular membranes and matrix components. These fragments were observed as electron dense structures (EDS) along glomerular basement membranes (GBM) and in the mesangial matrix, and shown to be terminal deoxynucleotidyl transferase-mediated dUTP nick end-labelling (TUNEL) positive, demonstrating that they contain nicked DNA. Furthermore, antibodies to components of chromatin, like DNA, histones or transcription factors, bind to antigens present in EDS in murine and human lupus nephritis *in vitro,* as demonstrated by co-localisation immune electron microscopy (IEM).

The main focus of paper I and paper II was to address the pathogenesis of lupus dermatitis at a molecular level, based on the experimental results in kidneys as described above. The primary focus was to determine whether dermo-epidermal immune complex deposits observed in lupus dermatitis have identical molecular compositions to EDS found in kidneys, thus trying to settle whether deposits in glomeruli and dermal deposits share a common pathogenetical pathway. Secondly, we wanted to address if chromatin fragments bind dermo-epidermal structures, and, finally, we intended to analyse whether immune

complex deposits in nephritic glomeruli predispose for accumulation of similar deposits in skin.

The third and fourth papers emphasize the role of DNaseI in the progression of lupus nephritis. Previous results from our group have demonstrated that exposure of extracellular chromatin in lupus nephritis is linked to a reduced ability of renal nucleases to degrade apoptotic or necrotic chromatin [1]. With low renal DNaseI enzyme activity, apoptotic chromatin may be inappropriately scavenged, resulting in its transformation into secondary necrotic chromatin fragments which are then released from apoptotic blebs. Subsequently, chromatin becomes exposed to the extracellular environment and may bind glomerular membranes.

A second focus was to understand the role of secreted matrix metalloproteinases (MMPs) and their potential to disintegrate glomerular basement membranes (GBM) and mesangial matrices by enzymatic degradation, which again may facilitate deposition of chromatin fragment-IgG complexes in GBM.

In our studies we demonstrated that EDS in skin do have a similar composition as EDS in glomeruli. Still, the presence of deposits in glomeruli did not predict similar deposits in skin. Further, we found that chromatin fragments bind dermal membrane components with high affinity and that chromatin deposition in skin is associated with increased local MMP activities.

In the studies concerning the role of DNaseI, our work demonstrated that accumulation of chromatin fragment-IgG complexes correlated with an acquired loss of renal DNaseI mRNA at the time when nephritis transforms into end-stage organ disease, suggesting that chromatin fragments found in GBM of nephritic kidneys are most likely derived from within the kidneys, due to loss of renal DNaseI. We observed no changes in the expression of

genes responsible for nuclease activity in skin. This may point to yet another mechanism for deposition of chromatin in dermal structures.

Data presented in this study indicate that lupus nephritis is a biphasic disease; the first phase depends on production of anti-dsDNA antibodies which promote mild mesangial nephritis, while the second phase seems to be linked to acquired loss of DNaseI, ultimately promoting the deposition of immune complexes in the GBM with subsequent progression to end-stage lupus nephritis.

5 Introduction

5.1 Systemic lupus erythematosus - a brief historical perspective

Historically, the cutaneous manifestations of lupus were the first to be bespoken. Already in medieval literature, the Latin term *lupus,* meaning *wolf,* was proposed to describe the erythematous, facial butterfly rash (resembling a wolf bite) typically seen in discoid lupus erythematosus [2]. In the late 19th century, William Osler was credited as the first to scientifically describe the diverse visceral manifestations of SLE into a vaguely defined clinical entity [3-5]. The hypothesis suggesting systemic lupus erythematosus (SLE) as an autoimmune disorder was launched in the early 20th century [6], followed by the detection of antinuclear antibodies (ANAs) by Friou in 1958 [7]. Further milestones in the understanding of SLE included the knowledge about the human leucocyte antigen (HLA) system and the role of B- and T cells in autoimmunity. The advances at the molecular and genetic levels in recent years have been tremendous. Yet, a unifying concept of this enigmatic set of disease manifestations remains to be settled. However, in the quest for understanding the initiation and pathogenesis of SLE, four main directions must be mentioned; i) infectious diseases in which cross-reactivity with self-antigens triggers an autoimmune reaction (via e.g. molecular mimicry or superantigens) [8], ii) disturbances in lymphocyte regulation that generate a state

of autoimmunity, iii) environmental exposures [9] and iv) genetic aberrations involving various aspects of organ homeostasis. Genome-wide association studies have failed to identify single gene defects [10] (exept in rare cases of single gene deficiencies (e.g. C1q [11]), but rather suggest that several genes, or gene combinations, contribute to the risk of developing SLE [12].

5.2 Autoimmunity and disease

Distinguishing self from non-self is fundamental to the immune system. Autoimmune diseases are caused by a breakdown of immunological tolerance against self-antigens. Lymphocytes reactive against self structures are erroneously activated and allowed to operate to an extent that causes sustained self-reactivity and tissue damage. The inflammation is initiated by the presence of either autoantibodies or self-reactive T cells, leading to organ- or tissue damage afflicted by autoantibodies or secondary activation of inflammatory cells. However, the mere existence of autoantibodies is not sufficient to evoke an autoimmune disease. Several clinical conditions that clearly involve new or mutant proteins evoke autoimmune phenomena without eliciting autoimmune disease; pregnancy, malignancies, infections or immune deficiencies [13]. Matzinger`s danger model suggests that most autoimmune states do not stem from deficiencies in the immune response by itself, but rather from defects in the normal physiology of tissues. In other words; the immune system is more concerned with damage than foreignness, and becomes activated by alarm signals from injured tissues (pathogens, toxins, mechanical damage), rather than by the recognition of non-self [13]. A sustained self-reactivity and subsequent tissue damage (as opposed to a transient activation of lymphocytes in the course of e.g. an acute infection) compromise the fundamental core of the danger model; that autoreactivity *per se* is a normal aspect of the inflammatory process of tissue inflammation, whereas the defining sentinel of pathological

autoimmunity (e.g. autoimmune disease) is the persistence of autoimmunity caused by a continuous provision of danger signals and autoantigens. Matzinger defines danger signals as structures released or produced by cells or tissues undergoing stress or abnormal cell death, or even normal cell death, if the cells are not properly scavenged and instead go into secondary necrosis. The resulting pathogens call the resting antigen presenting cells (APCs) into action.

As a disease process, lupus nephritis may be fully explained by the danger model of Matzinger, with extracellular exposed secondary necrotic chromatin as the initiating danger signal. Furthermore, aside from providing danger signals, chromatin act as the central auto-antigen complex [13-15] that is targeted by induced autoantibodies. This process creates the immune complexes responsible for the inflammatory process (as discussed below).

5.3 Systemic lupus erythematosus and autoimmunity

SLE is a multiorgan disease elicited by yet incompletely understood molecular and cellular aberrations of the immune systems. A triggering pathogenetic factor (infection, sun exposure, medications) combined with a genetic susceptibility and a compromised immune system distort the equilibrium between a physiological condition and the development of disease. The development of autoimmunity in SLE leads to a variety of autoantibody specificities [16]. A dysregulation of the apoptotic process and inadequate removal of apoptotic cells and nuclear debris will expose the immune system to chromatin material and stimulate the activation of autoantibodies [17-19]. The binding of autoantibodies may elicit cell- and tissue injury by initiating inflammation. In the kidneys, intrinsic antigens such as extracellular chromatin fragments may serve as targets for antibody binding and autoantibody – chromatin complex formation [20]. Fc receptor- (presented by macrophages) and complement activation further increase the inflammatory and cytotoxic response and eventually lead to the development of manifest organ disease [21].

5.4 Autoantibodies

The potential to produce autoantibodies is an inherent property of the normal immune system. Normal, healthy individuals may have detectable levels of autoantibodies without any clinical disease. Advancing age is associated with an increase in the number and titers of autoantibodies in the absence of clinical symptoms, and these may merely reflect loss of immune tolerance over time [22] Healthy siblings of patients with autoimmune disorders, such as SLE, also have increased numbers of autoantibodies, without necessarily exhibiting clinical disease. Instead, this may reflect an underlying genetic predisposition for autoantibody development.

Activation of self-reactive B cells that have escaped central tolerance and become activated by an eliciting autoantigen results in extrafollicular formation of short-lived plasma cells that produce autoantibodies. The differentiation of B cells into plasma cells may be either T cell dependent [23] or T cell independent [24]. T cell responses are usually required for this pathway to take place [25]. Autoreactive B cells can also enter germinal centers where they undergo somatic hypermutation and affinity maturation of their B cell receptors with resulting Ig class switching and generation of long-lived autoreactive memory B cells [25]. Autoreactive memory and germinal center B cells can further differentiate into long-lived plasma cells secreting high affinity autoantibodies. These processes normally occur in secondary lymphoid structures such as in the spleen and lymph nodes, but can also form at sites of inflammation.

5.4.1 Anti-dsDNA antibodies

Shortly after their detection in 1957, [26-28] antibodies to dsDNA were associated with renal manifestation of SLE. Furthermore, anti-dsDNA antibodies have been eluted from affected glomeruli [29-32]. The mechanisms underlying the presence of circulating anti-nuclear

autoantibodies, and anti-dsDNA autoantibodies in particular, are probably one of the key issues in understanding the pathogenesis of SLE. Even in patients with overt autoimmune disorders, presence of high titer autoantibodies can precede clinical symptoms by decades [25]. More than a 100 autoantibodies have been identified in lupus patients. However, their pathogenicity differ relative to their antigenic specificity [33].

Numerous publications report autoantibodies against DNA and DNA-containing particles (nucleosomes/chromatin) as a major constituent of glomerular immune complex deposits [34-37]. Release and exposure of relevant antigens recognised by nephritogenic anti-dsDNA antibodies must therefore be crucial events in making target molecules available for the antibodies, and subsequently render the autoantibodies pathogenic [38].

Pathogenic anti-DNA antibodies are believed to represent high avidity IgG antibodies binding dsDNA. These antibodies proceed through somatic hypermutations and affinity maturation due to repeated stimulation with immunogenic DNA [39]. Some patients with SLE produce antibodies against dsDNA and nucleosomes but do not develop lupus nephritis. Thus, it is conceivable that there are distinct pathways or selection principles that determine their pathogenic impact. Two mechanisms have received attention over the years; antibodies may cross-react with native glomerular structures and induce inflammation [32;40-45], or alternatively, the nephritogenic effect of anti-dsDNA antibodies depend on binding to chromatin fragments associated with glomerular basement membranes (GBMs), leaving antibodies non-pathogenic in the absence of exposed chromatin [20;46;47].

It is almost impossible to stimulate an immune response to intact, native DNA, ribosomal RNA or tRNA unless certain biochemical modulations have taken place (as discussed in [48]). Thus, DNA is only regarded immunogenic when complexed with DNA-binding peptides of a non-self [49-57] or self origin [58;59] .

A hapten is a small molecule that can elicit an immune response only when attached to a large carrier such as a protein. The hapten-carrier model was launched to explain how weak immunogens, such as DNA, can become targets of an immune response when in complex with a more potent immunogenic structure. According to this model, the hapten adjoins in the antibody-mediated internalisation of the carrier in APCs or in hapten-specific B cells. The carrier peptides are then presented and serve as T cell targets [39]. It has been demonstrated that nucleosome- and histone-specific T cells in both murine and human SLE have the potential to provide cognate help for DNA–specific B cells [56;58;60]. DNA–peptide complexes stimulate DNA-specific B cells and peptide-specific T cells analogous to the hapten carrier model for induction of anti-hapten antibodies. A consequence of sustained stimulation with dsDNA–peptide complexes is affinity maturation of the induced anti-dsDNA antibodies [61-65].

There is no incongruity between the danger model and the hapten-carrier principle. The fundamental virtue of a hapten-carrier complex is that it introduces a protein antigen that is not effectively displayed on host cells, analogous to 'altered self' antigens as presented in the danger model. Also, there is the possibility that certain hapten structures may represent danger signals on their own. For instance, High mobility group box protein 1 (HMGB1), a well-recognized endogenous danger signal, has been shown to provide an effective adjuvant, boosting the immune response against nucleosomes [66;67].

The strength of binding between an antibody and a single epitope is termed affinity, whereas involvement of multiple binding sites by a single antibody yields functional affinity, called avidity. Whether antibody avidity is essential for antibody binding *in vivo* is controversial, but it is assumed that high avidity of anti-dsDNA antibodies contributes to pathogenicity [68-71]. The intrinsic affinity of circulating versus glomerular *in vivo*–bound anti-dsDNA antibodies in individual nephritic (NZB×NZW)F1 mice has been studied [72].

Affinity was higher in antibodies eluted from kidneys compared to affinity of circulating antibodies in individual mice. However, affinity of antibodies in renal eluates from *different* nephritic (NZB×NZW)F1 mice with severe proteinuria varied considerably, from low affinity to very high [72]. These data suggest that intrinsic antibody affinity is not a key nephritogenic parameter.

Dual specificity of antibodies for dsDNA and naturally exposed glomerular constituents represents the most logical explanation for why some but not all anti-dsDNA antibodies are pathogenic [32;42]. According to this model, only antibodies that cross-react with glomerular antigens will act as pathogens. In support of this, serum anti-DNA antibodies recognize inherent non-DNA/non-nucleosomal renal antigens [29;32;42;73-75], but dual specificity *per se* does not identify which of the cross-reactive renal ligands that actually bind these antibodies *in vivo*.

An alternative model suggests that availability of chromatin fragments in circulation or in glomeruli are required for anti-chromatin antibodies to exert full pathogenic effect [76-79]. Electron-dense structures (EDS) associated with GBM and the mesangial matrix were initially described in the 1960-70s [80-82]. Recently, these were demonstrated to constitute chromatin constituents, a major target for *in situ*–bound antibodies in both murine [46] and human lupus nephritis [30]. Binding of antibodies to other glomerular structures was not observed [20;30;46].

5.4.2 The role of chromatin - anti-DNA antibody complexes in induction and progression of lupus nephritis.

Both molecular and genetic processes have been identified as initiators in the production of anti-dsDNA and anti-chromatin antibodies. These include the above-mentioned hapten-carrier model, [49;51;52;83-88] single gene mutations/gene alterations [89-94] and gene deficient mouse models [95-98]. Despite the yet unsettled mechanism accounting for autoantibody production *in vivo*, the availability of an antigen is imperative for an autoantibody to become pathogenic [48;99]. Whereas the pathogenicity of lupus autoantibodies most likely are linked to recognition of chromatin, the transformation of apoptotic into secondary necrotic chromatin is considered central in the pathogenesis of the disease. An alternative source of chromatin could be the increased levels of microparticles (MP) containing DNA, RNA and nuclear proteins found in SLE patients [100]. MPs are secreted by dying cells [101;102] and have been attributed to both induction of tolerance in normal immunity and as supplier of self antigen in autoimmune responses [101].

A *tet*-regulated T antigen–transgenic mouse model may provide an alternative explanation for how chromatin fragments are released and how they serve as target structures for nephritogenic antibodies. *De novo* expression of polyomavirus large T antigen in a binary *tet-off* regulated T antigen transgenic mouse model resulted in activation of $CD8^+$ and $CD4^+$ T cells and in sustained production of antibodies to dsDNA, nucleosomes and T antigen [55]. In T antigen–expressing mice, EDS were observed in GBMs [48;103]. These EDS bound experimental antibodies to T antigen, dsDNA, histones and transcription factors as demonstrated by co-localization immune electron microscopy. This demonstrates that they contain chromatin fragments in complex with T antigen. In addition, these observations indicate that chromatin fragments may have been released from T antigen–expressing cells secondary to $CD8^+$ T cell–mediated killing of cells that express that T antigen [103].

In situations in which high levels of exposed chromatin coincide with presence of anti-chromatin antibodies, an immune complex-mediated process emerges. Such immune complexes may, either *in situ* in the organ, or in circulation, impose inflammation and subsequent organ damage. Deposition of immune complexes in tissue will further activate complement and induce cytokine secretion and inflammation [104]. Whether these immune complexes are formed in circulation or *in situ* is yet unknown. In this scenario however, it is conceivable that chromatin fragments act both as inducers of potentially nephritogenic autoimmunity and at the same time represent target structures for the autoimmune response.

5.4.3 SLE and the clearance of dead cells

To maintain a state of homeostasis, phagocytes administer the clearance of dead cells. These are recognized as dead by "eat me" signals exposed on their surface, mostly in a phospatidylserine-dependent manner. The cell and nucleus condense and become fragmented and subsequently engulfed by phagocytes [105]. After internalisation, the dead cell is transferred to lysosymes where their cellular components are degraded, metabolised and re-used [106;107]. A hallmark of apoptosis is DNA fragmentation, the cleavage of chromosomal DNA into multimers of approximately 200 base pair nucleosomal units with a 3`-hydroxyl group; the mononucleosome [108;109]. This group is identified by the TUNEL assay, a widely used marker to detect apoptotic cells *in vivo* and *in vitro*. The enzyme that degrades DNA of apoptotic cells in lysozymes in macrophages, is DNaseII [110]. DNaseII is ubiquitously expressed in various tissues, particularly in macrophages [111]. In case of incomplete phagocytic clearance, either caused by impaired engulfment or due to the fact that the number of apoptotic cells overwhelms the capacity of the phagocytes, the cell`s plasma membrane ruptures and its cellular content is released into the microenvironment in a process called secondary necrosis [105]. This is a process able to provide the same danger signals by

non-infectious self, as infectious non-self structures (as proposed by Janeway [112]) or in Matzinger`s danger model [13;14].

For the specific and efficient engulfment of apoptotic cells, the dead cells discharge molecules ("find me signals"), to recruit phagocytes. When necrotic cells interact with or are engulfed by macrophages, the macrophages produce pro-inflammatory cytokines [113], leading to a state of inflammation. The most important "find me signal" is probably lysophospatidylcholine (LPC), but the activated complement system is also involved [114]. Complement factors have been shown to mediate efficient clearance of apoptotic cells by means of macrophages [11;115] or immature dendritic cells [116]. Furthermore, it has been demonstrated that knockout of the C1q gene resulted in manifest nephritis as a result of reduced removal of apoptotic cells [11].

To promote their engulfment, several proteins have been identified as recognition molecules to phosphatidylserine ("eat me signals") on the surface of apoptotic cells. Milk fat globule EGF factor 8 (MFGE8) is expressed on antigen presenting cells [117;118]. MFGE8-deficient female mice, particularly of the B6/129 background, develop an age dependent SLE phenotype of autoimmune disease [117]. These mice produce high concentrations of anti-dsDNA antibodies and suffer from glomerulonephritis.

As non-engulfed apoptotic cells are present in the germinal centres of lymph nodes of certain SLE patients, and macrophages from these patients often show reduced ability to engulf apoptotic cells, a deficiency in the clearance of apoptotic cells has been proposed to be a central mechanism in the pathogenesis of SLE [119]. If the clearance of apoptotic cells is reduced, [18;46;77;78;120;121] it may explain the observed increase in the number of dead cells in glomeruli [46] and hence the initial loss of immune tolerance related to lupus nephritis.

Toll like receptors (TLRs) are pivotal modulators of the innate immune response due to their ability to recognise conserved molecular patterns that are either microbe

specific or endogenously released danger signals [122]. Chromatin fragments released from apoptotic blebs are consumed by immature dendritic cells and bind to TLR7-9 through exposed RNA structures or CpG DNA motifs, respectively. Because TLR7-9 are confined to endosomal compartments, their activity depends on the internalisation of their respective ligands by other receptors recognizing chromatin constituents [123]. Mammalian DNA binds to TLR9 through CpG motifs [124], and peptides derived from chromatin may be processed and then presented on the cell surface within MHC class II molecules. Signalling through TLRs induce dendritic cell maturation, upregulation of the co-stimulatory molecules CD80/86, and processing and presentation of antigenic peptides derived from chromatin. Such activated dendritic cells prime e.g. nucleosome-specific or Sm/RNP-specific T-helper cells that provide help to DNA- or RNP-specific B cells to be transformed into antibody-producing plasma cells [13;14;18;120;125;126]. This scenario may be sufficient to activate innate and adaptive immune systems to produce anti-dsDNA or anti-nucleosome antibodies.

An alternative pathway that directs chromatin into macrophages and permits interaction with TLRs has been described. Means *et al.* [127;128] demonstrated a novel functional interaction between Fc receptors and TLRs. This interaction follows a pathway in which CD32 (FcγRIIa) transfers anti-dsDNA antibody–dsDNA-containing immune complexes to TLR9 in lysosomes, an interaction which induces plasmacytoid dendritic cell activation and IFNα production.

5.4.4 Murine models in SLE

Animal models provide a powerful tool to study disease mechanisms under well-defined conditions. Classical models of spontaneous and investigator-induced murine lupus, as well as more novel transgenic and recombinant knockout lineages have been particularly useful. One of the most widely used spontaneous model is the (NZBxNZW)F1 hybrid strain initially developed by Helyer and Howie [129], a F1 crossbreed progeny of the New Zealand black

and the New Zealand white strain originally described by Bielschowsky *et al.* [130]. The female (NZBxNZW)F1 offspring spontaneously develop an autoimmune phenotype with proteinuria, glomerulonephritis, splenomegaly, arthritis and anti-dsDNA autoantibodies from about 20-35 weeks of age, until they eventually die from end stage renal failure or cardiovascular disease [131].

The MRL-lpr/lpr is another widely used spontaneous model, initially described by Murphy and Roths in the late 1970s [132;133]. The MRL-lpr/lpr strain expresses a homozygote mutation for the apoptosis-inducing ligand Fas-lpr. The lpr mutation results in an alteration in the Fas gene and a defect of apoptosis, resulting in abnormal lymphoproliferation, anti-DNA antibody production, lupus-like nephritis and dermatitis [134;135]. In the MRL-lpr/lpr strain, autoreactive B cells are activated in a T cell independent but TLR and B cell receptor dependent manner [136; 137].

5.5 Lupus dermatitis

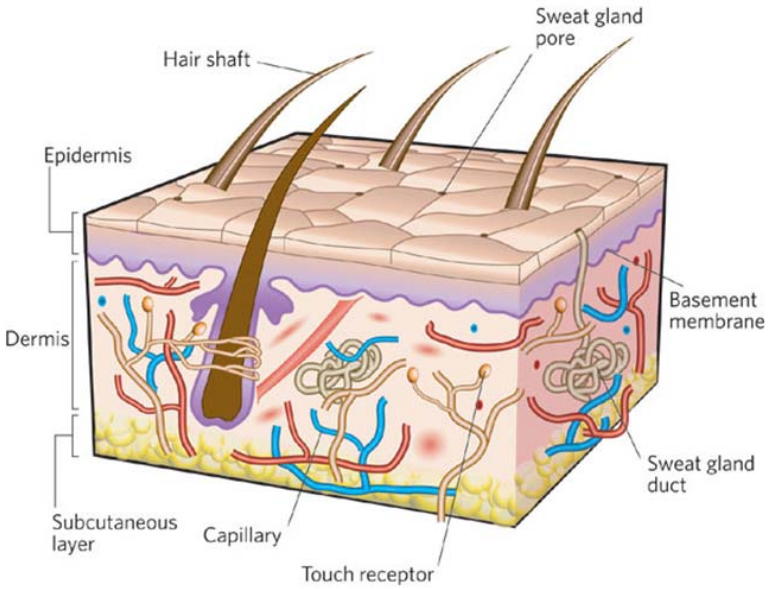
Cutaneous lesions occur in a majority of lupus patients and constitute three of the 11 American College of Rheumatology's (ACR) criteria for the classification of SLE [138]. Lupus dermatitis has traditionally been classified into three clinical subtypes: chronic, subacute and acute [139]. Discoid lupus erythematosus (DLE) is the most common subtype in chronic cutaneous LE, and is rarely associated with systemic disease. DLE is longstanding, heals with scarring and most commonly occurs as a localized process above the neck in sun exposed areas. Generalised DLE with lesions both above and below the neck is more frequently associated with SLE.

Subacute cutaneous LE (SCLE) persists for weeks or months and typically heals without atrophy or scarring [140], although there may be postinflammatory hypopigmentation or teleangiectasias. The patients normally present with papulosquamous, psoriasiform or

annular-polycyclic plaques. SCLE was described in 1979 as a separate subset, intermediate in severity between DLE and acute cutaneous LE [140]. The distribution is normally widespread, and patients frequently have mild systemic disease [140;141]. About 50% of the patients fulfil the ACR criteria, and these patients commonly have high titers of anti-SSA or anti-SSB antibodies [142]. Druginduced SCLE is not infrequent [143]. Acute cutaneous LE (ACLE) is evanescent, heals without scarring, and is usually associated with active systemic disease. Considerable histological overlap exist between DLE and SCLE and differentiation between them cannot be established from the histological picture alone [144]. The typical clinical presentation of acute cutaneous LE (ACLE) is malar erythema, but other clinical manifestations as widespread morbiliform or exanthematous eruptions in sun exposed areas, or bullous or toxic epidermal necrolysis –like skin lesions may occur.

The subtypes of lupus dermatitis are clinically but not necessarily histopathologically distinct, but recognising the different subtypes has traditionally been a helpful tool in predicting the likelihood of underlying systemic disease. By direct immunofluorescence, a granular band-like array of localized immunoglobulins and/or complement components can be found at the dermal-epidermal junction (for skin anatomy, see figure 1). The test is characteristic, but not pathognomonic for cutaneous lupus. It can yield positive results in skin of patients with any subclass of cutaneous lupus erythematosus as well as in normal, non-diseased skin. Furthermore, it has been observed that as many as 20% of healthy young adults do have a positive lupus band test in sun exposed skin regions, whereas virtually none are positive in fully sun proctected non-lesional sites [145]. Still, the lupus band test is of clinical importance in differentiating DLE from SLE, and in differentiating LE from clinically similar skin disorders. It is also useful in separating SLE from other connective tissue disorders. A positive lupus band test has also been observed in skin of lupus prone MRL-lpr/lpr and (NZBxNZW)F1 mice [146].

Fig 1 **The structure of human skin.**



MacNeil S. Nature 2007; 445:875 © Nature Publishing Group 2007. With permission

The pathogenesis of lupus dermatitis is not fully elucidated. An increased level of apoptosis or a defective clearance of apoptotic cells are possible mechanisms, and several studies do suggest an abnormal or delayed clearance of apoptotic cells in lupus skin [147-155]. In lupus dermatitis, the cells targeted for immunological damage through apoptosis are probably the basal keratinocytes [156]. It is well known that lupus dermatitis may be exacerbated if the skin is exposed to UV irradiation [157-159]. However, UV irradiation of normal skin may also induce apoptotic processes [160-162]. Other factors that have been associated with the onset or triggering of cutaneous lupus erythematosus include viruses, drugs, trauma and hormones [163].

Interestingly, high mobility group box protein 1 (HMGB1) is demonstrated to be expressed extracellularly in cutaneous lesions of SLE. Extracellular HMGB1 expression within developing lesions of CLE peaked at 48 h after UVB irradiation of human skin and correlated with IL-1b and TNF expression [164]. HMGB1 is a DNA-binding pro-

inflammatory cytokine released by monocytes and macrophages. This molecule can also be released by necrotic cells and late apoptotic cells. HMGB1-containing nucleosomes from apoptotic cells were recently shown to be able to induce secretion of IL-1, IL-6, IL-10 and TNF and expression of co-stimulatory molecules in macrophages and DCs.

In 1970, Grishman and Churg published a study on electron microscopy examination of skin specimens from clinically normal and lesional skin of SLE patients. They found electron dense granular deposits below the epidermal basal membrane, in vessel walls and along collagen bundles. These deposits were similar to those found within the kidney and thought to represent immune-complexes [165]. The presence of immune deposits at the dermo-epidermal junction has been interpreted as evidence for tissue injury to be mediated by immune complexes. The nature of the deposits that define a positive lupus band test seem to be constituted by nucleosomes or chromatin and antibodies bound to them. Grootscholten *et al.* demonstrated by immunofluorescence presence of DNA and histones in the basement membrane zone [166] and results from the first paper of this thesis supports this finding at a molecular level, demonstrating that i*n vivo*–bound autoantibodies co-localized with experimental chromatin-binding antibodies, including those specific for dsDNA, histones and the chromatin-associated transcription factor TATA-box binding protein (TBP) in sub-epidermal EDS [167].

The antigenetic specificities of autoantibodies demonstrated by the lupus band test have not been determined, but these may well represent anti-chromatin antibodies that bind chromatin released from apoptotic cells. It has been demonstrated that nucleosomes possess high affinity for dermal basemement membrane constituents – which again can explain why released chromatin fragments deposit here [167]. Chromatin-containing immune complexes have similarly been observed sub-epidermally in lesional skin from MRL-lpr/lpr mice, and the presence of such complexes seemed to be associated with increased matrix metalloproteinase

activity [168]. MMP2 and MMP9 are both gelatinases involved in the remodelation and collagen turnover in the dermis [169;170]. An increase in MMP2 and MMP9 activities is hypothesised to disintegrate skin membranes (vascular and basal), potentially resulting in increased access for chromatin containing immune complexes [168].

5.6 Lupus nephritis

5.6.1 Clinical considerations and the ISN/RPS classification system for lupus nephritis.

Lupus nephritis is a major cause of morbidity in SLE patients. Most commonly it develops early in the course of disease, but it can also present as a late complication [171]. Renal involvement is common, and up to 60% of all patients will develop lupus nephritis either as an initial manifestation of SLE or at some point during the course of the disease [172]. 10–15% of the patients who develop lupus nephritis will progress to end-stage renal disease [173].

One of the major clinical problems in diagnosing and treating lupus nephritis is that both clinical symptoms as well as laboratory finding have a low predictive value in identifying patients at risk of developing the more severe subtypes of the disease. Monitoring serum levels of anti-dsDNA and other groups of autoantibodies have provided unsatisfactory low sensitivity and specificity [69;174-176]. A proper diagnosis of lupus nephritis generally requires histopathological evaluation by means of renal biopsy, with a considerable procedural risk for the patient. Unfortunately, the prognostic information provided by a biopsy is limited by the fact that the renal affection may in early phases of disease be confined to focal pathology [177-179]. Still, due to the diversity of the clinical manifestations and the diversity of disease severity, the subtypes of lupus nephritis are primarily classified based on morphologic examination.

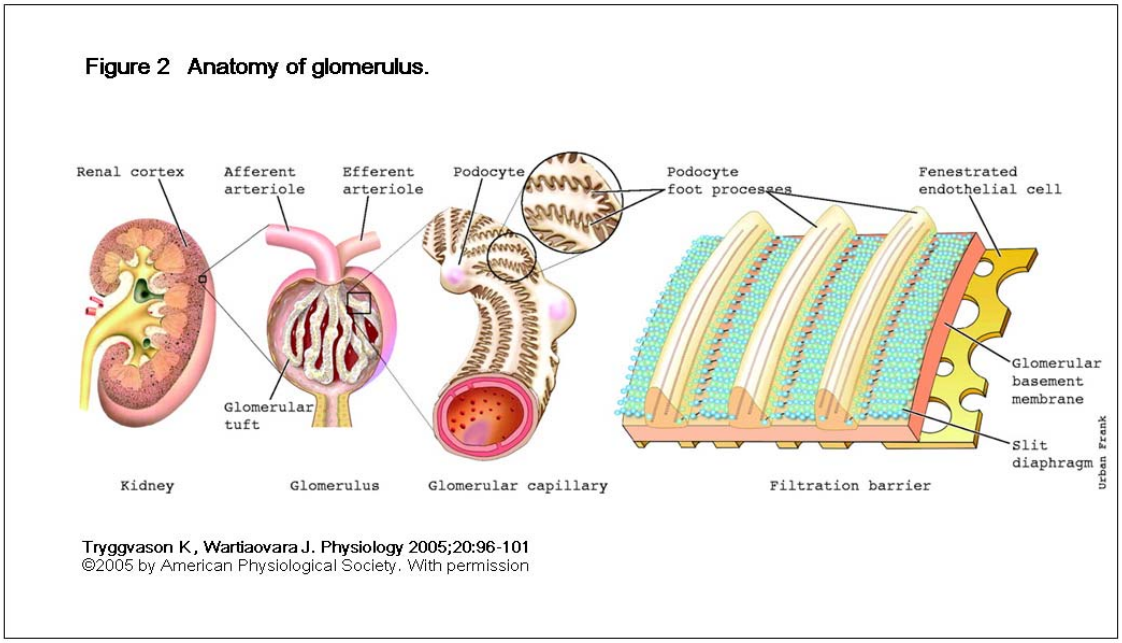
The WHO classification of lupus nephritis was refined in 1982 with further modifications in 1995 [180]. It defined six major classes and a vast number of subclasses

which made an exact diagnosis complicated in daily practice. In a consensus conference in 2003 a new International Society of Nephrology/Renal Pathology Society (ISN/RPS) classification system was proposed [181;182] (see Appendix for the ISN/RPS classification system). Both classification systems integrate the three modalities of light microscopy, immunofluorescence and electron microscopy. The ISN/RPS classification system divides lupus nephritis into six major classes, with the dominating pattern of cellular proliferation and matrix expansion defining the characteristics of major impact. Generally speaking, class I and II changes are considered mild renal disease defined by mesangial deposits in absence of glomerular capillary deposits. Subclasses III and IV are associated with rapidly progressive glomerular damage. Class V is associated with increased occurrence of thromboembolic glomerulonephritis, whilst class VI represents end stage nephritis with advanced sclerosis.

5.6.2 The pathogenesis of lupus nephritis

It is believed that a combination of systemic autoimmunity and local tissue response to immune injury underlies renal involvement in SLE. Substantial progress has been made to identify the pathogenic triggers that result in autoantibody production, yet many details are still lacking in the knowledge about the pathogenesis of the proliferative processes that lead to irreversible glomerular damage and compromised renal function. To further complicate this issue, the patterns of glomerular injury in human lupus nephritis are diverse (as discussed above). A histopathological hallmark is the appearance of immune complex deposits along glomerular membranes (for overview of renal anatomy, see figure 2) and within the mesangium of the glomerulus. Besides, mesangial and endothelial cell proliferation and leucocyte infiltration are frequent findings of active disease. The immune complexes typically consist of nucleosome-anti nucleosome immune complexes, but other antigens have also been identified, as complement factor C1q, Sm, SSA/SSB, ubiquitin and ribosomal proteins [183].

The predominance of chromatin-associated antigens points at deficiencies in the processing and elimination of chromatin as central factors in the pathogenesis of the disease [78;120;184-186].



Whether chromatin-containing immune complexes are formed *in situ* by binding of circulatory autoantibodies to membrane bound chromatin fragments [46], or if this is due to cross-reactivity with GBM components such as laminin [42;187], α–actinin [32;74] or cell surface structures [44;188], is an issue of debate. Accumulating evidence supports the former view. This is supported by observed immune complexes in mesangial matrix and GBM of the kidneys containing DNA, histones and other DNA-binding proteins [20;100;189-191]. Nucleosomes have also been shown to bind GBM components such as laminin and collagen IV with high affinity [47].

Glomerular autoantibody deposition is pivotal in lupus glomerulonephritis. The mere presence of anti-DNA antibodies is not sufficient to initiate the development of lupus

nephritis [192]. However, the levels of anti-nucleosome antibodies and anti-DNA antibodies correlate with the development of lupus nephritis while anti-α actinin antibodies do not [30;193]. Anti-DNA antibodies eluted from nephritic kidneys showed higher affinity for DNA compared with affinity of anti-DNA antibodies found in the circulation of BW mice [72]. This is further substantiated in experiments where monoclonal anti-DNA antibodies were repeatedly injected into normal BALB/c mice. This caused an increased accumulation of chromatin deposits in the mesangial matrix [194], and helps explain the renal consequences of glomerular binding of affinity-matured anti-dsDNA antibodies *in situ*; their chromatin-associated targets are exposed in glomeruli [46]. Thus, anti-dsDNA antibodies execute a potential nephritogenic mechanism by complex formation with chromatin in glomeruli [20;46].

The source of the deposited chromatin found in glomerular matrices and basement membranes are not known (as discussed above), but recent studies have demonstrated that the fragmentation of chromatin in nephritic kidneys is impaired as demonstrated by experimental induction of apoptosis in *ex vivo* kidneys [1]. Reduced fragmentation was later shown to be linked to reduced DNaseI activity [195-197]. Improperly sequestered chromatin fragments seem not be cleared appropriately by phagocytes. These fragments may instead bind to membranes and be targeted by anti-DNA antibodies in the organs where they are released from dead cells.

Immune complex deposits in the mesangium and in the GBM also induce complement activation and subsequent chemokine release and influx of inflammatory cells. Activation of renal endothelial cells, chemokines and infiltration of dendritic cells and macrophages are associated with onset of proteinuria [198], together with activation of the complement system [199]. This again leads to cellular proliferation with generation of urinary sediments, proteinuria and progression of the disease.

T- and B cells are vital cellular components of the adaptive immunity and central in the etiopathogenesis of lupus nephritis (reviewed in [200]). T cells were previously considered mainly to assist in B cell production of nephritogenic autoantibodies. During the past several years, the focus on the role of T cells in lupus nephritis has been changed to the emphasis of these cells as effectors of mediating tissue injury, as autoreactive T cells modulate T helper cell and effector functions via immunmodulatory cytokines and infiltration of renal parenchyma, thus contributing to tissue injury both directly (via cytotoxicity) and indirectly through activation and recruitment of macrophages and natural killer cells (reviewed in [201-203]).

An important finding confirming the crucial role of B cells in the intiation of lupus nephritis was elegantly illustrated in a study by Shlomchik *et al.*, demonstrating that nephritis and vasculitis did not develop in B cell-depleted MRL-lpr/lpr J heavy chain (JHC) knockout mice [204], whereas development of glomerulonephritis was unaffected in JHD-MRL-Fas-lpr mice bearing genetically manipulated B cells incapable of secreting antibodies [205]. In general, B cells are mostly acknowledged for autoantibody release, but B cell production of regulatory cytokines and direct interactions with T cells and dendritic cells have significant impact on cellular immune responses (reviewed in [206;207]). B cells are highly efficient antigen presenting cells, and autoantigen presentation directly activates autoreactive T cells primed by professional APCs. B cells modulate T cell memory and regulate activation and development of dendritic cells. Subsets of differentiated effector and regulatory B cells produce immune modulatory cytokines such as IL-10 and TNFβ in particular [208].

Recent advances in the field of epigenetics also require attention, as accumulating evidence point at epigenetic factors as central in the onset and progression of SLE. The traditional mechanisms of epigenetic regulation include DNA methylation and histone modifications. DNA methylation involves the addition of a methyl group to the pyrimidinyl

ring of cytosine, primarily within CpG pairs, and is catalyzed by DNA methyltransferases (DNMTs) [209]. Methylation of CpG islands in promoter regulatory regions is associated with transcriptional inactivation of the corresponding gene, while demethylation of these regions creates a permissive transcriptional environment [210]. Recent data suggest that miR-126 regulates DNA methylation in CD4(+) T cells and contributes to T cell autoreactivity in SLE by directly targeting DNA methyltransferase 1 [209] .

MicroRNAs (miRNAs) belong to a family of short non-coding RNAs. A recently published comprehensive analysis of miRNA expression patterns in renal biopsies of lupus nephritis patients further demonstrates that miRNAs are involved in the pathogenesis of lupus nephritis [211].

5.7 The role of acquired renal DNaseI deficiency in lupus nephritis.

Enzymatic DNA fragmentation by the activation of different endonucleases is significant in both the process of apoptosis (reviewed in [212;213] and the elimination of DNA from necrotic cells (reviewed in [190;212]. The reduced clearance of apoptotic cell debris is assumed to play a causal role in necrotic transformation of apoptotic chromatin and in deposition of chromatin in glomeruli [18;77;120;214]. Secondary to this, chromatin fragments are inappropriately degraded and subsequently released into the local environment where they bind glomerular membranes and the mesangial matrix [78;99] with high affinity[47]. Until recently, there was no apparent explanation for the reduced clearance of chromatin [77;78;119]. Zykova *et al.* observed that nucleosomal DNA fragmentation in camptothecin-induced apoptotic cells in freshly isolated kidneys from nephritic (NZB×NZW)F1 mice was markedly reduced compared with the effective fragmentation in similarly induced apoptotic cells in kidneys from non-autoimmune mice [1].

The endonuclease DNaseI, expressed primarily in tubular and to a lesser extent in glomerular mesangial cells, represents the major renal nuclease [215]. Reduced DNA fragmentation coincides with reduced levels of DNaseI mRNA and a near-absent DNaseI enzyme activity in nephritic kidneys [1;197]. Loss of DNaseI activity is not observed in kidneys from prenephritic (NZB×NZW)F1 or age-matched non-autoimmune mice [196;197]. Notably, as described in paper II, we found no reduction of DNaseI mRNA or enzyme activity in skin of nephritic autoimmune mice. This indicates that there is not a systemic loss of DNaseI activity. DNaseI activity has also been investigated in liver and spleen of lupus prone mice, demonstrating identical results as in skin and further confirming the loss of renal DNaseI as an organ specific process (Seredkina et al., manuscript under revision). By quantitative PCR analyses of DNase I, DNase II, endonuclease G, DNA fragmentation factor subunit, and cell death–inducing DNA fragmentation factor subunit–like effector B, the expression of DNaseI mRNA was the single nuclease dramatically reduced in the kidneys of proteinuric (NZB×NZW)F1 mice [1]. The strong association between the deposition of chromatin-containing immune complexes within the GBM and the abrupt downregulation of DNaseI offers an attractive hypothesis to explain how tissue- specific changes in gene expression may induce antigens in the form of immune complex deposition [79;186]. It also suggests a potential link between systemic autoimmunity and single-organ disease.

6 Aims of the thesis

The aims of paper I and paper II were to investigate whether the molecular composition of immune complexes found in skin of lupus patients are identical to the composition of complexes in glomeruli. Secondly, we wanted to address whether the mechanism resulting in deposition of chromatin fragments in dermal structures are similar to what is seen in lupus nephritis. Thirdly, we aimed to determine if deposition in one of the organs (kidney) predicted

deposition in the other (skin). These studies were performed in both lupus patients and lupus prone mice.

The main focus in paper III was to evaluate the impact of anti-dsDNA antibodies and their relation to renal DNaseI and matrix metalloproteinase (MMP) mRNA levels and enzyme activities in early and late murine lupus nephritis. Secondly, the studies were designed to analyse how regulation of DNaseI, MMP2 and MMP9 mRNA levels and enzyme activities correlate with each other, with production of antibodies to dsDNA, with successive deposition of EDS in the mesangial matrix and in GBM, and finally with progressive proteinuria, all important characteristics in lupus nephritis. Thirdly, we wanted to analyse if these factors in any way were interrelated, and if changes in their expression can explain basic processes accounting for the different stages of lupus nephritis, from mild to severe disease. In paper IV, we addressed similar issues as for paper III, translated to human lupus nephritis. We further investigated whether DNaseI downregulation resulted in exposure of chromatin and initiation of a signalling cascade potentially leading to upregulation of pro-inflammatory cytokines and matrix metalloproteinases.

7 Summary of experimental results

7.1. Paper I. Circulating chromatin-anti-chromatin antibody complexes bind with high affinity to dermal-epidermal structures in murine and human lupus nephritis.

Electron dense structures (EDS) containing IgG and chromatin are found in glomerular basement membranes (GBM) in murine and human lupus nephritis. Nucleosomes, the main constituent of chromatin, are known to have a high affinity for GBM, which could explain the accumulation of nucleosome-containg EDS in the GBM. It is well known that EDS are also found in relation to the basement membranes in lupus dermatitis. However, the ultrastructural

compositon of these EDS had not been described. In this study we did a comparative analysis of the molecular composition of immune complex deposits in paired skin and renal biopsies from patients and lupus prone (NZBxNZW)F1 and MRL-lpr/lpr mice by immune electron microscopy (IEM) and co-localization terminal deoxynucleotidyl transferase dUTP nicked-end labelling (TUNEL) immune electron microscopy. IEM analyses demonstrated that antibody deposits were confined to EDS in glomerular capillary membranes and the mesangial matrix, and subepidermally in skin. Presence of EDS in glomeruli however, did not predict similar deposits in skin. Chromatin-anti chromatin antibody complexes were found in capillary lumina in dermis and in glomeruli of nephritic patients and also in diseased mice. *In vivo*–bound autoantibodies co-localized with experimental chromatin-binding antibodies, including those specific for dsDNA, histones, or the chromatin-associated transcription factor TATA-box binding protein. To confirm that the experimental anti-chromatin antibodies used in the co-localization IEM assay were not cross-reactive, we applied the co-localization TUNEL IEM assay to demonstrate the presence of nicked endogenous extracellular DNA and its co-localization with *in vivo*–bound autoantibodies in membrane–associated EDS. This result is in harmony with the fact that chromatin fragments bind GBM and mesangial matrix with high affinity, as demonstrated by surface plasmon resonance (SPR). SPR analyses also demonstrated that chromatin constituents do indeed have a high affinity for epidermal basement membrane components. We therefore proposed that the chromatin-anti-chromatin antibody complexes possibly access skin through circulation, presumably dependent on other biological factors, to deposit within dermal structures other than blood vessels.

7.2. Paper II. Deposition of chromatin-IgG complexes in skin of nephritic MRL-lpr/lpr mice is associated with increased local matrix metalloproteinase activities.

Chromatin-IgG complexes are observed in glomerular basement membranes (GBM) of nephritic patients and in nephritic lupus prone mice. Similar deposits appear in skin and in capillary lumina. Surface plasmon resonance (SPR) has demonstrated that chromatin fragments possess high affinity to epidermal basement membrane components, but high affinity alone is not sufficient to promote extracellular deposition in tissues. This indicates that other factors play a role for the immune complexes to deposit in relation to dermal membranes. Recent studies from our group demonstrated upregulation of matrix metalloproteinase (MMP2 and MMP9) activities and decreased levels of DNaseI in murine nephritic kidneys. Our hypothesis was accordingly that an increased expression of epidermal basement membrane components and their disintegration of matrices by MMPs could result in an increased binding capacity for chromatin fragments. In this study we compared the composition of immune complex deposits in dermatitis and nephritis by immune electron microscopy and investigated whether glomerular deposits in nephritic kidneys predicted similar deposits in skin of lupus prone mice. Analyses were performed on lupus prone (NZBxNZW)F1 and MRL-lpr/lpr mice in advanced stages of the disease. The expression of dermal basement membrane encoding genes, dermal MMPs and DNaseI mRNA levels were analyzed by qPCR. Activity of MMPs and DNaseI were correlated with immune complex deposition. The results demonstrated that immune complex deposition in murine dermatitis did not necessarily coincide with immune complex deposits in nephritic kidneys, even though their molecular compositions were similar. DNaseI levels and total nuclease activity in skin were stable during disease progression in contrast to the decline observed in nephritic kidneys. Depositions of chromatin-containing immune complexes were found sub-epidermally in skin lesions of nephritic MRL-lpr/lpr mice. This was associated with high activity levels of MMPs.

Hence we postulate that elevated dermal MMP2 and MMP9 activities may be important in the process of making extracellular matrices accessible for immune complex deposition.

7.3. Paper III Anti-dsDNA antibodies promote initiation, and acquired loss of renal DNaseI promotes progression of lupus nephritis in (NZBxNZW)F1 mice.

Deposition of chromatin-IgG complexes in the mesangial matrix and glomerular basement membranes (GBM) morphologically define stages of lupus nephritis. Further, the levels of circulating anti-DNA antibodies have been associated with disease activity in lupus nephritis. In the present study we addressed the impact of antibodies to dsDNA, renal DNaseI and matrix metalloproteinase (MMP) mRNA levels and enzyme activities in early and late events in lupus nephritis in (NZB x NZW)F1 mice. The major focus was to analyse if these factors were interrelated, and if changes in their expression could explain basic processes accounting for lupus nephritis. The correlations were based upon circulating anti-DNA antibodies, degree of proteinuria, morphological changes assessed by immune electron microscopy and renal mRNA levels and corresponding enzyme activity/protein expression. We found that early phases of nephritis were associated with chromatin-IgG complex deposition in the mesangial matrix. A striking observation was that this event correlated with appearance of anti-dsDNA antibodies and mild or clinically silent nephritis. These events preceded down-regulation of renal DNaseI. Later, renal DNaseI mRNA level and enzyme activity were reduced, while MMP2 - and to a lesser extent MMP9 - mRNA level and enzyme activity increased. Another striking observation was that reduced levels of renal DNaseI were temporally associated with deficient fragmentation of chromatin from dead cells. Large fragments were retained and accumulated in GBM. These scenarios may help explain the basis for deposition of chromatin-IgG complexes in glomeruli in early and late stages of nephritis, loss of glomerular integrity and finally renal failure.

7.4. Paper IV Renal up-regulation of Trap1 and p62/SQSTM1 is associated with DNaseI down-regulation during progression of murine and human lupus nephritis .

Recent findings have demonstrated that transformation of mild glomerulonephritis into end-stage organ disease coincided with an abrupt decrease in the renal expression of DNaseI in (NZBxNZW)F1 hybrid mouse. The acquired reduction in DNaseI resulted in reduced chromatin fragmentation and a consequent deposition of chromatin fragments in glomerular basement membranes accessible for nephritogenic anti-chromatin antibodies. In this translational study we have applied identical methods as those used in paper III, to kidney biopsies from patients with lupus nephritis. We further addressed how exposure of chromatin presumably initiates a signalling cascade that lead to the upregulation of pro-inflammatory cytokines and matrix metalloproteinases. Data generated suggest two possible mechanisms accounting for DNaseI reduction. One mechanism is most likely due to transcriptional interference of the Trap1 gene. The heat-shock molecule Trap1 is encoded in the opposite direction of DNaseI, and the two transcripts overlap in their 3'- untranslated regions. Transcription of one may hence result in suppression of the other. The other pathway is yet unclear, but the possible effect of regulatory RNAs is discussed. As a probable link to the loss of DNaseI we demonstrate activation of Toll like receptors 7-9 and Clec4e receptor, with a subsequent upregulation of TNFα and IFNγ, and the MMP 2 and 9 in (NZBxNZW)F1 mice. The upregulation of the signalling factors were less convincing in human nephritis, compared to non-treated nephritic (NZBxNZW)F1 mice. It must be taken into consideration that all patients were under treatment with immunosuppressant and anti-inflammatory treatment, possibly inhibiting the activation of TLRs. In either case, the loss of renal DNaseI seems to initiate a cascade of inflammatory signals which eventually lead to the upregulation of matrix metalloproteinases responsible for disintegration of matrices and membranes.

8 Discussion

8.1 Immune complex deposition and its importance in disease progression of lupus nephritis and lupus dermatitis

It is well established that clearance of apoptotic cells is reduced in SLE [77;99;120;216]. This aberration transforms the silent, non-inflammatory removal of apoptotic cells into the potential extracellular release of chromatin structures. Chromatin fragments generated by nucleases in the context of apoptosis are normally encapsulated within blebs presenting "eat me" signals. This ensures clearance in a fast and silent manner. In SLE, retained and exposed extracellular chromatin may break this silence and initiate inflammation via secondary necrosis [216]. According to Matzinger's danger model, [13-15] this provides danger signals that may initiate dendritic cell maturation via Toll-like receptor engagement. In addition, apoptosis-induced changes in chromatin exposure may enhance immunogenicity and pathogenicity of chromatin in vivo [217]. Thus, the combination of danger signals and secondary structural alterations linked to apoptosis and necrosis contribute to inflammation and activation of dendritic cells with the potential to activate nucleosome-specific T cells which further may provide cognate help for DNA-specific B cells [13;14;56;59;120;121].

Eluted antibodies from diseased kidneys have demonstrated that these generally bound DNA and nucleosomes similar to or much better than they bound membrane constituents as α-actinin, laminin or collagen [29;30;32]. This harmonises with the finding that *in vivo*-bound antibodies co-localise with experimental antibodies against chromatin components and with TUNEL-positive DNA in capillary membranes and mesangial matrix, both central loci for immune complex deposits [20;46]. Exposure and accessibility of chromatin *in situ* are consistent with nucleosomes as target structures for *in vivo*-bound antibodies, and analyses by

SPR confirm that nucleosomes possess high affinity for glomerular constituents as laminin and collagen IV [47].

In the circulation of SLE patients, nucleosomes are found as mono- and oligonucleosomes; in other words considerably smaller than the chromatin fragments observed in association with glomerular membranes [218-220]. Combining these data, it is reasonable to conclude that chromatin fragments exposed in glomeruli represent incompletely degraded nuclei due to renal nuclease deficiencies, in this context deficiency of renal DNaseI, as opposed to properly degraded chromatin into nucleosomes in circulation by normally expressed nucleases in organs other than kidneys [197](Seredkina, manuscript under revision) .

The hypothesis that nucleosomes enter the glomerular mesangium via the circulation cannot, however, be excluded for at least two reasons. There are published data showing nucleosome-containing constituents (DNA, TBP, Histone H3) within capillary lumina in nephritic kidneys [167]. Furthermore, Schiffer *et al.* suggest that early onset of proliferative glomerulonephritis and proteinuria is associated with activation of the renal endothelium, by upregulation of the chemokine CCL20 expressed on inflamed endothelial cells [198]. This may speak in favour of the importance of circulatory immune complexes in the pathogenesis of early lupus nephritis. Also, activation of resident macrophages in early lupus nephritis [198] may have a role in allowing nucleosome-containing immune-complexes to enter the mesangium, as activation of TLRs may explain glomerular membrane disintegration through increased local secretion of MMPs. The effect of bifunctional TLR7/9 inhibitors [221;222] as blockade on SLE disease progression also supports this hypothesis, as inhibitory oligonucleotides may prevent local MMP secretion and thereby leaving the glomerular membranes intact. Our finding in paper III [197] that early, mesangial lupus neprhitis

coincides with the presence of circulating anti-dsDNA antibodies, further supports this possibility.

Large chromatin fragments due to acquired renal DNaseI shut-down, similar to those observed in $CAD^{-/-}$ [223] or $DNaseI^{-/-}$ mice [189;224], remain in the tissue in where they are released from dead cells. In that situation, exposure of chromatin fragments may explain why chromatin-specific autoantibodies gain pathogenic potential, and may furthermore explain why certain organs, such as kidneys, suffer from this pathophysiological autoimmune process: Provided the production of relevant anti-chromatin autoantibodies, the organ in which chromatin fragments are not handled appropriately will subsequently develop immune complexes.

Another indication that points to the kidney as the site of origin of chromatin fragments is the finding that polyomavirus large T antigen is associated with these fragments in lupus nephritis [225]. The kidney represents the major host organ for polyomaviruses such as BK virus, and productive polyomavirus infection is regularly observed in lupus nephritic kidneys (reviewed in [83]). It is noteworthy that the expression of T antigen is the event that initiates and maintain productive polyomavirus infection (reviewed in [226]). This is further supported by the finding of Fenton *et al.,* demonstrating that glomerular EDS in human nephritic kidney samples contain T antigen, DNA and histones, indicating that renal, extracellular chromatin may originate from polyomavirus-infected renal cells [225]. This is consistent with the fact that T antigen binds firmly to host chromatin in cells where polyomaviruses replicate [226].

For lupus dermatitis, we could not demonstrate fluctuations in the DNaseI expression during the progression of the disease in our mice models [168]. The finding of intravascular chromatin containing immune complexes, together with the finding of T antigen

in dermal EDS and an increased MMP expression may all contribute to the hypothesis that the complexes may in fact originate in the kidneys when it comes to lupus dermatitis. However, other authors have found an increased apoptotic rate in diseased skin, supporting the idea of *in situ* formation of dermal immune complexes [227].

8.2 Acquired loss of DNaseI activity and the role of matrix metalloproteinases

Both caspase-activated DNase (CAD) and DNaseI are instrumental in degrading nuclear chromatin when apoptosis is initiated [212;213]. Subsequently, apoptotic cells are engulfed by macrophages, and CAD/DNaseI-fragmented chromatin is further degraded by DNaseII, Endonuclese G and DNaseIl1-3 in the lysosomes of macrophages. Thus, CAD, as well as DNaseI, have important roles as initiators of chromatin fragmentation during apoptosis, whereas other secondary nucleases account for progressive degradation of chromatin into nucleosomes (reviewed in [212;213]). Although there are several mediators of apoptosis and different apoptotic pathways (reviewed in [228]), the main focus in this context is an aberrant apoptotic (or secondary necrotic) process linked to acquired or sustained nuclease deficiencies that eventually results in exposure of chromatin fragments. The finding of downregulation of DNaseI in the kidney after initiation of anti-dsDNA antibody production indicates that loss of renal nuclease activity is not responsible for the appearance of anti-chromatin autoimmunity.

Analyses of nuclear autoimmunity in a DNaseI knockout mouse model with the 129 × C57Bl/6 mixed genetic background indicated that normal DNaseI activity may protect against an anti-DNA autoimmune response [189;224], as experimental deletion of the DNaseI gene correlated with production of anti-dsDNA antibodies and development of lupus nephritis. In different genetic backgrounds, however, this lupus-like phenotype was not observed. This may indicate that a single deficiency of a given nuclease is not sufficient to induce potentially pathogenic autoimmunity to chromatin.

The idea of analysing collected longitudinal data based on progression of nephritis in a (NZBxNZW)F1 model was based on previous findings in our group demonstrating that renal DNaseI mRNA expression and enzyme activity were significantly reduced in mice with symptoms of lupus nephritis [1,195]. The temporal relationship between the acquired loss DNaseI activity and the occurrence of anti-dsDNA antibodies does not favour the role of DNaseI in the production of anti-DNA antibodies, nor in the initiation of the disease. However, the reduced expression of DNaseI mRNA correlated with the appearance of GBM-associated immune complex deposits, consistent with the pattern seen in mebranoproliferative lupus nephritis [197]. The data presented in paper III also demonstrated a decrease in mRNA and protein level expression of DNaseI in nephritic mice with ultrastructurally confirmed immune complex deposits within the GBM, whereas mice with immune complexes purely confined to the mesangial matrix had renal DNaseI expression comparable to that of normal BALB/c mice. We therefore proposed a theory that anti-dsDNA antibodies are responsible for the initiation of mild nephritis, whereas the acquired loss of DNaseI may be important in the progression into more advanced stages of the disease (for illustration of this mechanism, see figure 3).

The mechanisms controlling DNaseI expression are yet to be fully understood, but promising results concerning transcriptional interference with the Trap1 gene (Thiyagarajan et al., manuscript submitted, Fismen et al., manuscript in preparation) and advances in the field of regulatory microRNAs are presumably important. Identifiying the exact mechanism by which DNaseI mRNA expression is downregulated will undoubtedly become important in context of therapeutic tools to restore the gene expression of DNaseI to its original level. Based on the apparent importance of DNaseI in the context of intracellular DNA processing, such inventions could potentially contribute to the removal of extracellular chromatin, e.g. prevent exposure of the antigen potentially responsible for triggering this serious pathological

cascade. Even though attempts to restore serum DNaseI activity in BW mice have been generally disappointing [229;230], targeting the intracellular tissue-specific DNaseI has yet to be tested.

Figure 3

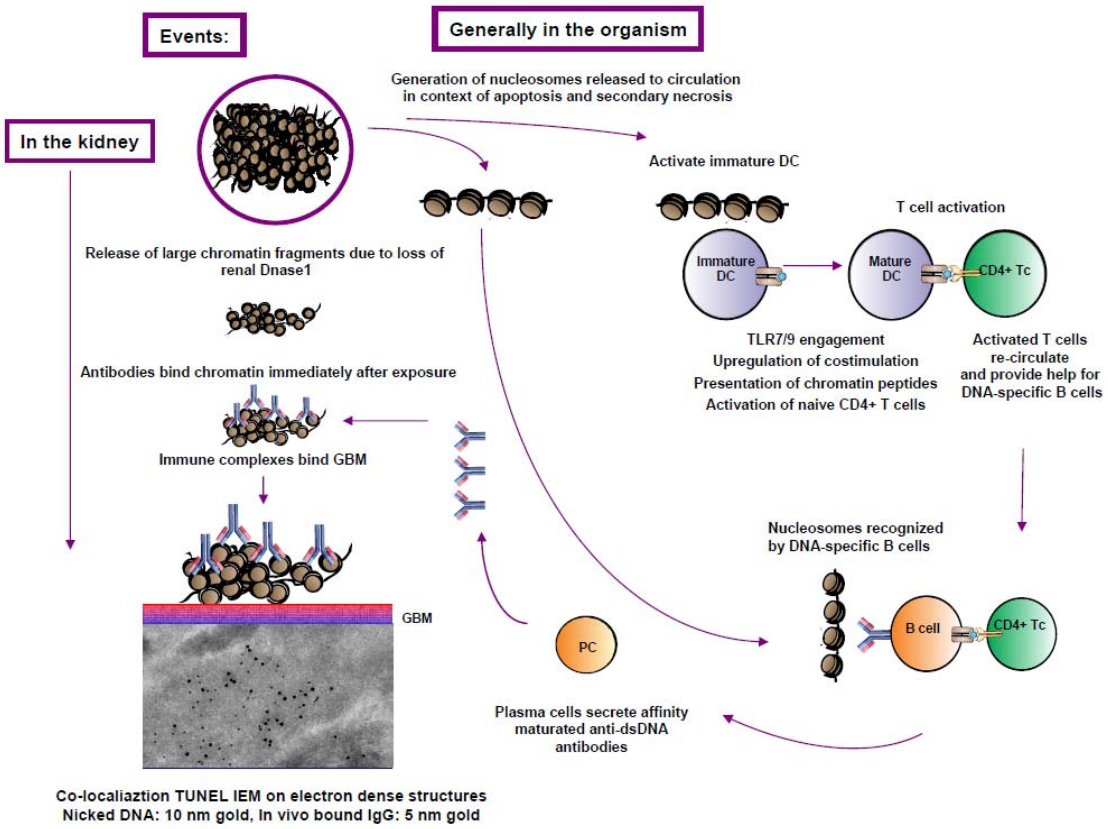


Figure 3. Exposed, extracellular chromatin is a central factor in the evolution of lupus nephritis—a model. In normal situations, chromatin is effectively removed in context of apoptosis. When chromatin fragments are not appropriately cleared they may be exposed in tissue or in circulation. Exposure of chromatin may have impact on the immune system. Chromatin may re-circulate as oligo-nucleosomes, and eventually activate dendritic cells. These cells present chromatin-derived peptides and upregulated co-stimulatory molecules to naı¨ve peptide-specific CD4+ T cells. Activated T cells may subsequently re-circulate and provide help to DNA- or nucleosome-specific B cells to be transformed into antibody-secreting plasma cells. In this situation the antibodies are potentially pathogenic, but to exert this potential, they must to bind exposed chromatin fragments. This may happen in the kidneys when Dnase-1 is downregulated, and may have an immense impact on the pathogenic effect of the autoantibodies. Chromatin in cells dying from, for example, apoptosis may, due to loss of Dnase-1, not be degraded, and instead of clearance, they become exposed as secondary necrotic chromatin in, for example, glomerular membranes, where they are targeted by induced anti-chromatin antibodies. Thus, chromatin fragments may exert two effects with fatal consequences for the kidneys: they may induce autoimmunity (nucleosomes) and they represent targets for the induced autoantibodies (chromatin fragments).

Modified from Fismen et al. Immunology and Cell Biology (2011) 89, 90–99. With permission.

The reason why chromatin fragments get access to GBM (defining end-organ disease) may be due to the fact that released secondary necrotic chromatin fragments trigger local secretion of matrix-degrading enzymes, including MMPs, upon interaction with, for example, TLR9 [231;232] in infiltrating macrophages and dendritic cells. MMPs may degrade barriers constituted by membranes [233;234] and thereby allowing chromatin to associate with disintegrated membranes, and even to penetrate the GBM. Furthermore, engagement of TLR7 and TLR9 by chromatin structures is instrumental in generation of autoimmunity to chromatin components [122;235-237]. The findings of Lenart *et al.* and Duramad *et al.* showing that inhibitory oligonucleotides can block TLR7/9-dependent activation of primary macrophages and progression of the disease may support these assumptions [221;238].

As a consequence of the interaction of chromatin with TLRs, a chain reaction may be initiated that implies activation of the innate immune system, upregulation of co-stimulatory molecules and possibly activation of chromatin-derived peptide-specific T cells. This may in the end activate B cells and induce the production of affinity-maturated anti-dsDNA and anti-chromatin antibodies, at least in an autoimmune-prone background.

8.3. The unsettled role of TRAP1 and the need for further studies

Silencing of the DNaseI gene may theoretically be caused by several regulatory pathways. Today, there is little information to explain this process. One mechanism might be the possible effect of methylation of the DNaseI gene. One focus in our laboratory has been to investigate the role of regulatory RNAs and the role of transcriptional interference with the anti-sense gene Trap1 (Thiyagarajan et al., manuscript submitted, Fismen et al., manuscript in preparation). Data presented in this study indicate that DNaseI indeed may be silenced

through expression of the Trap1 gene, demonstrated both by inverse mRNA expression level and by confocal microscopy analyses of nephritic kidneys in BW mice. However, in several kidney samples of severe lupus nephritis, expression of Trap1 and DNaseI mRNA and protein levels were both reduced. This may point at other regulatory mechanisms than transcriptional interference such as microRNAs targeting the overlap region of the two transcripts. Thus, at least two different mechanisms could be involved in DNaseI gene silencing in context of lupus nephritis. Further studies on these issues are currently in progress in our laboratory.

8.4 Limitations of the studies

Like SLE in general, both lupus dermatitis and lupus nephritis are heterogenous disease entities. The flares and relapses further complicate studies on human subjects. The scant availability of human biopsies in a small research laboratory like ours makes it a challenge to get statistically significant results.

On the opposite scale, a caveat of the animal studies – as for all researchers utilizing experimental animal models – this model does not capture the totality of *human* SLE – even though a reasonable number of objects are attainable. For example; the lupus phenotype of the MRL-lpr mouse is caused by a genetic defect in Fas/APO-1(CD95) or its ligand (Fas L), a genetic aberrancy absent in most lupus patients [239]. However, the strength of a murine model is the opportunity to investigate the role of particular cells or molecules in inflammatory pathways to help solve interconnected pieces of the puzzle. Further, animal studies allow detailed investigation of the progession of disease and of temporal associations of events in the pathogenesis. Still, the validity of the animal data relies on the assumption that the changes taking place in the kidneys are attributable to human conditions. Furthermore, the invasive nature of human kidney biopsies contributes to complicate translational research.

Regardless of the limitations mentioned above, follow-up data of this work should include verification on a significant number of human kidney samples.

9 Concluding remarks

During recent years pathophysiological mechanisms behind SLE have been considerably elucidated. On the cellular level, there have been advances in the knowledge about interaction between immune complexes and the organ of deposition. We know that glomerular immune complexes are largely confined to mesangial matrix and glomerular membranes, containing undigested chromatin particles. On the genetic level, the number of aberrations known to be associated with human lupus has increased by fivefold since 2007, emphasising the complexity of inheritance that contributes to disease pathogenesis [240]. Approximately 35 genes associated with lupus have either been replicated in multiple samples or are near the threshold for genome-wide significance. Some are rare variants that convincingly contribute to lupus in specific subgroups only. Strong associations have been found with genes in the major histocompatibility complex region, with Fcγ receptors and with genes coding for complement components (reviewed in [240]). Examples of newly discovered genes include Integrin αM [241], STAT4 [242] and MECP2/IRAK1 [243;244].

Epigenetics is another field of immense development and worldwide interest. Posttranscriptional modifications in the form of miRNAs or posttranslational modifications involving acetylations/methylations will undoubtedly shed new light upon the pathogenesis of lupus nephritis. Recent studies highlight the significance of epigenetic alterations in aberrant expression of immune factors (reviewed in [245]). Despite significant advances, the scarcity of novel therapies continues. Patients with lupus nephritis are currently treated with non-specific immunosuppressive drugs. Although morbidity and mortality have improved

significantly over the past decades, treatment-related morbidity remains a major problem [246].

The debate concerning SLE as a single disease, or rather a set of separate organ diseases, will hopefully be settled based on scientifically proven evidence. The clinical heterogeneity of the disease, the multiplicity of the involved pathogenic mechanisms and the lack of reliable biomarkers all contribute to the absence of clinical consensus. It should not be omitted that there seems to be an increasing support to the theory that SLE simply cannot represent a single disease entity, but rather a set of overlapping entities broadly linked by the presence of antinuclear antibodies [39;235;247]. SLE is undoubtedly a product of multiple and stepwise failures of immune regulation, leading to diverse and complex clinical scenarios. The consequent interpretation of data discussed in this thesis is that lupus nephritis is a principally biphasic organ disease in which each phase has a distinct pathogenesis [186;197]. With increasing insight into the origins of the symptoms comprising the ACR criteria in the classification of SLE, this syndrome as it is defined today may in the future be split up into more restricted sets of disease entities characterized by new criteria, which may be aetiologically unrelated.

10 References

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APPENDIX

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