

Toll-like receptors in the development of renal injury in systemic lupus erythematosus

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Abstract

Systemic lupus erythematosus is a complex, multisystem autoimmune disease characterized by the production of pathogenic autoantibodies to nucleoproteins and DNA, self antigens that probably accumulate as a result of apoptosis defects. The level of autoantibodies correlates with disease severity and the deposition of these immune complexes in the kidney is thought to contribute to disease pathogenesis. Recent studies in mouse models have drawn attention to the involvement of Toll-like receptors (TLRs) in the generation of autoreactive immune responses. TLRs7 and 9 are activated either in immune or non-immune cells by immune complexes containing self RNA and DNA, respectively. These endogenous TLR ligands act as adjuvants providing a stimulatory signal together with the autoantigen, thus contributing and perpetuating the autoimmune disease. In this review we will summarize recent data on TLR7 and TLR9 in the development of renal injury in SLE.

Key words: SLE, lupus nephritis, TLR7, TLR9.

Introduction

Systemic lupus erythematosus (SLE) is an incurable autoimmune disease of unknown aetiology that affects more than one million individuals each year, primarily young and middle-aged women [1, 2]. It is characterized by a wide array of clinical manifestations, from skin to mucosal lesions, to severe injuries in the central nervous system, kidney and other organs [2]. The presence of high titres of autoantibodies against nuclear components, elevated circulating immune complexes and complement consumption are the common and defining features of the disease [3]. The exact cause of SLE is unclear but emergence of active disease may depend upon environmental factors, such as sunlight, drugs and infections that initiate and/or contribute to the development of the systemic autoimmune disease in genetically susceptible individuals [4]. Multiple susceptibility alleles that interact to cause a profound dysregulation of the immune system have been identified in both humans and animal models of SLE [1].

Pathogenesis

Systemic lupus erythematosus is characterized by abnormal B cell activation and differentiation that lead to the production of antibodies directed against various nuclear autoantigens, particularly against double-stranded (ds-) DNA, histones, nucleosomes and ribonucleoproteins [5, 6]. In these years several investigations have been conducted to understand how these

antigens became immunogens capable of triggering and maintaining a strong and prolonged auto-antibody response in SLE.

These lupus autoantigens are highly diverse in terms of structure and location in control cells but become clustered at the membrane of cells undergoing apoptosis [7]. It has been suggested that either impaired immune cell-apoptosis or defects in the clearance of apoptotic cells are major events in the breakdown of tolerance and in the induction of SLE [8-10]. Apoptosis is an active, programmed and regulated cell-death process which occurs under both physiological and pathological conditions in all tissues. It consists of an enzymatic chain reaction that leads to the activation of several intracellular proteases and DNase, which finally degrade intracellular material in an orderly way. The membrane of apoptotic cells shows characteristic and important changes in the arrangement of phospholipids and sugars and during this process it remains intact, thereby preventing the release of intracellular components [11, 12]. These morphological and biochemical changes are extremely important for clearance of dying cells to the extent that if they are not cleared on time by the phagocytic system, they lose their membrane integrity and become secondarily necrotic, thereby releasing large amounts of modified nuclear and cytoplasmic materials. These modified nuclear autoantigens are thus exposed to the immune system in a "danger" way and recognized as non-self antigens. In this case, the activation of antigen presenting cells, T cell-mediated autoimmune response and the formation of pathogenic immune complexes may occur [10, 13, 14].

The evidence for the relation between impaired apoptosis and SLE came from mouse models bearing defects in proteins involved in apoptosis [15]. Mice lacking proteins such as Fas or Fas-ligand [16, 17], mice heterozygous for *phosphatase-10* with an impaired Fas response phenotype [18], mice overexpressing Bcl2, an inhibitor of apoptosis [19], or mice lacking Bim, a Bcl-2 inhibitor [20, 21], or mice with increased B cell survival through overexpression of B lymphocyte stimulators (BLys) [22] or loss of TACI, a BLys inhibitor [23, 24], all developed auto-antibodies and immune-complex deposits in the kidney. In human SLE the evidence for disturbed apoptosis is less evident; however, a polymorphism in *programmed cell death 1* gene, located in an SLE susceptibility locus, was found in a Nordic multi-case family of SLE patients [25].

In addition to an aberrant apoptosis, it was reported that the clearance of apoptotic material is impaired in both lupus mice and patients with SLE. Mice deficient for DNase1 and C1q [26], C4 [27], serum amyloid proteins [28], IgM [29, 30] and c-Mer

[31, 32], molecules required for proper opsonization and clearance of apoptotic cells, develop autoimmune disease with characteristics of SLE, including autoantibodies and renal inflammatory disease. Moreover, there is a strong association between deficiencies of the complement components involved in the classical pathway (C1q, C1s, C1r, C4 and C2) and SLE in humans [33-35].

Once tolerance toward autoantigens is overcome, the response is propagated by activated immune cells (T, B and dendritic cells) and soluble mediators (pro-inflammatory cytokines, chemokines). It is well established that both lupus T and B cells have a series of intrinsic abnormalities that eventually result in the expansion of autoreactive clones [36]. In SLE, T cells display increased spontaneous apoptosis and decreased activation-induced cell death [36]. B cell defects include abnormal expression or function of key signalling molecules, dysregulation of cytokines with key B cell effects and perturbations in B cell development subsets [37].

Lupus nephritis

Once B cell tolerance is broken and auto-antibodies are formed some, but not all, patients develop immune deposits within glomeruli, initiating inflammation and disease. Renal involvement in SLE, which affects over half of the SLE population, remains a major cause of morbidity and end-stage renal disease and is associated with a greater than four-fold increase in mortality [38, 39]. While therapies such as corticosteroids, cyclophosphamide and mycophenolate mofetil have improved outcomes, a significant proportion of patients have refractory disease or are unable to tolerate these agents [40]. This organ manifestation has traditionally been characterized by the World Health Organization classification criteria, which focused on histological parameters. This classification system of lupus nephritis has recently been revised by the International Society of Nephrology and the Renal Pathological Society [41, 42]. The organ disease is separated into six different classes from subclinical (class I, mild proteinuria) to end stage disease (class VI). A cardinal feature of lupus nephritis is the deposition of autoantibodies in the kidneys where they initiate the inflammatory response by activating resident tissue cells that secrete cytokines and chemokines [43]. These secreted chemokines recruit dendritic cells, neutrophils, and monocytes, amplifying the inflammatory response. Among self nuclear-autoantibodies, anti-nucleosome [44-46] and anti-DNA antibodies [47, 48] are thought to play a major role in lupus nephritis.

The mechanisms through which autoantibodies mediate renal injury remain controversial and three well-known theories have been claimed to explain the mechanism behind their localization in the kid-

ney. The first is the formation of immune complexes that can deposit in the kidney and initiate an inflammatory cascade, interfere with the normal filtration barrier and cause proteinuria [49-51]. The second theory suggests that autoantibodies deposit in the kidney by reacting with nucleosomes trapped in the glomerular basement membrane (GBM). Interaction with positively charged histone tails exposed at the surface of nucleosomes with negatively charged GBM components such as heparan sulfate or collagen IV has been proposed as the cause of trapping of nucleosomes in the GBM [52-55]. The third mechanism is based on the possibility that autoantibodies cross-react with kidney antigens such as α -actinin [56], laminin [57] or cell membranes [58] or other inherent glomerular constituents [58, 59]. In this context, it has been shown in both *in vivo* [60] and *in vitro* [61-63] studies that the process of anti-DNA binding to resident renal cells is associated with alteration of cellular function and induction of the inflammatory cascade [47]. Administration of monoclonal anti-DNA antibodies derived from lupus prone mice to normal mice resulted in their penetration into resident renal cells at the nuclear level, triggering renal abnormalities such as glomerular hypercellularity and proteinuria [60]. Polyclonal anti-DNA antibodies isolated from serum of patients with biopsy-proven lupus nephritis were able to bind and to be internalized by cultured mesangial and renal tubular epithelial cells causing decreased cell viability and increased synthesis of proinflammatory cytokines [61, 64].

It is possible that all three above mechanisms contribute to the development of lupus nephritis.

Toll-like receptors

The immune system consists of two interlocking parts: an evolutionary ancient and relatively non-specific innate immune system and a more sophisticated and recently evolved antigen-specific adaptive immune system. For many years, investigations into the pathogenesis of SLE focused on the role of the B and T cells of the adaptive system. In recent years it has become increasingly well accepted that the innate immune system plays an important role in triggering an adaptive autoimmune response in SLE. Apoptotic blebs and immune complexes containing RNA and DNA are endogenous ligands for Toll-like receptors (TLRs) which can provide costimulatory signals for autoreactive B and dendritic cells, thereby converting potential autoreactivity into systemic autoimmune disease.

TLRs are an evolutionarily conserved family of type I transmembrane receptors that play an important role in innate immunity [65]. Individual TLRs recognized distinct pathogen-associated molecular patterns (PAMPs) that have been evolu-

tionarily conserved in a specific class of microbes. To date, 10 human TLRs have been identified. TLR1, TLR2, TLR4, TLR5, and TLR6 are expressed on the cell surface, where they serve to recognize bacterial products such as triacylated lipopeptides (TLR1/2 heterodimer) [66], diacylated lipopeptides (TLR2/6 heterodimer) [67, 68], lipopolysaccharide (TLR4) [69, 70], and flagellin (TLR5) [71]. TLR3, TLR7, TLR8, and TLR9 are localized to intracellular compartments and serve to recognize viral products such as dsRNA (TLR3) [72], ssRNA (TLR7/8) [73, 74], and unmethylated CpG DNA from bacteria and viruses (TLR9) [75]. Upon ligand binding, TLRs trigger a signal transduction cascade beginning with the recruitment of MyD88, IRAK, and TRAF6, which activate downstream mediators such as mitogen-activated protein kinase and NF- κ B. Finally, the activation of transcription factors leads to the production of chemokines and cytokines that amplify the immune response [65]. TLRs 1, 2, 4, 5 and 6 specialize in the recognition of products that are unique to bacteria and not made by the host. Their detection therefore affords a straightforward self/non-self discrimination. TLRs 3, 7, 8 and 9, in contrast, specialize in viral detection and recognize nucleic acids, which are not unique to the microbial world. In this case, self/non-self discrimination is mediated not so much by the molecular nature of the ligands but rather by their accessibility to the TLRs. These TLRs are localized in endosomal vesicles not accessible for self nucleic acids while reachable by pathogens within a circumstance which allows TLRs to detect them. An additional level of specificity seems to be provided by the fact that vertebrate nucleic acids have several modifications that functionally reduce the probability of activating TLRs. Modified nucleosides that are commonly present in vertebrate RNA reduce the activation of TLR3, TLR7 and TLR8, and the methylation of CpG dinucleotides in vertebrate DNA prevents it from having the same immune stimulatory activity as bacterial DNA [65].

These barriers, however, are not infallible and can be breached [76]. Indeed recent *in vitro* experiments have demonstrated that autoimmune complexes containing self RNA and DNA can bind endosomally localized TLRs7 and 9 in B lymphocytes and in plasmacytoid dendritic cells (pDC) [77-84]. Ledbetter *et al.* [80] presented data showing a B cell receptor (BCR)-TLR9 dual engagement via DNA-containing immune complexes. In this system the BCR first recognized the Fc-region of the autoantibody and triggered endocytosis of the immune complexes into endosomes where TLR9 is localized. B cells are thus stimulated to increase their production of immunoglobulins, cytokines and expression of co-stimulatory molecules [80]. Elegant studies have shown that immune complexes associated

with self DNA and RNA can also directly activate pDC to produce interferon- α (IFN- α) [79, 81, 82], a cytokine which has a direct role in lupus pathology [85, 86]. The recognition by TLRs on pDC is facilitated by Fc γ RII receptor allowing efficient uptake of self-nucleic acid into the TLR-containing endosomal compartment [83]. A very recent study suggests that the mechanism by which immune complexes containing dsDNA activate the TLR9-dependent response implies the binding of High-mobility-group-box 1 protein (HMGB1) to extracellular DNA. The resultant complex stimulates pDC cytokine production through a TLR9-MyD88 pathway involving the multivalent receptor Receptor-of-Advanced-Glycation-End [84]. Thus, self antigens that are endogenous TLR ligands accumulate in SLE, providing a costimulatory signal eventually contributing to breaking peripheral tolerance.

Role of TLRs in murine models of lupus nephritis

Despite there being no single animal model that reproduces the various abnormalities of human SLE disease, mouse models have provided valuable insights into the pathogenesis of lupus. These models can be divided into three groups depending on how they were generated: spontaneous, congenic and engineered mouse models of lupus [87]. Some of the best characterized spontaneous murine lupus models include the hybrids of New Zealand black and New Zealand white F1 (NZB \times NZW) mice and the related inbred NZM2410 strains [88, 89], the MRL^{*lpr/lpr*} mice which carry the *lpr* mutation of Fas on the lupus-prone MRL background [90], and BXSB/*Yaa* mice bearing the *Y-linked autoimmune disease accelerator (Yaa)* gene on the lupus prone BXSB background [91]. Linkage analyses in these spontaneous mouse strains have identified numerous lupus susceptibility loci (such as *Sle1*, *Sle2*, *Sle4* and *Sle3/5*) that are associated with different disease phenotypes [92-94]. By congenic dissection strategy, each of these loci has been individually introgressed onto the genome of lupus-resistant strains, such as C57BL/6 (B6), thus allowing one to study phenotypes contributed by each locus separately [87]. Engineered mouse models of lupus, obtained by single-gene knockout technology, represent a very powerful tool for studying the roles of individual genes in the development of autoimmunity [87]. A lupus-like syndrome can also be reproduced in mice by deleting a single protein belonging to different pathways that regulate the immune response. All three types of mouse models have been used for studying the role of TLRs.

TLR7

Strong evidence supports a crucial role of TLR7 in contributing to the development of lupus glomerulonephritis. These studies involve either

over-expression or deletion of TLR7 gene and both the activation and inhibition of TLR7 signalling in mouse models of SLE (Table I).

The association of increased expression of TLR7 with lupus nephritis arises from studies in mice carrying the lupus *Yaa* mutation. *Yaa* mutation is an *X-to-Y* translocation that results in a duplication of about 16 genes of the X chromosome onto the Y chromosome and in twofold gene over-expression. Within these genes TLR7 has been identified as a major candidate for causation of the autoimmune phenotype.

The addition of the *Yaa* mutation on congenic B6 strain mice carrying the autoimmune susceptibility locus *Sle1* (B6.*Sle1.Yaa*) causes the development of fatal lupus with numerous immunological aberrations and severe kidney pathology [95]. The inclusion of a single TLR7 deletion by introgressing a female TLR7-deficient allele onto male B6.*Sle1.Yaa* mice eliminates all the severe disease phenotypes associated with *Yaa* [96].

Yaa locus produces a striking acceleration of autoimmunity also when bred to other models of lupus such as the Fc γ R11B-deficient mouse, in which the loss of the IgG inhibitory Fc γ receptor R11B molecule results in the accumulation of pathogenic autoantibodies in the kidney with development of glomerulonephritis. The addition of the *Yaa* allele to these mice (Fc γ R11B^{-/-}*Yaa*) exacerbates renal disease and accelerates mortality [97]. Lowering *TLR7* gene dosage by breeding Fc γ R11B^{-/-}*Yaa* male mice with female mice bearing the *TLR7*-deficient allele results in increased survival and decreased renal disease. Indeed, Fc γ R11B^{-/-}*Yaa* mice deficient for *TLR7* died of a lupus-like syndrome at a later stage and at a lesser extent than did Fc γ R11B^{-/-}*Yaa* [98]. In addition, evidence is available that spontaneous autoimmunity and glomerulonephritis could be induced by *TLR7* overexpression in a dose-dependent manner in mouse strains that lack any genetic lesions, underscoring the fact that dysregulation of TLR7 on its own can break tolerance [98].

A few studies have also examined the effects of TLR7 activation in mouse models of autoimmunity. In lupus-prone MRL^{*lpr/lpr*} mice, treatment with the TLR7 agonist imiquimod given in young mice (8 weeks of age) does not accelerate the onset of lupus nephritis, but it aggravates existing disease in older mice (16 weeks of age) as documented by an increase in proteinuria and in the activity and chronicity scores of lupus nephritis [99, 100].

The role of TLR7 dysfunction in the spontaneous onset of disease and in the inflammatory renal pathology has also been investigated in TLR7 gene deletion and signalling-inhibition studies. Knocking out *TLR7* gene expression, by backcrossing *TLR7*^{-/-} mice to lupus prone MRL^{*lpr/lpr*} mice, results in ame-

Table 1. Studies on the role of TLR7 in mouse models of lupus

Gene overexpression	Strategy	Background	Effect on lupus nephritis	Ref.
B6. <i>Sle1.Yaa</i>	Introgressing <i>Yaa</i> mutation that confers double gene expression	B6 mice carrying <i>Sle1</i> susceptibility locus	Worsening	95
<i>FcγRIIB.Yaa</i>	Introgressing <i>Yaa</i> mutation that confers double gene expression	B6 mice with <i>FcγRIIB</i> deficiency	Worsening	97
B6.TLR7 transgenic	TLR7 transgenic (4-32 fold gene overexpression)	Non lupus prone B6 mice	Development and dose-dependent increase in pathology	98
Gene lowering in lupus mice bearing <i>Yaa</i> mutation				
B6. <i>Sle1.Yaa.TLR7^{-/-}</i>	Introgressing a female TLR7 deficient allele (TLR7 expression is limited to the single <i>Yaa</i> copy)	B6 mice carrying <i>Sle1</i> susceptibility locus and <i>Yaa</i> mutation	Amelioration	96
<i>FcγRIIB.Yaa.TLR7^{-/-}</i>	Introgressing a female TLR7 deficient allele (TLR7 expression is limited to the single <i>Yaa</i> copy)	B6 mice bearing <i>Yaa</i> mutation and <i>FcγRIIB</i> deficiency	Amelioration	98
Activation of TLR7 signalling by agonists				
	Imiquimoid administration to 8 week-old lupus mice (2 week treatment)	MRL ^{<i>lpr/lpr</i>}	Unchanged	99
	Imiquimoid administration to 16 week-old lupus mice (2 week treatment)	MRL ^{<i>lpr/lpr</i>}	Worsening	100
Gene deletion				
MRL ^{<i>lpr/lpr</i>} TLR7 ^{<i>-/-</i>}	Generation of TLR7-deficient MRL ^{<i>lpr/lpr</i>} mice	MRL ^{<i>lpr/lpr</i>}	Amelioration	101
Inhibition of TLRs signalling by antagonists				
	Synthetic ODN that block TLR7	MRL ^{<i>lpr/lpr</i>}	Amelioration	102
	Synthetic ODN that block TLR7/TLR9	MRL ^{<i>lpr/lpr</i>}	Amelioration	102
	Synthetic ODN that block TLR7/TLR9	NZB×NZW	Amelioration	103

lioration of lupus nephritis as documented by the reduction in the composite score of renal disease in TLR7 deficient mice as compared with wild type MRL^{lpr/lpr} mice [101]. Pharmacological inhibition of TLR7 attenuates glomerulonephritis in lupus mice. The administration of synthetic oligodeoxynucleotides (ODN), which specifically block signalling via TLR7 or via TLR7/TLR9 to MRL^{lpr/lpr} mice, is associated with significant reduction of the activity and chronicity indices for lupus nephritis, reductions of glomerular IgG and complement factor C3c deposition, and decreased intra-renal inflammation [102]. Similarly, simultaneous blocking of TLR7 and TLR9 in NZBxNZW mice leads to a reduction in the kidney damage and a significant reduction in proteinuria and mortality [103]. Altogether these findings clearly and extensively demonstrate that TLR7 is involved in the pathogenesis of lupus nephritis.

TLR9

At variance with TLR7, experimental studies, including TLR9 gene deletion and activation/ inhibition of TLR9 signalling in lupus mice, show controversial results (Table II). TLR9 knock-out studies in different murine models of lupus have documented that TLR9 deficiency resulted either in exacerbation or in amelioration of the SLE autoimmune disease. The first report on the effect of TLR9 deficiency was performed in the lupus prone MRL^{lpr/lpr} mice. In this study knocking out the *TLR9* gene resulted in loss of anti-DNA (anti-dsDNA and anti-chromatin) autoantibodies but there was no effect on the development of clinical autoimmune disease or nephritis [104]. In this study, however, confounding results may arise from the fact that mice of TLR9^{-/-} genotype were backcrossed only once or twice against mice of the MRL background, which likely allowed protective genes associated with non-lupus prone background to segregate with TLR9 deficiency [104]. Later the same authors examined sufficiently backcrossed TLR9 deficient MRL^{lpr/lpr} mice and observed the development of more severe clinical disease, confirming other reports on disease exacerbation in MRL^{lpr/lpr} mice lacking TLR9 [101]. In congenic B6^{lpr/lpr} mice that develop a lupus-like autoimmune disease characterized by massive lymphoproliferation, anti-DNA autoantibodies, immune complex deposition and glomerulonephritis, deficiency of TLR9 leads to increase in proteinuria and mesangial proliferation [105]. In another study, TLR9 deficient animals of both the MRL and the MRL^{lpr/lpr} backgrounds develop more severe lupus as judged by hypergammaglobulinaemia, lymphadenopathy, inflammatory infiltrates in kidney, proteinuria and mortality in comparison with their TLR9-sufficient littermates [106]. In these mice anti-nucleosome

antibody response is reduced while anti-dsDNA antibody is actually slightly increased.

Experiments on the autoimmune-prone mice Plcγ3A^{li5}, a lupus model in which a single point mutation in the phospholipase Cγ2 causes severe autoimmunity, confirm that the absence of TLR9 does not confer protection from glomerulonephritis. Indeed, the histopathological score of kidney disease activity is increased in Plcγ2B^{li5}+TLR9-deficient mice as compared to TLR9 sufficient littermates [107].

In contrast with the above studies, another report showed that TLR9-deficient 56R⁺FcγRIIB^{-/-} mice (a mouse model in which a transgene for the expression of anti-nucleosome VDJ heavy chain – termed 56R – is introduced in FcγRIIB^{-/-} mice to drive the emergence of anti-DNA-B cells in the periphery) do not develop kidney pathology and survive beyond 8 months of age in contrast with the premature mortality observed in TLR9 sufficient lupus mice [108]. However, the restricted autoantibody response in these triple mutant mice may alter the analysis of the influence of TLR9 on pathology in the FcγRIIB^{-/-} lupus model.

At variance with studies in TLR9 deficiency in lupus models, studies employing the use of synthetic ODN that either activated or blocked TLR9 signalling provide evidence for a pathogenetic, not a protective, role of TLR9 in lupus disease.

A short administration of TLR9 activating-CpG-DNA in 8-week old MRL^{lpr/lpr} mice (at early stage of the disease) triggers the early onset of lupus nephritis as defined by diffuse proliferative glomerulonephritis, glomerular IgG and C3 deposition and overt proteinuria, a process that could be prevented by co-injection of inhibitory DNA [99]. Accordingly, the administration of TLR9-activating CpG-ODN in MRL^{lpr/lpr} mice induces a more severe proteinuria associated with aggravation of glomerulonephritis, tubulointerstitial damage and fibrosis, an effect also mediated by direct binding of injected CpG-ODN to inflammatory cells in the kidneys [109]. Treatment of NZBxNZW lupus mice with CpG-ODN during the pre-active stage of the disease has an accelerating effect on lupus nephritis [110].

Similar results have been obtained in studies examining the effect of suppressive ODN on the spontaneous development of kidney disease in lupus prone mice.

A synthetic G-rich DNA, that blocks CpG-DNA effects, is administered to MRL^{lpr/lpr} mice from weeks 11 to 24 of age. At the end of the study, treated MRL^{lpr/lpr} mice show decreased lymphoproliferation, reduction in kidney inflammatory cell infiltration and decreased proteinuria compared with lupus mice treated with saline [111, 112], confirming a previous report in NZBxNZW lupus mice [113] in which treatment with suppressive ODN, even when started after

Table II. Studies on the role of TLR9 in mouse models of lupus

Gene deletion	Strategy	Background	Effect on lupus nephritis	Ref.
MRL ^{lpr/lpr} TLR9 ^{-/-}	Generation of TLR9 deficient MRL ^{lpr/lpr} mice (2 nd and 3 rd generation)	MRL ^{lpr/lpr}	Unchanged	104
MRL ^{lpr/lpr} TLR9 ^{-/-}	Generation of TLR9 deficient MRL ^{lpr/lpr} (8 th generation)	MRL ^{lpr/lpr}	Worsening	101
B6 ^{lpr/lpr} TLR9 ^{-/-}	Generation of TLR9 deficient B6 ^{lpr/lpr} mice	B6 mice carrying <i>lpr</i> mutation	Worsening	106
MRL TLR9 ^{-/-}	Generation of TLR9 deficient-MRL mice	MRL	Worsening	106
MRL ^{lpr/lpr} TLR9 ^{-/-}	Generation of TLR9 deficient MRL ^{lpr/lpr} mice	MRL ^{lpr/lpr}	Worsening	106
Plcγ2B ^{is/+} TLR9 ^{-/-}	Generation of TLR9 deficient Plcγ2B ^{is} mice	Al15 mice bearing a gain-of-function mutation in the phospholipase Cγ2 (Plcγ2) gene	Worsening	107
56R+FcγRIIIB ^{-/-} TLR9 ^{-/-}	Generation of TLR9 deficient 56R+FcγRIIIB ^{-/-} mice	B6 mice with FcγRIIIB deficiency and transgenic for 56R VDJ heavy-chain	Amelioration	108
Activation of TLR9 signalling by agonists				
	activating CpG-DNA administration to 8 week-old lupus mice (2 week treatment)	MRL ^{lpr/lpr}	Worsening	99
	activating CpG-ODN administration to 16 week-old lupus mice (2 week treatment)	MRL ^{lpr/lpr}	Worsening	109
	activating CpG-ODN administration to 2.75 week-old lupus mice (3.5 mo treatment)	NZB×NZW	Worsening	110
Activation of TLR9 signalling by antagonists				
	blocking DNA administration to 11 week old lupus mice (2 week treatment)	MRL ^{lpr/lpr}	Amelioration	111
	suppressive ODN administration either to 6 week- or to 7 month-old lupus mice	NZB×NZW	Amelioration	113

the onset of renal disease, significantly slowed the onset of glomerulonephritis and prolonged survival.

Role of local expression of TLR9 on renal injury in systemic lupus erythematosus

Lesions of lupus nephritis involve the renal glomerulus and tubulointerstitium. Tubulointerstitial changes are prominent and contribute substantially to the unfavourable long-term prognosis [114]. As mentioned above, proximal tubular epithelial cells exposed to anti-dsDNA produce cytokines that promote local recruitment of inflammatory cells [61]. We have recently studied whether TLR9 could be involved in the development of tubular damage and interstitial inflammation in lupus nephritis and found that TLR9 activation, at the tubular cell level, has a pathogenetic role in experimental and human lupus nephritis [115]. Strong TLR9 expression is detected in kidney from NZB×NZW lupus-prone mice at late stage of the disease and its staining is localized in proximal tubules, besides in inflamma-

tory cell infiltrate (Figure 1A and Figure 1B). Up-regulation of tubular TLR9 expression parallels the development of proteinuria (Figure 1C) and correlates with tubulointerstitial damage [115]. The finding that increased tubular TLR9 generation is associated with proteinuria provided the rationale for studying the effect on TLR9 expression of seliciclib (an inhibitor of cyclin-dependent kinases 2, 7, and 9), which effectively reduces proteinuria and ameliorates renal injury in this model [115, 116]. In kidneys of seliciclib-treated NZB×NZW mice, the intensity of tubular TLR9 protein staining is markedly reduced compared with that in untreated lupus mice of the same age (Figure 1D). To assess the relevance of animal data to the pathophysiology of interstitial lesions in human lupus, the expression of TLR9 in renal biopsy specimens from patients with lupus nephritis has been evaluated. Intense and diffuse staining for TLR9 is present in proximal tubules on renal biopsy sections from patients with class IV lupus nephritis (Figures 2B-C) and TLR9 protein expression corre-

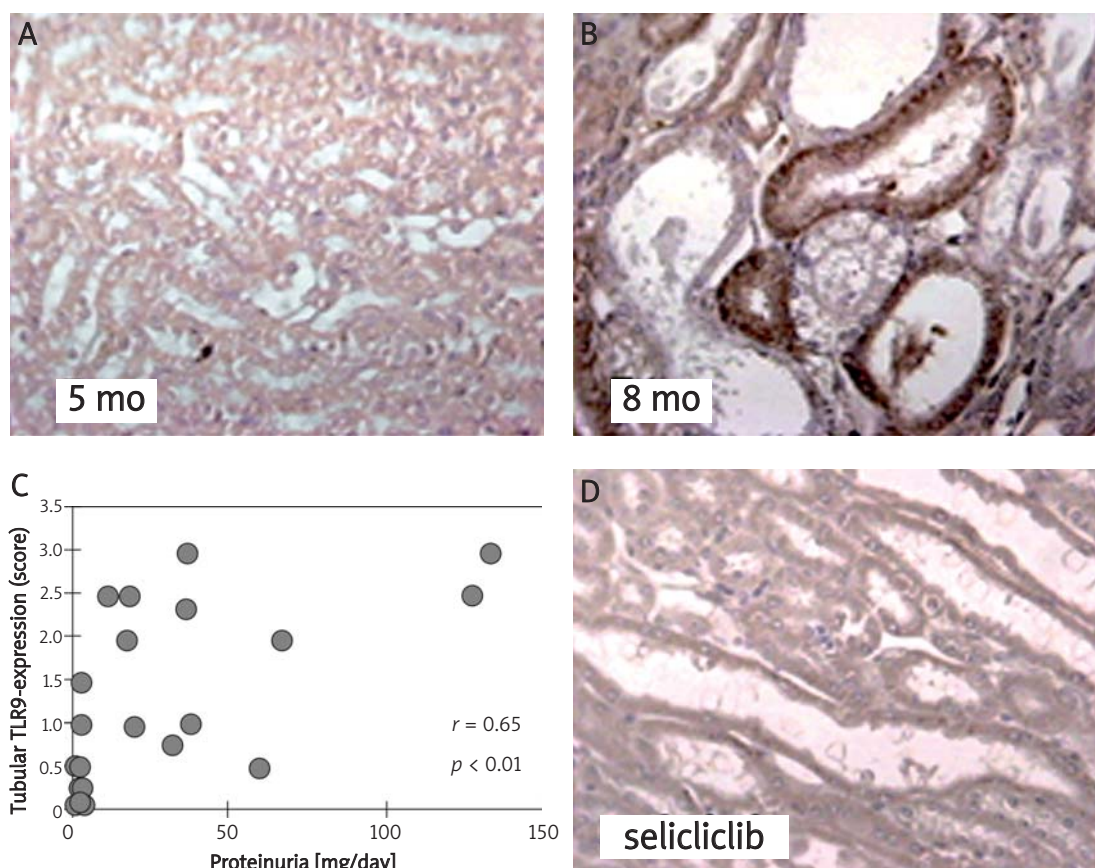


Figure 1. Renal TLR9 expression in NZB×NZW mice. Representative photomicrographs of TLR9 protein localization in kidney of NZB×NZW mice (A, B, D). TLR9 protein was almost undetectable in kidney from NZB×NZW mice at 5 months of age (A) whereas strong TLR9 staining was present in tubules of renal tissue from 8-month old NZB×NZW mice (B). Tubular TLR9 protein expression significantly correlated with proteinuria (C). TLR9 staining was dramatically decreased in seliciclib-treated NZB×NZW mice (D). Original magnification 250×

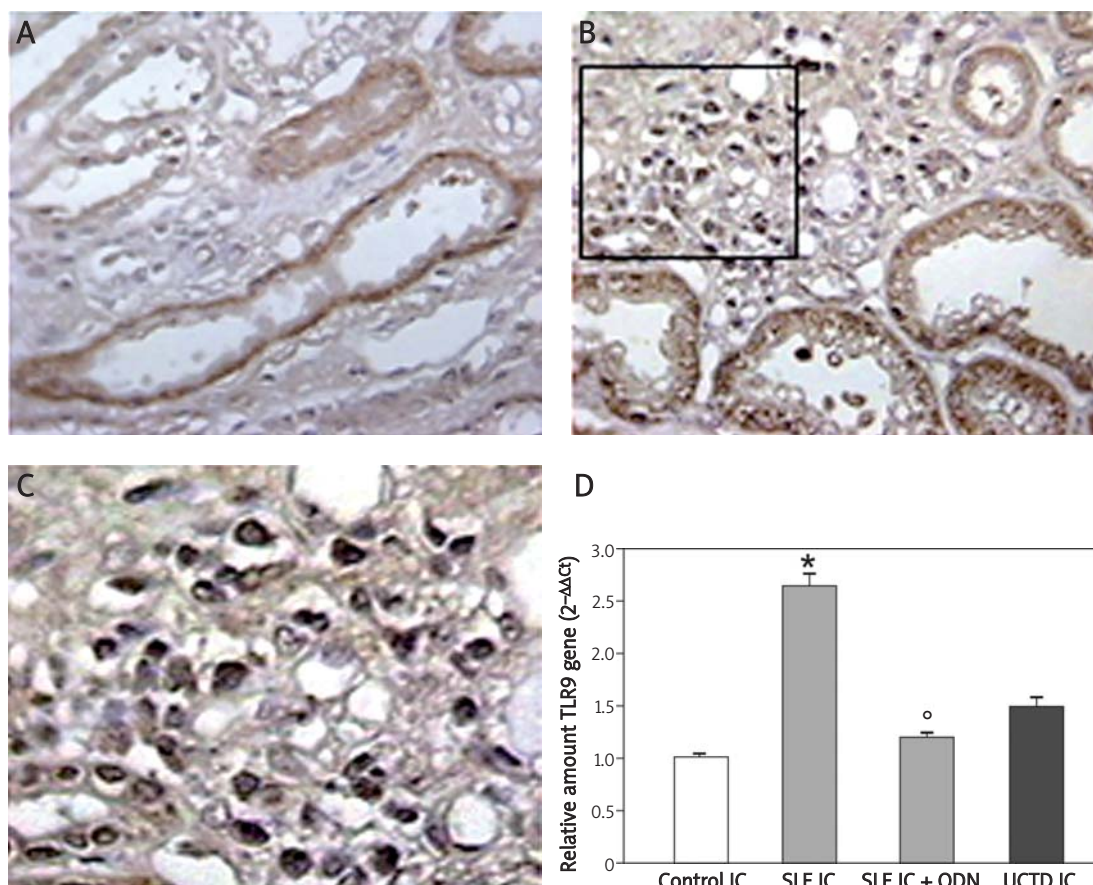


Figure 2. TLR9 expression in renal biopsy sections from lupus patients and in cultured tubular cells. Photomicrographs of TLR9 protein immunostaining on human renal biopsy from patients with class IV lupus nephritis (A-C). TLR9 positive tubules (A) and both tubules and interstitial inflammatory cells positive for TLR9 (B) were found in kidney of patients. C – boxed area in B at higher magnification showing positivity for TLR9 in cell infiltrates. D – Immune complexes purified from SLE sera stimulate TLR9 expression in cultured human proximal tubular epithelial cells. Cultured human proximal tubular epithelial cells were exposed to immune complexes purified from sera of healthy subjects (control IC), of SLE patients – either alone (SLE IC) or in the presence of TLR9 antagonist ODN (SLE IC + ODN) – or of undifferentiated-connective-tissue-disease patients (UCTD IC)
^{*}*p* < 0.01 vs. UCTD IC, ^o*p* < 0.05 vs. SLE IC

lates positively with tubulointerstitial injury, extending the significance of our observation to humans [115]. Furthermore, we found that anti-dsDNA-positive sera or serum-purified immune complexes from SLE patients induce TLR9 expression in cultured proximal tubular epithelial cells, a process that is inhibited by the addition of CpG DNA antagonist-ODNs (Figure 2D) [115]. Altogether these results led us to hypothesize that in immune-mediated glomerular diseases, which include lupus nephritis, alteration of size-selective properties of the glomerular capillary wall is associated with abnormal filtration of plasma proteins, which conceivably include immune complexes containing DNA. Ultrafiltered immune complexes then activate proximal tubular cells through TLR9, leading to cytokine and chemokine production and triggering tubulointerstitial inflammation and damage in lupus nephritis (Figure 3).

TLRs and human systemic lupus erythematosus

Data from human studies support an important role for TLRs in SLE. Increased expression of TLR7 and TLR9 was found in peripheral blood mononuclear cells from human lupus patients [117, 118]. A polymorphism in the IRF-5 gene was found to be a genetic risk factor for SLE development [119, 120]. IRF-5 is a signal transduction molecule downstream of TLRs. Moreover, antimalarials, such as hydroxychloroquine, have been shown to interfere with acidification of lysosomal compartments and inhibiting signalling through TLR7 and 9 [80]. This might be the molecular explanation for the observation of the effectiveness of hydroxychloroquine as SLE medication [121]. No genetic data are available to indicate that polymorphisms which cause inhibition of TLR9 function might be associated with protection from SLE. In contrast, a Japanese group recently

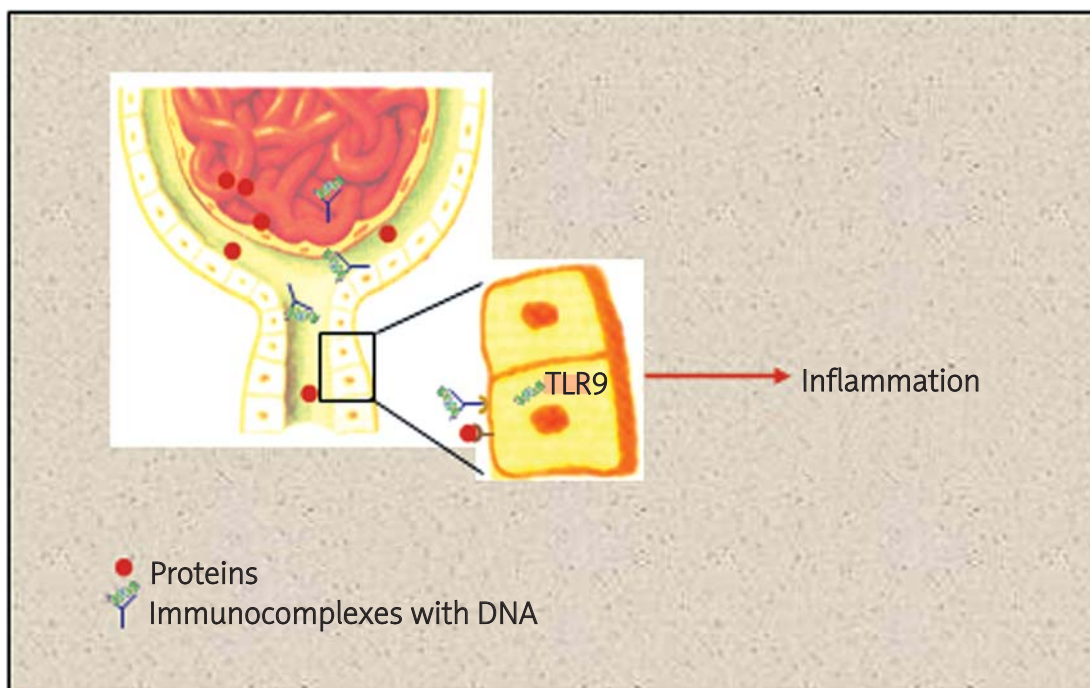


Figure 3. A possible pathogenetic role of tubular TLR9 expression in the progression of lupus nephritis. In immune-mediated glomerular diseases alteration of size-selective properties of the glomerular capillary wall is associated with abnormal filtration of plasma proteins. We hypothesize that in lupus nephritis ultrafiltered immune complexes including immune complexes containing DNA activate proximal tubular cells through TLR9 leading to cytokine and chemokine production, triggering tubulointerstitial inflammation and damage in lupus nephritis

described two alleles which down-regulated TLR9 expression in a reporter assay, but these are associated with increased SLE susceptibility [122].

Conclusions

The available data point to a prominent role for TLR7 in the pathogenesis of SLE and to a more complex role of TLR9. While results from TLR9 knock-out mouse models concur to indicate a protective role of TLR9 in the development of lupus nephritis, studies employing agents which interfere with TLR9 signalling in non-TLR9 deficient mice suggest a more pathogenetic role for TLR9.

One possibility for reconciling the contrasting observations is that counter-regulatory mechanisms, which normally occur to terminate the TLR9-induced immune response, may have an important protective role during the early phase of SLE autoimmunity [123-126], while in a condition in which the system is chronically activated, TLR9 signalling may concur to the pathogenesis of the disease.

Data obtained in studies of sepsis syndrome are a relevant example [127]. A direct link between lipopolysaccharide (LPS) poisoning and sepsis acting on TLR4 was derived from the observation that

TLR4 activation by LPS caused fever, shock and death, whereas Sultzzer's mutant mice, bearing non-functional TLR4, were protected against shock when given LPS [128]. However, when these mice were infected with gram-negative bacteria, which release LPS and pathogen-associated molecules, the manifestations of sepsis worsened and the rate of death increased. These paradoxical results can be explained if TLR4 either protects the host by sequestering infectious organisms or it causes manifestation of sepsis when the system is maximally activated and protection is overwhelmed.

On the basis of the reviewed evidence it is possible that chronic stimulation of TLR9 has a role in the perpetuation of the SLE autoimmune process that follows autoantibody formation, and when lupus nephritis and proteinuria ensue, tubular TLR9 triggers tubulointerstitial damage, worsening the nephritis toward end-organ disease.

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