TARGETING KIDNEY INFLAMMATION IN A MURINE MODEL OF ALPORT SYNDROME

by

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A thesis submitted in conformity with the requirements for the degree of Master of Science
Department of Physiology
University of Toronto

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Abstract

Alport Syndrome is a rare inherited kidney disease that is due to genetic mutations of type IV collagen. The mutations interfere with normal assembly of the glomerular basement membrane and this structural abnormality leads to a progressive rise in urinary protein excretion and eventually to end stage renal failure. Preliminary studies in our laboratory suggest that inflammation may play an early role in the pathogenesis of glomerular injury.

Thus, the relationship between inflammatory pathways and AS disease progression was examined in this study by inhibiting NF-κB activation in glomerular cells using MS417. I hypothesized that the early activation of NF-κB plays a key role in the initiation and progression of kidney injury in AS and the blockade of NF-κB-mediated gene expression can attenuate kidney injury in experimental AS. The findings from the analysis of gene expression in a murine model of AS identified NF-κB as an important target for treatment.
Acknowledgements

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<th>Description</th>
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<tbody>
<tr>
<td>αSMA</td>
<td>Alpha-smooth muscle actin</td>
</tr>
<tr>
<td>ACE</td>
<td>Angiotensin-converting enzyme</td>
</tr>
<tr>
<td>ADAS</td>
<td>Autosomal dominant Alport Syndrome</td>
</tr>
<tr>
<td>ADM</td>
<td>Adrenomedullin</td>
</tr>
<tr>
<td>AKI</td>
<td>Acute kidney injury</td>
</tr>
<tr>
<td>Ang II</td>
<td>Angiotensin II</td>
</tr>
<tr>
<td>ARAS</td>
<td>Autosomal recessive Alport Syndrome</td>
</tr>
<tr>
<td>ARB</td>
<td>Angiotensin receptor blocker</td>
</tr>
<tr>
<td>AS</td>
<td>Alport Syndrome</td>
</tr>
<tr>
<td>AT1R</td>
<td>Angiotensin II type 1 receptor</td>
</tr>
<tr>
<td>AT2R</td>
<td>Angiotensin II type 2 receptor</td>
</tr>
<tr>
<td>BET</td>
<td>Bromodomain and extra-terminal domain</td>
</tr>
<tr>
<td>BM</td>
<td>Bone marrow</td>
</tr>
<tr>
<td>BMP-7</td>
<td>Bone morphogenic protein-7</td>
</tr>
<tr>
<td>BRD4</td>
<td>Bromodomain-containing protein 4</td>
</tr>
<tr>
<td>BUN</td>
<td>Blood urea nitrogen</td>
</tr>
<tr>
<td>CCP-1</td>
<td>Chemokine receptor 1</td>
</tr>
<tr>
<td>CD45</td>
<td>Cluster of differentiation 45</td>
</tr>
<tr>
<td>CDK9</td>
<td>Cyclin-dependent kinase 9</td>
</tr>
<tr>
<td>CKD</td>
<td>Chronic kidney disease</td>
</tr>
<tr>
<td>COL</td>
<td>Collagen</td>
</tr>
<tr>
<td>CXCL10</td>
<td>C-X-C motif chemokine 10</td>
</tr>
<tr>
<td>CXCR3</td>
<td>C-X-C motif chemokine receptor 3</td>
</tr>
<tr>
<td>CYP1B1</td>
<td>Cytochrome P450 family 1 subfamily B member 1</td>
</tr>
<tr>
<td>DAMP</td>
<td>Damage-associated molecular pattern</td>
</tr>
<tr>
<td>DOX</td>
<td>Doxycycline</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
</tr>
<tr>
<td>EAST</td>
<td>Epilepsy, ataxia, sensorineural deafness, and tubulopathy</td>
</tr>
<tr>
<td>ECM</td>
<td>Extracellular matrix</td>
</tr>
</tbody>
</table>
ESCAPE  Evaluation Study of Congestive Heart Failure and Pulmonary Artery Catheterization Effectiveness
ESRD  End-stage renal disease
ESRF  End-stage renal failure
GAPDH  Glyceraldehyde 3-phosphate dehydrogenase
GBM  Glomerular basement membrane
GFR  Glomerular filtration rate
HBSS  Hank’s balanced salt solution
HIV  Human immunodeficiency virus
HIVAN  HIV-associated nephropathy
HK-2  Human kidney-2
HMG-CoA  3-hydroxy-3-methylglutaryl-CoA
HSPG  Heparan sulfate proteoglycan
IFN-γ  Interferon gamma
IgA  Immunoglobulin A
IkB  Inhibitor kappa B
IKK  IkB kinase
IL  Interleukin
IPA  Ingenuity Pathway Analysis
KCNJ1  Potassium voltage-gated channel subfamily J member 1; renal outer medullary potassium channel
KCNJ16  Potassium voltage-gated channel subfamily J member 16; inward rectifier potassium channel 16
KLK1  Kallikrein 1
KO  Knockout
LCC  Laminin coiled-coil
LG  Laminin globular
LM-521  Laminin-521
LincRNA  Long intergenic non-coding ribonucleic acid
LPS  Lipopolysaccharide
MCP-1  Monocyte chemoattractant protein-1
MMP  Matrix metalloproteinase
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>MSC</td>
<td>Mesenchymal stem cell</td>
</tr>
<tr>
<td>MT</td>
<td>Masson’s trichrome</td>
</tr>
<tr>
<td>MYD88</td>
<td>Myeloid differentiation primary response 88</td>
</tr>
<tr>
<td>NADPH</td>
<td>Nicotinamide adenine dinucleotide phosphate</td>
</tr>
<tr>
<td>NC</td>
<td>Negative control</td>
</tr>
<tr>
<td>NF-κB</td>
<td>Nuclear factor-kappa-light-chain-enhancer of activated B cells</td>
</tr>
<tr>
<td>NLR</td>
<td>Nucleotide-binding oligomerization domain-like receptor</td>
</tr>
<tr>
<td>PAMP</td>
<td>Pattern-associated molecular pattern</td>
</tr>
<tr>
<td>PAS</td>
<td>Periodic acid-Schiff</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate-buffered saline</td>
</tr>
<tr>
<td>PDGF</td>
<td>Platelet-derived growth factor</td>
</tr>
<tr>
<td>PI3K</td>
<td>Phosphoinositide 3-kinase</td>
</tr>
<tr>
<td>PRR</td>
<td>Pattern recognition receptor</td>
</tr>
<tr>
<td>P-TEFb</td>
<td>Positive transcription elongation factor b</td>
</tr>
<tr>
<td>RANTES</td>
<td>Regulated on activation, normal T cell expressed and secreted</td>
</tr>
<tr>
<td>RAS</td>
<td>Renin-angiotensin system</td>
</tr>
<tr>
<td>RC</td>
<td>Renal cortex</td>
</tr>
<tr>
<td>RCC</td>
<td>Renal cell carcinoma</td>
</tr>
<tr>
<td>RHD</td>
<td>Rel homology domain</td>
</tr>
<tr>
<td>RIN</td>
<td>Ribonucleic acid integrity number</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>Reverse transcription polymerase chain reaction</td>
</tr>
<tr>
<td>SFRP1</td>
<td>Secreted frizzled related protein 1</td>
</tr>
<tr>
<td>SM22α</td>
<td>Smooth muscle protein 22-alpha</td>
</tr>
<tr>
<td>TAGLN</td>
<td>Transgelin</td>
</tr>
<tr>
<td>TGF-β</td>
<td>Transforming growth factor beta</td>
</tr>
<tr>
<td>TLR</td>
<td>Toll-like receptor</td>
</tr>
<tr>
<td>TNF</td>
<td>Tumour necrosis factor</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Tumour necrosis factor alpha</td>
</tr>
<tr>
<td>TNFRSF12A</td>
<td>Tumour necrosis factor receptor superfamily member 12A</td>
</tr>
<tr>
<td>TNFSF12</td>
<td>Tumour necrosis factor superfamily member 12</td>
</tr>
<tr>
<td>Acronym</td>
<td>Description</td>
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<tr>
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<tr>
<td>TRIF</td>
<td>Toll-interleukin-1 receptor domain-containing adapter-inducing interferon-beta</td>
</tr>
<tr>
<td>TWEAK</td>
<td>TNF-related weak inducer of apoptosis</td>
</tr>
<tr>
<td>UAE</td>
<td>Urinary albumin excretion</td>
</tr>
<tr>
<td>UUO</td>
<td>Unilateral ureteral obstruction</td>
</tr>
<tr>
<td>Wnt</td>
<td>Wingless-related mouse mammary tumour virus</td>
</tr>
<tr>
<td>WT</td>
<td>Wildtype</td>
</tr>
<tr>
<td>XLAS</td>
<td>X-linked Alport Syndrome</td>
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Chapter 1

Introduction

1.1 Clinical features of Alport Syndrome

Alport Syndrome (AS) is a rare inherited form of chronic kidney disease (CKD) characterized by progressive nephropathy, sensorineural loss, ocular defects, and the development of end stage renal disease (ESRD)\textsuperscript{1-3}. It affects at least one in 50,000 individuals and it is caused by mutations in genes that encode α3-α4-α5(IV) chains of type IV collagen (Col4α3, Col4α4, and Col4α5, respectively), which interfere with the normal assembly of the glomerular basement membrane (GBM)\textsuperscript{2,4-6}. Mutations of the \textit{COL4A5} gene on chromosome Xq22 cause X-linked AS (XLAS), which accounts for approximately 85\% of cases, whereas recessive mutations of \textit{COL4A3} or \textit{COL4A4} genes on chromosome 2q36 constitute the remaining cases (autosomal recessive AS; ARAS)\textsuperscript{7-9}. In rare instances (<1\%), \textit{COL4A3} or \textit{COL4A4} mutations may follow an autosomal dominant pattern (autosomal dominant AS; ADAS)\textsuperscript{10}. Clinically, XLAS and ARAS have similar disease characteristics and prognosis, and are indistinguishable on clinical grounds or standard assessments of renal biopsy tissue, despite the very distinct patterns of inheritance\textsuperscript{6}.

Such genetic mutations in the type IV collagen give rise to microhematuria as the first renal manifestation of AS in childhood, which then gradually progress to proteinuria, hypertension, and eventually to kidney failure in all males with XLAS and in both sexes with ARAS\textsuperscript{4,11}. Males with X-linked inheritance may be affected with an early onset of the disease, generally accompanied by extrarenal features, and develop ESRD before the age of 30, or experience a late onset with renal failure after 30 years of age, commonly accompanied solely by hearing loss\textsuperscript{12,13}. High-tone sensorineural hearing loss occurs in 70\% of affected males and
lenticonus in approximately 30% of males with XLAS by age 40, when renal failure, hearing
loss, and retinopathy are already present\textsuperscript{14}. In contrast, the disease characteristics tend to be
predominantly milder in females with XLAS compared to males as they are heterozygous
carriers of the disease. Generally, XLAS female patients have minimal clinical manifestations
such as asymptomatic hematuria by age of 30-40 years, proteinuria (75%), ESRD (15% by age
of 60), hearing loss (40%), or peripheral retinopathy (40%). Lenticonus may not occur and
central retinopathy is rare\textsuperscript{15}. As well, X chromosome inactivation, the process by which the
unmutated copy of one X chromosome counters the effect of the mutation, contributes to
variability among female carriers of XLAS, lending to mosaic disease symptoms\textsuperscript{16}.

Autosomal recessive AS is suspected where the disease is found in only a single
generation or a consanguineous family, where males and females in a family are affected with
equal frequency and severity\textsuperscript{1,17}. In autosomal recessive AS, patients exhibit an early onset of
renal failure, including persistent microhematuria that progresses to significant proteinuria and
ultimately to ESRD, before 30 years of age in both sexes. Individuals with ARAS typically also
exhibit juvenile onset of hearing loss with no gender differences in the incidence of hearing
deficit. Ocular lesions stemming from ARAS are present in 30-40% of individuals with more
frequent occurrences in affected males than females\textsuperscript{18}. Contrastingly, in individuals with
autosomal dominant AS, ESRD is frequently delayed until later adulthood than in those with
XLAS or ARAS, with late onset of cochlear manifestations and rare occurrence of ocular
complicity\textsuperscript{19,20}.

In addition to nephropathy, cochlear and ocular abnormalities observed in AS result from
an absence of the collagen IV networks in the ear and eye\textsuperscript{14}. Hearing loss in AS is never
congenital with defective adhesion of the organ of Corti to the basilar membrane possibly
underlying the hearing deficit observed in AS patients. Pathognomonic ocular feature of AS is bilateral anterior lenticonus in which the anterior surface of the lens assumes abnormal conical shapes and protrude into the anterior chambers of the eyes, bringing about progressive distortion of the lens accompanied by increasing myopia and astigmatism. Other typical ocular abnormalities include dot-and-fleck retinopathy, consisting of whitish or yellowish flecks or granulations in the retina, corneal disorders, such as recurrent corneal erosion and posterior polymorphous corneal dystrophy, which can cause severe ocular pain.

Atypical clinical features of AS include gastroesophageal leiomyomatosis, a condition characterized by visceral smooth muscle overgrowth leading to benign tumours within the respiratory, gastrointestinal, and female reproductive tracts. Leiomyomas are rarely associated with AS; however, when present, it is pathognomonic for AS and affected patients carry a large deletion in the 5’ ends of the COL4A5 and COL4A6 genes, which is normally expressed in smooth muscle cells of the gastroesophageal tract. The pathogenetic correlation between the deletions of the COL4A5 and COL4A6 genes and the incidence of leiomyomas has not been elucidated. One study by Dahan et al. (1995) suggests that cell proliferation in leiomyomatosis could be due to the misalignment that results from the occurrence of repeated sequences in the large COL4A5 and COL4A6 introns. Arterial abnormalities, such as aneurysms in adolescence, have also been reported in patients with AS, pronouncing the importance of Col4α5 and Col4α6 chains in the maintenance of vascular integrity.

The pathologic lesions for renal biopsies from AS patients are variable when examined by electron microscopy; however, typically renal biopsies exhibit podocyte hypertrophy and stiffness of the capillary wall, periodically associated with the presence of tubular red blood cell casts. Thereafter, gradual glomerular basement membrane thickening and splitting with
progressive enlargement of mesangial stalks can be observed\textsuperscript{20,35}. This development of longitudinal splitting of the lamina densa of GBM bestowing a lamellated appearance is diagnostically relevant to AS\textsuperscript{36}. The GBM lesion can be intermittent, alternating between segments of thick or abnormally thin GBM, which is particularly prevalent in children with AS or XLAS females\textsuperscript{37}. GBM lamellation is also accompanied by fragmentary podocyte foot process effacement, exhibiting progressive podocyte depletion, a common mechanism in glomerular diseases that further drives progression\textsuperscript{38–41}. Furthermore, there is segmental or diffuse glomerulosclerosis, tubular atrophy, and interstitial fibrosis present, along with interstitial infiltration with lymphocytes and macrophages, correlated with progressive renal failure\textsuperscript{42,43}.

1.2 Genotype-phenotype correlation

The correlation between clinical presentations and the underlying mutations that bring about AS has been extensively studied and reviewed in order to not only better understand the mechanisms of disease, but also to potentiate the prognostic value of efficient and noninvasive screening methods, such as genetic testing\textsuperscript{44}. Extensive studies have been performed globally to characterize the natural history of AS and it has been established that there is a strong association between the genetic variants and the renal disease severity\textsuperscript{45–47}. Specifically, patients with large deletions and nonsense mutations demonstrate more severe manifestations of the disease with earlier progression to ESRD compared to those with missense mutations, while those with splice site mutations present with an intermediate disease progression\textsuperscript{48}. Thus, the risk of ESRD by 30 years of age for male XLAS patients with missense mutations, splice site mutations, and large deletions and nonsense mutations are 50%, 70%, and 90%, respectively, with the average age of ESRD being 37, 28, and 25 years, respectively\textsuperscript{12}. Correspondingly, large deletions, splice site, or
truncating mutations confer a significantly higher risk of developing hearing loss and ocular lesions in patients than among those with missense mutations\textsuperscript{12,44,48}. Furthermore, Bekheirnia \textit{et al.} (2010) demonstrated that there was an association between the mutation position and the disease outcome, with an earlier onset of ESRD related to pathogenic mutations at the 5’ end of the gene; the development of hearing impairment and ocular changes showed a similar association with the mutation location. Though the genotype-phenotype correlation has only been established in XLAS males, clinical courses of XLAS and ARAS are similar\textsuperscript{47}. In contrast, no correlation between the degree of the disease progression and the type of underlying mutation could be substantiated in female carriers of XLAS\textsuperscript{15,49}.

Currently several animal models of Alport Syndrome have been developed and published in the literature\textsuperscript{50,51}. Such experimental models have yielded significant insight into the molecular basis of AS as well as the structure and biosynthesis of type IV collagen, thus making animal models invaluable in the study of pathogenesis and treatment of disease\textsuperscript{52}. So far, these models are limited to spontaneous AS in dogs and transgenic models of AS generated in mice\textsuperscript{51}. XLAS models include \textit{COL4A5}-mutant Samoyed dogs and Navasota dogs, with English cocker spaniel modelling ARAS, and Bull terrier for ADAS\textsuperscript{53–57}. Murine models are comprised of \textit{COL4A3} knockouts and tg/tg mice\textsuperscript{58–60}. These animal models offer opportunities for investigation of the molecular pathophysiology of the disease, the roles of type IV collagen chains in the pathobiology, as well as the applicability of the model in the treatment of AS\textsuperscript{51}.

Understanding the source and extent of the genetic variation underlying AS is crucial in the management of the disease as clinical care and treatments rely on prognosticating phenotypes from genetic polymorphisms. Knowledge about the type and location of the mutations adds significant information about the progress of the renal and extrarenal manifestations of the
disease, which could potentially aid in clinical counselling and evaluation of therapeutic approaches.

1.3 Glomerular basement membrane

The glomerular basement membrane (GBM) is a non-cellular meshwork of extracellular matrix (ECM) proteins that plays a central role in the glomerular filtration barrier, separating the vasculature from the urinary space\textsuperscript{61}. It is situated between the interdigitated podocytes (visceral epithelial cells) and fenestrated endothelial cells, formed during glomerulogenesis by the fusion of these two cellular membranes\textsuperscript{62}. It provides not only selective permeability across the glomerular filtration barrier, but also contributes integral structural support for the glomerular capillaries\textsuperscript{62}. Moreover, it imparts morphogenic cues that determine the polarization of subcellular constituents and harbours ligands for cell receptors and transporters on the surface of adjacent endothelial cells, podocytes, and mesangial cells\textsuperscript{62}. As with all basement membranes, the four major components of the GBM are type IV collagen, laminin, nidogens, and heparan sulfate proteoglycans (HSPG), which conjunctively assemble an interwoven meshwork that imparts size and charge-selective properties\textsuperscript{63}. However, the GBM is unusually thicker than most other basement membranes and consists of ECM macromolecule isoforms that vary from other basement membranes (laminin-521, collagen $\alpha_3\alpha_4\alpha_5$(IV), and agrin), presumably due to its unique functional properties in establishing and maintaining the glomerular filtration barrier\textsuperscript{64}.

1.3.1 Laminin

Laminin is a ubiquitous basement membrane component that is secreted as heterotrimers of alpha, beta, and gamma chains\textsuperscript{65}. There are five $\alpha$ chains, four $\beta$ chains, and three $\gamma$ chains that
assemble to form 15 different heterotrimeric macromolecules. Each distinct laminin trimers are named based on the specific $\alpha\beta\gamma$ chain composition; for instance, laminin $\alpha5\beta2\gamma1$ is referred to as laminin-521 (LM-521)\textsuperscript{66}. The laminin heterotrimeric glycoprotein is structurally cross-shaped with the lower long arm of the cruciform assembled by the association of all three $\alpha$, $\beta$, and $\gamma$ chains intertwining with one another via coiled-coil interactions and disulfide bonding, forming laminin coiled-coil (LCC) domains\textsuperscript{67}. The laminin globular (LG) domain is situated at the distal end of the long arm, consisting exclusively of carboxyl-termini of $\alpha$ chains. The LG domains mediate interactions between laminin trimers and neighbouring cells by serving as ligands to cell surface receptors, such as integrins and dystroglycan\textsuperscript{68}. The three other short arms are formed by alternating globular and rod-like domains, including the amino-terminal globular domain, called LN domain, which mediates the trimer-trimer interactions in the extracellular space that bring about laminin polymerization and the initiation of basement membrane assembly\textsuperscript{64,65,69}.

The major laminin trimer found in the mature GBM is LM-521, which is secreted by both podocytes and endothelial cells\textsuperscript{69}. In the early stages of glomerulogenesis, LM-111 and LM-511 are the major laminin components in the nascent GBM; however, as the GBM matures, major transitions in the laminin trimer deposition occurs in which LM-111 is initially replaced by LM-511 and then eventually by LM-521 in the mature glomeruli, the composition of which persists in the GBM throughout life\textsuperscript{70}. The molecular mechanisms responsible for these developmental laminin isoform substitutions have yet to be elucidated, but this composition transition is crucial for glomerulogenesis\textsuperscript{71}. The importance of these laminin transitions is particularly highlighted by the effects of mutations that prevent them from occurring. For example, a null mutation of laminin $\alpha5$ in mice prevents the developmental switch in laminin $\alpha$ chain deposition in which $\alpha5$ replaces $\alpha1$ in the GBM during glomerular morphogenesis\textsuperscript{70}. Thus, this gives rise to an aberrant
GBM and the subsequent failure of glomerular vascularization. The GBM breakdown most likely occurs due to the lack of a sufficient concentration of polymerized laminin trimers, as the laminin network is required to maintain basement membrane integrity. Furthermore, *Lama5*−/− mice were shown to exhibit either renal agenesis in one or both kidneys or if both present, the kidneys were much diminished in size in comparison to those of the littermate controls. The absence of laminin α5 leads to progressively atypical metanephrogenesis and eventually result in embryonic lethality.

### 1.3.2 Type IV collagen

Type IV collagen is the most abundant protein found in the GBM, composed of six distinct alpha chains 1 through 6, designated Col4α1-Col4α6. The encoded proteins, α1(IV)-α6(IV) assemble to form three different heterotrimers referred to as protomers: α1α1α2; α3α4α5; and α5α5α6. Collagen α1α1α2(IV) is synthesized by endothelial cells, mesangial cells, as well as podocytes of immature glomeruli, while collagen α3α4α5(IV) originates solely from podocytes. Like all collagen chains, type IV collagen chains are characterized by a long collagenous domain of Gly-X-Y amino acid triplet repeats, with glycine being the only residue small enough to fit inside the collagen helix. However, unlike other collagen types, type IV chains consist of multiple noncollagenous interruptions, which impart flexibility to the protomers and the overall interwoven network they form in the GBM. The small noncollagenous amino-terminal domain of all collagen IV α chains is termed 7S and the larger carboxyl-terminal noncollagenous domain is called NC1, with both domains involved in promoter interactions and promoting collagen IV network assembly. The compositions of the protomers are
predetermined by the sequence and structure embedded in the NC1, ensuring that only the three types of heterotrimers form\textsuperscript{76}.

During embryonic development, the $\alpha_1\alpha_1\alpha_2$(IV) network is the most abundant collagen IV network in the GBM, and it is gradually replaced by the $\alpha_3\alpha_4\alpha_5$(IV) heterotrimer in the glomerulus, cochlea, and eye\textsuperscript{77}. This transition in the composition of collagen IV chains occurs concurrently with the transition of trimeric laminin chains, which switches from laminin-511 to laminin-521, as well as the formation of the capillary loops within the maturing glomeruli\textsuperscript{76}. The molecular mechanisms for the laminin and collagen IV isoform substitutions in the GBM are unknown. However, the mature $\alpha_3\alpha_4\alpha_5$(IV) network is more heavily crosslinked with disulfide linkages and resistant to proteolytic degradation than the immature $\alpha_1\alpha_1\alpha_2$(IV) network, which consists of more proteolytic cleavage sites than the $\alpha_3\alpha_4\alpha_5$(IV) network\textsuperscript{24}. This isoform switch may be critical for the establishment and maintenance of the permselective barrier properties in the glomerulus, especially when accommodating the increased blood pressure in the adult\textsuperscript{74}. In AS, mutations in type IV collagen genes \textit{COL4A3}, \textit{COL4A4}, and \textit{COL4A5}, which encode the $\alpha_3\alpha_4\alpha_5$(IV) chains, interfere with the assembly of the $\alpha_3\alpha_4\alpha_5$(IV) collagen network in the GBM and hinder the developmental switch from $\alpha_1\alpha_1\alpha_2$(IV) to $\alpha_3\alpha_4\alpha_5$(IV) network\textsuperscript{77}. This persistence of the nascent GBM results in abnormal membranes and clinical features characteristic of AS, illustrating the importance of the $\alpha_3\alpha_4\alpha_5$ type IV collagen network in the GBM.

1.3.3 Nidogen

Nidogens are two homologous basement membrane glycoproteins, designated nidogen-1 and nidogen-2, that are able to bind to the short arms of laminin $\gamma_1$ chain as well as to type IV
collagen. Thus, they are thought to act as a molecular bridge between the laminin and collagen IV networks in the basement membrane. However, the loss of either or both isoforms of nidogen have no effect on the basement membrane formation, suggesting that nidogens may provide extra stability to basement membranes under increased stress, but are not required for their initial development. Both nidogen-1 and -2 are present in the GBM, but not much is known about their developmental expression patterns.

1.3.4 Heparan sulfate proteoglycans

Heparan sulfate proteoglycans (HSPGs) are glycoproteins with a protein core to which heparan sulfate side chains are covalently linked. These sulfated glycosaminoglycan side chains can impart a highly negative charge, which corresponds to the anionic sites within the GBM that are detectable by cationic probes. Whilst perlecan is the most prominent HSPG in most basement membranes and in the mesangial matrix, agrin is the major HSPG in the mature GBM. During glomerulogenesis, perlecan and agrin are uniformly distributed; thereafter, perlecan is substituted by agrin and confined to the subendothelial side of the GBM.

Perlecan, produced primarily by glomerular endothelial cells, is anchored in the basement membranes by interactions with other constituents, binding to nidogen via its core protein, and to laminin and collagen IV via its heparan sulfate side chains. On the other hand, agrin is derived mainly from podocytes and its N-terminal domain binds to the laminin γ1 subunit, while the C-terminal LG domain binds to cell surface receptors, such as dystroglycans and integrins. Thus, agrin may play a role in cell-matrix adhesion through the affinity of its domain for laminin, dystroglycan, and integrin.
HSPGs in GBM were originally thought to confer charge selectivity in the glomerular filtration barrier; however, this concept has been recently challenged\textsuperscript{81}. Mice with podocyte-derived agrin knockout did not lead to any structural or functional defects in the glomerulus, despite the loss of GBM negativity\textsuperscript{76}. Furthermore, deletions of both agrin and heparan sulfate side chains on perlecan had little, if any, effects on permselectivity with no signs of proteinuria\textsuperscript{82}. Hence, no critical role for HSPGs, agrin and perlecan, in the GBM has been proven thus far.

1.3.5 Glomerular basement membrane in Alport Syndrome

Mutations of \textit{COL4A3}, \textit{COL4A4}, or \textit{COL4A5} interfere with the assembly of the \(\alpha3\alpha4\alpha5(IV)\) network in the GBM, resulting in the impediment of the collagen switch from \(\alpha1\alpha1\alpha2(IV)\) to \(\alpha3\alpha4\alpha5(IV)\) network during development\textsuperscript{4}. This persistent distribution of the primordial \(\alpha1\alpha1\alpha2(IV)\) network in the Alport GBM produces abnormal membranes that are less structurally sound due to fewer intra- and interheterotrimer crosslinks and more proteolytic cleavage sites than the \(\alpha3\alpha4\alpha5(IV)\) network\textsuperscript{24,83}. Thus, the \(\alpha1\alpha1\alpha2(IV)\) isoform is more susceptible to biomechanical strain caused by intraglomerular hypertension and ultrafiltration, endogenous proteolysis, and eventually results in the characteristic degenerative GBM splitting\textsuperscript{84,85}. This disposition to proteolytic degradation at the site of glomerular filtration is augmented by ectopic deposition of laminin chains, \(\alpha1\) and \(\alpha5\), resulting in an overall disruption of the GBM architecture\textsuperscript{86}. LM-511 increases in regions of focally thickened GBM, which are more permeable to protein\textsuperscript{71}. Furthermore, the lack of collagen IV and laminin isoform substitutions in the GBM bring about altered podocyte orientation, podocyte foot process effacement, and disruption of slit diaphragms. Affected podocytes can transmit this pathology and injury to adjacent healthy podocytes, giving rise to glomerulosclerosis with an
accumulation of ECM, as well as kidney fibrosis due to fibroblast formation by epithelial-mesenchymal transition, a physiological repair response to injury\textsuperscript{87,88}. Dysregulation of matrix metalloproteinases (MMPs) in glomerular mesangial cells has also been evidenced in AS, with their induction shown to contribute to the mechanism of pathobiology, while the blockade of specific MMP activities has been shown to ameliorate the disease progression\textsuperscript{89,90}. In particular, MMP-2, MMP-3, MMP-9, MMP-12, and MMP-14 have been most frequently associated with inflammatory diseases and fibrosis in the literature\textsuperscript{89–93}. Furthermore, a study by Zeisberg \textit{et al.} (2006) identified a dichotomous role for MMPs in the progression of AS in \textit{Col4α3\textsuperscript{-/-}} mice, in which an MMP upregulation was significantly correlated to deterioration of glomerular function; however, after the induction of proteinuria and the emergence of fibrosis, MMPs were found to be renoprotective against disease progression\textsuperscript{91}. Thus, changes in the integrity of the GBM, brought about by the mutations in the genes encoding collagen type IV α chains, affect cell signalling mechanisms, and further the pathogenesis of Alport glomerulopathy.

1.4 \textbf{Current clinical management options}

Currently the mechanisms responsible for kidney injury in AS have not been fully elucidated and the standard of care is limited to nonspecific therapeutic approaches that reduce renal fibrosis and delay the onset of ESRD\textsuperscript{94}. To date, several treatment options have been demonstrated to improve outcomes in animal models of AS, including renin-angiotensin system (RAS) blockade, such as angiotensin-converting enzyme inhibitors (ACE inhibitors) and angiotensin receptor blockers (ARBs), cyclosporine A, matrix metalloproteinase (MMP) inhibitors, transforming growth factor beta 1 (TGF-\textit{β1}) inhibitors, HMG-CoA reductase inhibition, chemokine receptor 1 (CCP-1) blockade, bone morphogenic protein-7 (BMP-7), stem
cell transplantation, and bone marrow irradiation. Such studies have not only shed light on an array of potentially effective therapies for AS, but have also contributed significantly in the understanding of the mechanistic pathways mediating the progression of AS towards ESRD.

Despite the lack of definite treatment for AS, ACE inhibitors are the recommended first line of treatment for non-immunological therapy of proteinuric glomerular disease. ACE inhibitors work by preventing the conversion of angiotensin I to angiotensin II, a growth factor involved in activating fibroblasts, leading to increased synthesis of matrix proteins, as well as functioning as a profibrotic cytokine, activating mononuclear cells and increasing proinflammatory mediators. The downstream proinflammatory effects of angiotensin II are mediated via the angiotensin type 2 receptor (AT2R) and the TGF-β pathway, which has been shown to be important in the renal fibrosis in AS. Thus, ACE inhibition has been shown to have antiproteinuric and antifibrotic nephroprotective effects in AS in a time-dependent manner, with earlier introduction of therapy yielding delayed onset of ESRD and prolonged life expectancy. Extensive clinical data on the efficacy and safety of ACE inhibitors in patients with progressive nephropathies are available; for instance, the Evaluation Study of Congestive Heart Failure and Pulmonary Artery Catheterization Effectiveness (ESCAPE) trial showed that ACE inhibition with ramipril resulted in low incidence of adverse effects in children with chronic kidney disease. However, in the case of patients experiencing adverse reactions to ACE inhibitors, such as coughs, angioedema, hyperkalemia, as well as decreased glomerular filtration rate (GFR), ARBs may be used. ARBs inhibit angiotensin II activity specifically by antagonizing its action on the angiotensin II type 1 receptor (AT1R). Long-term treatment with losartan was found to confer sustained antihypertensive and renoprotective effects with high tolerance in children with nephropathies. Thus, angiotensin antagonism has been shown to
be effective in preserving renal function via its capacity to reduce glomerular hypertrophy and sclerosis, as well as tubulointerstitial inflammation and fibrosis. This recommended approach to treatment is well-tolerated and is widely used in patients with AS; however, it does not prevent ESRD\textsuperscript{105}. Its efficacy is limited and most pronounced when administered at early stages of the disease, before the onset of microalbuminuria, thus making it a partially effective treatment option with limitations.

Calcineurin inhibitor, cyclosporine A, has also been experimentally and clinically studied as a potential treatment option of AS\textsuperscript{94}. Originally introduced into clinical practice as an immunosuppressant medication, it has been found to have beneficial effects on proteinuria and renal function when applied in the therapeutic management of AS. A study in a canine model of XLAS by Chen et al. (2003) showed that cyclosporine induced a significant delay in the onset and rate of progression of glomerulosclerosis as well as in the deterioration of renal function, although treatment did not affect the amount of interstitial fibrosis nor yield any beneficial effects on proteinuria\textsuperscript{99}. In contrast, the examination of the therapeutic efficacy of cyclosporine in small clinical trials have demonstrated a substantial reduction in proteinuria in Alport patients. However, cyclosporine usage was also associated with decreased GFR and significant renal lesions related to cyclosporine nephrotoxicity, thus precluding long-term administration\textsuperscript{113–115}. Though the mechanisms of actions of cyclosporine in AS have yet to be elucidated, its antiproteinuric effect seems to stem from its ability to stabilize the podocyte actin cytoskeleton, independent of its immunosuppressive action\textsuperscript{116}. This discovery sheds further light on the role of calcineurin signalling in the biology of proteinuric kidney diseases and provides novel calcineurin substrates, such as synaptopodin, as auspicious starting points for the identification of antiproteinuric drugs that evade the chronic adverse effects of long-term cyclosporine treatment.
Other experimental pharmacological therapies that have been studied with limited clinical experience include broad spectrum MMP inhibition, TGF-β1 blockade, inhibition of HMG-CoA reductase, CCP-1 antagonist, and BMP-7. These therapeutic interventions start to give insight into the pathophysiology of AS and highlight the importance of understanding the underlying molecular mechanisms of disease before initiating treatment. For instance, the pharmacologic ablation of MMP-2, MMP-3, MMP-9, and MMP-12, which are responsible for the proteolytic degradation of GBM, before the onset of proteinuria in Col4α3−/− mice have led to a significant attenuation in disease progression, marked by delayed proteinuria and prolonged survival.91,92 Likewise, a significant elevation in MMP expression levels were observed in a canine model of XLAS, implicating their induction with ECM dysregulation and interstitial fibrosis in Alport kidneys.117,118 Thus, MMPs have a contributory role in the pathobiology of Alport renal disease and their inhibition ameliorate GBM damage, restoring its ultrastructure and function.119 TGF-β1 has also been shown to have a potential role in Alport renal disease pathogenesis. A study by Sayers et al. (1999) observed an induction of TGF-β1 mRNA and proteins in both human and murine podocytes following the establishment of proteinuria.120 Similarly, HMG-CoA reductase inhibitors, also referred to as statins, which are potent inhibitors of cholesterol synthesis, have been recognized to have nephroprotective effects in Col4α3−/− mice.1 Beyond its role on lipid reduction, HMG-CoA reductase inhibitor’s pleiotropic effects have been recognized, in which, for instance in the kidney, it is able to inhibit mesangial cell proliferation, mesangial matrix deposition, as well as moderate monocyte infiltration and production of TGF-β.101 Thus, the administration of HMG-CoA reductase inhibitor brought about an attenuation in the expression of TGF-β, as well as concomitant decreases in proteinuria, renal fibrosis, and inflammatory cell infiltration, in addition to a prolongation of the lifespan of the KO mice. Other Col4α3−/− mice
studies have further illustrated CCP-1 antagonist and the upregulation of BMP-7 as potential new therapies for use in AS. The usage of BX471, a small molecule CCR-1 inhibitor, was associated with improved survival of KO mice, as well as decreased interstitial macrophages, tubular atrophy, and interstitial fibrosis\textsuperscript{100}. Treatment of KO mice with exogenous recombinant human BMP-7 led to significant decrease in the expression of profibrotic molecules, improved renal function, histology, and survival in mice, illuminating an overall renal protective effect of BMP-7\textsuperscript{102}.

Biological interventions, including cell-based therapies and bone marrow irradiation, are emerging techniques that have been extensively evaluated as a prospective therapeutic options in AS patients. Cell-based regenerative therapy aims to repair the underlying defect; in the case of AS, the defective assembly of the $\alpha_3$-$\alpha_4$-$\alpha_5$(IV) chains of collagen in the GBM\textsuperscript{121}. Thus far, replacement of the mutated genes via gene therapy have not been successful in humans\textsuperscript{122}. In Alport mice, however, it has been reported that stem cell therapy has yielded therapeutic benefits, promoting its application for clinical trials. For instance, Ninichuk \textit{et al.} (2006) presented that injections of primary bone marrow (BM)-derived multipotent mesenchymal stem cells (MSCs) into Col4a3 KO mice reduced interstitial fibrosis compared to saline-injected mice, though marks of renal function, including blood urea nitrogen (BUN), creatinine levels, proteinuria, and survival were not affected by MSC administration\textsuperscript{96}. Interestingly, MSC was found to localize to kidneys of the KO mice after injection, but their differentiation into renal cells was not detected. Similarly, studies by Prodromidi \textit{et al.} (2006) and Sugimoto \textit{et al.} (2006) have independently illustrated that whole BM transplantation is able to ameliorate the chronic renal injury caused by Col4$\alpha_3$ deficiency in KO mice via differentiation of stem cells into podocytes that then secrete the missing collagen $\alpha_3$-$\alpha_4$-$\alpha_5$(IV) chains\textsuperscript{103,104}. This is paralleled by
decreased serum urea and creatinine levels, reduced interstitial fibrosis, and improved renal histology. Nonetheless, the extent of podocyte cell engraftment by BM cells was low, suggesting that some other effect of BM transplantation may be contributing to the functional disease recovery\textsuperscript{122}. Furthermore, despite reported improvements in renal function as well as glomerular architecture defects in treated Alport mice compared to control groups, the age at ESRF, a meaningful endpoint of the disease, was not tested in either studies\textsuperscript{94,122–124}. Contrastingly, Katayama \textit{et al.} (2006) reported that BM transplant after lethal irradiation with either WT or KO BM prolonged the lifespan with similar efficiencies, denoting that the amelioration of disease observed is conferred most significantly by the irradiation. A positive correlation between the irradiation dosage and survival time was noted. The work by Katayama \textit{et al.} challenges the observations published by Prodromidi and Sugimoto groups and these differences may be derived from the dissimilar age at the time of BM transplant as well as the varying genetic backgrounds of the experimental Alport mice, in which Katayama’s study was performed in AS mice on the 129 SvJ background, while Prodromidi and Sugimoto’s studies were generated in C57BL/6J strain of Col4a3 KO mice\textsuperscript{95,103,104}. Thus, the different backgrounds and age at which BM transplantations were completed could have imparted different outcomes, attributable to the varied severity of GBM defects. Our current knowledge about gene- and cell-based therapies and their possible efficacies in the treatment of AS is yet insufficient and many experimental questions need to be addressed before clinical studies are considered in Alport patients\textsuperscript{125}. Nonetheless, biological interventions, such as regenerative medicine, provide new hope for a means to restore structural and functional integrity of the diseased kidney in the treatment of AS and other inherited renal glomerular diseases.
Currently there is a lack of therapies that can effectually change the course of disease progression in patients with AS\textsuperscript{94}. Promising experimental treatments have been developed and studied that promote the elucidation of the renal pathology of AS. However, despite a variety of treatment options available, none of these are causal therapeutic strategies that have been as effective in prolonging survival as ACE inhibition; they merely attenuate and do not prevent the progression to ESRD\textsuperscript{1}. Hence, for patients with AS who have progressed to renal failure, dialysis or renal transplantation are the only therapeutic options\textsuperscript{104}. There remains a gap in our understanding of the early mechanisms that lead to progressive kidney injury in AS which limits efficacious treatment. Accordingly, the Alport Foundation has issued a call for new studies in the development of novel treatment approaches to AS.

1.5 Renal inflammation blockade via BRD4 inhibition

The role of inflammation as a major driver in the pathophysiology of CKD has been widely and compellingly recognized in recent years\textsuperscript{126}. Inflammation contributes to progressive kidney damage by inducing the release of proinflammatory cytokines and adhesion molecules, which eventually brings about a loss of glomeruli, tubular atrophy and fibrosis, accompanied by a decrease in glomerular filtration rate\textsuperscript{127}. Therefore, an understanding of the molecular pathways driving renal inflammation is indispensable in the elucidation of the pathogenesis of chronic renal diseases as well as for the development of novel, targeted therapeutics in hopes of preventing ESRD in renal pathology\textsuperscript{128}.

During the initial stages of renal disease, inflammatory changes are detected with the concomitant involvements of several inflammatory cytokines, such as tumour necrosis factor-alpha (TNF-$\alpha$) and various interleukins (IL-1, IL-6, IL-18), which then activate a number of
signalling pathways\textsuperscript{129,130}. The expression of such inflammatory mediators is regulated by the transcription factor, nuclear factor-kappaB (NF-κB), which has been shown to activate a number of proinflammatory genes in both human and experimental models of progressive nephropathies\textsuperscript{126}. The mammalian NF-κB family consists of five members, RelA (p65), RelB, c-Rel, NF-κB1 (p50; precursor p105), and NF-κB2 (p52; precursor p100), which all share a highly conserved DNA-binding domain in the N-terminal region called the Rel homology domain (RHD)\textsuperscript{131}. Through the association of the RHD, the NF-κB proteins are able to form various homo- and heterodimers with each other, with the most common and best characterized dimer being the p65/p50 heterodimer\textsuperscript{132}. In addition, the RHD also binds with the inhibitory κB proteins (IκB), through which the NF-κB heterodimer is sequestered as an inactive complex in the cytoplasm. In response to a stimulus, such as cytokines, oxidative stress, or bacterial endotoxin (LPS), the IκB subunit is rapidly phosphorylated by IκB kinase (IKK) and ultimately degraded\textsuperscript{133}. The resulting release of the NF-κB dimers and their subsequent translocation to the nucleus promotes the transcription of target proinflammatory genes, such as RANTES, IL-1, IL-2, IL-6, MCP-1, TNF-α, as well as adhesion molecules, thereby promoting downstream renal inflammation\textsuperscript{126}.

Recently, it has come to light that the acetylation of the RelA subunit of NF-κB at lysine-310 is crucial for the transcriptional activation of NF-κB and the succeeding expression of inflammatory genes (Figure 1)\textsuperscript{134}. Stimulus-coupled acetylation of RelA by p300/CBP at lysine-310 promotes a direct interaction with bromodomain-containing protein 4 (BRD4) via its two bromodomains, which then recruits the acetylated NF-κB to the promoter regions of its target genes\textsuperscript{134,135}. The binding of the BRD4 to acetylated lysine-310 also promotes the kinases within the positive transcription elongation factor b (P-TEFb) complex, such as cyclin-dependent kinase
9 (CDK9), to phosphorylate RNA polymerase II for the transcriptional activation of NF-κB target genes, thus driving transcription of proinflammatory genes\textsuperscript{136,137}. This masterly epigenetic regulator, BRD4, belongs to the bromodomain and extra-terminal domain (BET) family, consisting of BRD2, BRD3, BRD4, and BRDT. A family of transcriptional coactivators and elongation factors, BET proteins are characterized by the presence of two conserved tandem bromodomains and an extra-terminal domain\textsuperscript{138}. The N-terminal bromodomains of BET proteins are able to recognize and bind to acetylated lysine residues on histones and other nuclear proteins in the regulation of gene transcriptional activity of cellular proliferation and differentiation processes, while the extra-terminal domain interacts with histone modifiers to bring about changes in the chromatin structure, thus effecting epigenetic modulations via chromatic dynamic and nucleosome assembly modifications\textsuperscript{139}.

Of all the mammalian BET proteins, BRD4 is the most extensively studied isoform and is broadly implicated in the regulation of renal inflammation\textsuperscript{140}. Given its role in the regulation of the innate and adaptive immune responses, however, a selective and temporal inhibition of NF-κB’s proinflammatory activity has been explored as of late, as a complete suppression of NF-κB may lead to adverse effects\textsuperscript{141}. Recent studies have demonstrated that targeted inhibition of NF-κB transcriptional activity brings about an attenuation of inflammation in experimental models of kidney injury. For instance, Zhang \textit{et al.} (2012) have shown that BET-specific bromodomain inhibitor, MS417, designed to block BRD4 from binding to the acetylated NF-κB, significantly downregulates the expression of proinflammatory cytokine and chemokine genes in human renal tubular epithelial cells, as well as reducing proteinuria, improving renal function, and effectively ameliorating NF-κB acetylation and in parallel, the expression of its target genes in Tg26 mice, an established animal model for HIV-associated nephropathy (HIVAN)\textsuperscript{140}. Likewise,
administration of the same NF-κB inhibitor, MS417, in db/db mice was shown to attenuate proteinuria and podocyte injury in diabetic kidney disease\textsuperscript{142}. Hence, these findings strongly suggest that the selective inhibition of NF-κB activity via BET bromodomain blockade, represents a new therapeutic approach for treating NF-κB-mediated inflammation and kidney injury.

Currently the specific effects of NF-κB signalling in experimental AS have not been extensively studied in the literature. However, recent findings involving the usage of this aforementioned novel compound, MS417, in the blockade of NF-κB activation in other renal disease models seem to be promising\textsuperscript{140,142}. The inhibition of BRD4 not only illustrates the pathophysiologic functions of NF-κB, but it also highlights the significant role of inflammation in mediating renal disease progression. Thus, these data motivate a pivotal need to investigate the collaborative roles of NF-κB and BRD4 in the regulation of proinflammatory activation in the pathogenesis of AS in hopes that targeting epigenetic regulators such as BET proteins may contribute in the elucidation of the etiology and progression of AS.
Figure 1. Molecular mechanism of BRD4-regulated inflammation and the inhibition of inflammatory gene transcription with MS417. Inflammatory stimulus consisting of the binding of ligand to a cell surface receptor, such as Toll-like receptor 4 (TLR4), leads to the recruitment of I kappa B kinase (IKK) complex, which in turn leads to the phosphorylation and degradation of the IκB inhibitor. This frees and activates the NF-κB dimers, p65 and p50. Active NF-κB is translocated to the nucleus, where it binds to consensus sequences in the regulatory elements of gene promoters. The transcription factor recruits BRD4 and together NF-κB and BRD4 control the transcription of inflammatory response genes. BRD4 also helps recruit RNA polymerase II to initiate gene transcription. However, MS417, a BRD4 inhibitor, prevents BRD4 from binding to NF-κB, limiting the recruitment of RNA polymerase II to the transcriptional complex \(^{140,142}\). Thus, the blockade of BRD4 binding to the acetylated NF-κB using MS417 can effectively attenuate NF-κB transcriptional activation of proinflammatory genes.
1.6 Experimental design and hypothesis

Sterile inflammation is an innate immune response that is vital for tissue and wound repair in response to trauma or ischemia-reperfusion injury in the absence of any microorganisms\textsuperscript{143}. Similar to pathogen-induced inflammation, sterile inflammation is marked by the recruitment of neutrophils and macrophages, bringing about the production of proinflammatory cytokines and chemokines, such as TNF and IL-1\textsuperscript{144}. Although inflammation is important in the resolution of the initial insult, chronic inflammation may lead to emergence of disease, contributing to the pathophysiology\textsuperscript{127}. Therefore, this study sought to examine the pathways that result in sterile inflammation in experimental AS, in particular the impact of NF-κB inhibition via the administration of a BRD4 antagonist.

In order to discern the changes in the patterns of gene expression in the Alport kidney, a microarray was performed using 4-week-old KO and WT glomeruli as described in Chapter 2. IPA approach was subsequently used to analyze the differential expression data in hopes of better understanding the biological changes that occur with the onset of the disease, as well as to predict the upstream transcriptional regulators to uncover potential therapeutic targets. Once the molecular alterations of the disease were determined, pharmacologic inhibitor of BRD4 was administered in HK-2 cells and in KO mice to better define the role of NF-κB in the pathogenesis of AS.

In summary, I hypothesize that NF-κB will attenuate kidney injury in mice with AS.
Chapter 2

Materials and Methods

2.1 Animals

*Col4a3*<sup>+/+</sup> (wildtype; WT) and *Col4a3*<sup>-/-</sup> (KO) mice of 129/SvJ background were purchased from The Jackson Laboratory (Bar Harbor, ME, USA)<sup>58</sup>. All animals were housed at the Division of Comparative Medicine at the University of Toronto, and fed standard chow diet with free access to food and water. Only male mice were used for experiments to avoid the potential confounding effects of female sex hormones. All experiments were conducted under the guidelines of the University of Toronto Animal Care Committee.

Male mice were followed from 4 weeks of age until sacrifice and tissue harvest at 7 weeks of age. 24-hour urine samples were obtained at 7 weeks of age by placing animals individually in metabolic cages in order to perform urinary albumin measurements. At sacrifice, mice were anesthetized with inhaled isoflurane and their body weights and kidney weights were recorded. Blood samples were collected from the carotid artery and both kidneys were dissected out. The kidneys were cross-sectioned into 3 parts, with two polar sections snap-frozen in liquid nitrogen and stored at -80°C until use. Middle sections of kidneys were fixed in buffered formalin and embedded in paraffin for histological analysis. The genotypes of study mice were verified by tail clip genotyping PCR via the usage of the following primers: common, 5’-CCAGGCTTAAAGGGAATCC-3’; WT reverse, 5’-TGCTCTCTCAAATGCACCAG-3’; and mutant reverse, 5’-GCTATCAGGACATAGCGTTGG-3’.

2.2 MS417 treatment

Male WT and KO mice were administered with either a control vehicle (0.1% DMSO in
PBS) or MS417 by daily oral gavage at a dosage of 0.0016 mg/kg. MS417 was provided by Dr. John Cijiang He (Mount Sinai School of Medicine, New York, NY, USA). The mice were fed this compound daily from the age of 4 weeks to sacrifice at 7 weeks of age. The mice were euthanized at 7 weeks of age for blood, urine, and kidney collection. Body weight was recorded daily from the start of the experiment till sacrifice for all experimental groups.

For the glomerular microarray study, male WT and KO mice were administered with either a control vehicle (0.1% DMSO in PBS) or MS417 by daily oral gavage at a dosage of 0.0016 mg/kg. The mice were fed this compound daily from the age of 4 weeks till they were sacrificed 10 days later.

2.3 Blood biochemistry

Blood samples were collected from carotid artery with Microvette (Sarstedt Inc., Montreal, QC, Canada) according to the manufacturers’ protocol at the time of sacrifice. Serum was isolated by centrifuging blood samples at 2000 x g for 5 minutes at room temperature and was then stored at -80°C until use. Levels of blood biochemical parameters, including blood urea nitrogen, creatinine, sodium, potassium, chloride, phosphorus, calcium, and total proteins, were measured at the Toronto Centre for Phenogenomics (Toronto, ON, Canada).

2.4 Urinary albumin excretion

24-hour urine samples were obtained by placing experimental animals individually in metabolic cages (Nalgene, Nalge Nunc International, Rochester, NY, USA). Urine samples were centrifuged at 8000 x g for 5 min at room temperature and the supernatants were collected and stored at -80°C until use. Albumin levels were determined with Albuwell M Kit (Exocell,
Philadelphia, PA, USA) following the manufacturers’ guide. Urinary albumin levels were used to calculate the 24h urinary albumin excretion (UAE) rate.

2.5 Isolation of mice glomeruli

The glomerular isolation procedure was adapted from a magnetic bead-based isolation method described by Takemoto et al. (2002) and Schwarz et al. (2013). An illustrated flow chart of the protocol is shown in Figure 2.\(^{145,146}\)

M-450 tosylactivated, 4.5 \(\mu\)m diameter Dynabeads (Invitrogen, Carlsbad, CA, USA) were inactivated according to manufacturer’s instructions. Congenic male WT and KO mice at 4 weeks of age were used. At the time of kidney glomeruli harvest, mice were anesthetized and perfused with 10mL of Hank’s balanced salt solution (HBSS; Invitrogen, Carlsbad, CA, USA) and then 10mL of \(8 \times 10^7\) magnetic Dynabeads diluted in HBSS through the left ventricle of the heart at a constant perfusion speed of 2.3ml/min using Syringe Infusion Pump (Harvard Apparatus, Holliston, MA, USA). Once the perfusion was completed, kidneys were removed, minced into 1mm\(^3\) pieces, and digested with 1mg/ml collagenase A (Roche Diagnostics, Indianapolis, IN, USA) in 3ml of HBSS at 37°C for 30 minutes with gentle agitation. The digested tissue was passed through a 100 \(\mu\)m cell strainer (Thermo Fisher Scientific, Waltham, MA, USA) using a flattened syringe pestle and the cell strainer was then washed 3 times with 1 ml of PBS. The cell suspension containing intact glomeruli with trapped paramagnetic Dynabeads was placed into a magnetic particle concentrator (Invitrogen, Carlsbad, CA, USA) for 7 minutes. The glomeruli containing Dynabeads were gathered on the wall of tubes; the supernatant (containing smaller tubular fragments, single tubular cells, a variety of polymorphic interstitial cells, and blood cells) and pellets (containing larger tubular fragments) were then
carefully pipetted into separate tubes and stored on ice. The glomeruli and larger tubular fragments were washed for at least three times with PBS. All procedures were performed on ice except the collagenase digestion. The purity of the glomerular and tubular isolates was verified under light microscopy. To prove the glomerular and tubulointerstitial origin of separated tissue fractions, the mRNA expression levels of glomerular (Nphs1 for podocyte) and tubular (Fxyd2 for distal tubules) markers were also examined (Figure 3). Isolated glomeruli samples were resuspended in QIAzol Lysis Reagent and stored in -80°C.
Figure 2. Flow chart of glomeruli isolation from Col4a3−/− mice. Anesthetized mice are perfused with Dynabeads (depicted by blue circles) diluted in HBSS through the left ventricle of the heart. The kidneys are removed, minced into 1mm³ pieces, and then chemically digested in collagenase for 15min at 37°C. The collagenase-digested tissue is then gently filtered through a 100µm cell strainer. The supernatant is discarded and the cell pellet resuspended in HBSS. The glomeruli containing Dynabeads are then collected by a magnetic particle concentrator¹⁴⁵,¹⁴⁶.

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Figure 3. RT-PCR of podocyte and distal tubular markers for microarray sample selections. Representative gels illustrating whether glomeruli RNA samples isolated from WT and KO mice had tubular contamination was evaluated via RT-PCR using podocyte (Nphs1) and tubular (Fxyd2) markers. The first column shows an isolated glomeruli sample along with cDNA controls from glomeruli, tubules, renal cortex (RC) sample from a 7-week-old mouse, and RC sample from a 4-week-old mouse, along with a negative control (NC).
2.6 Microarray data and bioinformatics analysis

By using Affymetrix Mouse Gene 2.0 ST Arrays (Affyetrix, Santa Clara, CA, USA), the global gene profiling of glomerular samples from male WT and KO mice at 4 weeks of age (n = 8 per group) was performed. Then in order to test whether the early genetic changes observed in the disease model could be reversed by the anti-inflammatory drug, MS417, the global gene profiling of glomerular samples from male vehicle-treated WT mice (n = 5), vehicle-treated KO mice (n = 9), and MS417-treated KO mice (n = 2) was performed. For all microarray studies, the total RNA containing small RNA was extracted using RNeasy Mini Kit (Qiagen Inc., Mississauga, ON, Canada) following the manufacturer’s protocol with an on-column DNA digestion step to minimize genomic DNA contamination. The sample integrity of the RNA was assessed using the RNA 6000 Nano Assay on 2100 Bioanalyzer (Agilent Technologies, Inc., Santa Clara, CA, USA) to ensure that RNA integrity number (RIN) was greater than 7.

Microarray experiments were performed at the Microarray Analysis and Gene Expression Facility at The Hospital for Sick Children (Toronto, Ontario, Canada). Following the manufacturer’s protocol, 125 ng of total RNA was labelled using the GeneChip WT PLUS Reagent Kit (Affymetrix, Santa Clara, CA, USA). Fragmented and biotin-labelled ss-cDNAs were then hybridized to GeneChip Mouse Gene 2.0 ST Arrays for 16 hours at 45°C. GeneChip Mouse Gene 2.0 ST Arrays feature probe sets for 28,137 coding transcripts, 7,103 non-coding transcripts, 2,000 long intergenic non-coding transcripts (lincRNA), with an average of 21 unique probes across each gene. Hybridized arrays were then stained and washed in the Affymetrix Fluidics Station 450. Thereafter, the arrays were scanned on an Affymetrix GeneChip Scanner 3000 and the image (.DAT) files were preprocessed using the Affymetrix GeneChip Command Console (AGCC) software v.4.3 to generate cell intensity (.CEL) files. After image
processing, the array data was uploaded to the Affymetrix Expression Console software v1.4.1 for further processing and quality control. All quality assessment metrics, including spike-in controls during target preparation and hydridization, were found within the boundaries. The probe set signal intensities were then extracted and normalized using the robust multi-array average (RMA) algorithm embedded in the Expression Console software, which consists of convolution background correction, quantile normalization, and median polish summarization. Downstream supervised statistical analysis was carried out via Partek Genomics Suite 6.6 (Partek Inc., Chesterfield, MO, USA) to determine differentially expressed genes affected by the genotype and treatment using a one-way ANOVA and a false discovery rate (FDR) equal to or less than 10%. Ingenuity Pathway Analysis (IPA; Qiagen Silicon Valley, Redwood City, CA, USA) was used to determine enriched biological processes, disease or toxicological functions, signalling and metabolic canonical pathways, upstream regulators, and gene pathways and networks in the context of the gene expression changes in experimental Alport Syndrome.

2.7 Cell culture and NF-κB activity assay

Human kidney-2 cells were sub-cultured in 6-well plates for 24 hours and co-transfected with 0.4 ug/well of pNF-κB-Luc plasmid (Stratagene, Agilent Technologies, Inc., Santa Clara, CA, USA) and 3 ug/well of pRL-TK reporter vector (Promega Corp., Madison, WI, USA) using the Effectene Transfection Reagent Kit (Qiagen, GmbH, Hilden, Germany) according to the manufacturers’ guides. Cells were incubated for 17 hours then subjected to Angiotensin II (10^{-7} mol/L) treatment with or without MS417 (1 uM) for 24 hours. Luciferase activity was determined with the Dual-Luciferase Reporter Assay System (Promega Corp., Madison, WI, USA) and the light emission was read by FB12 Luminometer (Titertek-Berthold, Berthold...
Detection System GmbH, Pforzheim, Germany). Total protein measured with Bradford Assay (Bio-Rad, Hercules, CA, USA) was used to normalize the activity results.

2.8 Real-time RT-PCR

Total RNAs from cortical and glomerular samples were isolated using RNeasy Mini Kit and reverse transcribed into cDNA with the QuantiTect Reverse Transcription Kit (Qiagen Inc., Mississauga, ON, Canada). For the cortical samples, the mRNA expression levels for TNF-α, TGF-β1, IL-6, and IL-1β were quantified by real-time PCR (TaqMan; Thermo Fisher Scientific, Waltham, MA, USA) by using a sequence detection system (ABI Prism 7900; Applied Biosystems, Foster City, CA). GAPDH was used as internal control. For the glomerular samples, the mRNA expression levels for Fxyd2 and Nphs1 were quantified by semi-quantitative PCR.

2.9 Statistical analysis

All results are shown as mean ± S.E. Statistical analyses were performed using GraphPad Prism 5 (GraphPad Software, Inc. La Jolla, CA, USA). For multiple group comparisons, one-way ANOVAs with Tukey’s range test were performed, and for two-group comparisons, unpaired t-tests were performed. Statistical significance was set at a p-value of less than 0.05.
Chapter 3

Results

3.1 Characterization and breeding of murine model of Alport Syndrome

In order to better understand the molecular pathways associated with renal disease progression and address the call for new therapeutic options, a well-characterized experimental murine model of AS with a Col4a3 knockout raised on a 129/SvJ genetic background was employed for the study. To produce a cohort of mice for experiments, two heterozygous mutant mice were bred together and succeeding heterozygous progenies were used for further breeding. The genotypes of study mice were verified by PCR and only male Col4a3\(^{-/-}\) mice and wildtype (WT) littermate control mice were used for the study (Figure 4). The developmental collagen IV switch has been demonstrated in the Col4a3\(^{-/-}\) mice between 4 to 7 weeks of age (Figure 5). It is evident that by 7 weeks of age, the kidneys of Col4a3\(^{-/-}\) mice fail to undergo the normal collagen IV network switch; Figure 5 illustrates the markedly decreased expression levels of collagen IV \(\alpha3, \alpha4, \text{ and } \alpha5\) genes in the Col4a3\(^{-/-}\) kidneys, while the expressions of both \(\alpha1\) and \(\alpha2\) genes were increased compared to that of the age-matched WT controls.

The structural and functional manifestations of the renal pathology of Col4a3\(^{-/-}\) mice have been comprehensively reviewed in the literature, establishing the experimental disease model extensively and solidly\(^{58,147-154}\). Histopathologic kidney injury was pronounced in the 7-week-old Col4a3\(^{-/-}\) mice on Periodic acid-Schiff- and Masson’s trichrome-stained sections, along with tubular dilatation, tubulointerstitial scarring, and fibrosis compared with the WT mice (Figure 6). Furthermore, Col4a3\(^{-/-}\) mice exhibited albuminuria with a significant increase in urinary albumin excretion (UAE) as early as 4 weeks of age compared to WT mice (Figure 7A). Serum creatinine levels were progressing in an increasing trend in the 7-week-old Col4a3\(^{-/-}\) mice, however, the
difference did not reach statistical significance (Figure 7B, $p = 0.18$). Thus, the macroscopic and microscopic renal examinations illustrated how this $Col4a3$ knockout model mirrors that of the human AS.
Figure 4. *Col4a3* KO mice breeding scheme and PCR genotyping. The breeding scheme for generating experimental groups as well as the maintenance of the mice colonies were effectuated via pairing of two heterozygous mutants (A). Only *Col4a3*<sup>+/−</sup> and *Col4a3*<sup>−/−</sup> mice were utilized as experimental groups for the study. Panel B then shows the representative PCR blot of the genotypes of mice used for the study as well as in generation of the *Col4a3*<sup>−/−</sup> mouse line verified by PCR (B). The first loaded sample is of *Col4a3* KO sample with WT sample loaded next. The third column is of the heterozygous sample<sup>152</sup>. 
Figure 5. Heat map of collagen IV α1-α5 expression levels in Col4a3−/− kidneys. The gene expression levels for collagen IV α1-α5 in kidney tissue derived from the microarray analysis of 4- and 7-week-old WT and Col4a3−/− mice (latter 7-week-old dataset highlighted with a green box). It is evident that there is an impaired collagen IV network switch in the KO mice between 4 to 7 weeks of age. In the 7-week-old KO kidney, there is an absence of Col4a3, Col4a4, and Col4a5, indicated by the blue squares, as well as a persistence of Col4a1 and Col4a2, indicated by the red squares, compared to the age-matched WT kidney. Each column represents an individual mouse sample and each row represents an individual collagen gene. Red colour indicates upregulation and blue represents downregulation.152
Figure 6. Histopathologic injury in Col4a3⁻/⁻ and WT kidneys. The renal phenotypes of Col4a3⁻/⁻ (KO) and WT mice at 4 and 7 weeks of age presented in Periodic acid-Schiff-stained and Masson’s trichrome-stained sections. The KO mice display minimal glomerular abnormalities and normal tubulointerstitium compared to WT mice at 4 weeks of age. However, by 7 weeks of age, moderately severe glomerular and tubulointerstitial scarring was evident in the KO but not WT mice. Similarly, there was very little fibrosis evident in 4-week-old KO kidneys compared to the age-matched WT kidneys. By 7 weeks of age, the KO kidneys exemplify extensive tubulointerstitial fibrosis compared to WT mice evidenced by the green protein casts in MT-stained samples. Insets in PAS-stained sections show the glomerulus. Original magnification: 100x, insets 200x.
Figure 7. Functional manifestations of renal pathology of Col4a3−/− mice. Urinary albumin excretion (A) and serum creatinine levels (B) in WT and KO mice at 4 and 7 weeks of age. At 4 weeks of age, a slight increase in urinary albumin excretion, but normal serum creatinine levels in the KO mice as compared to control WT mice. However, by 7 weeks of age, there was a marked increase in albuminuria excretion measure in KO mice, as well as an increase trend in plasma creatinine level, although it was not statistically significant (p = 0.18) Data are expressed as means ± SEM. *p < 0.05 versus WT152.
3.2 Early microarray studies in glomeruli

To elucidate the molecular pathways associated with renal disease progression, total RNA was extracted from glomeruli of 4-week-old male wildtype (WT) and Col4a3−/− (KO) mice (n=8/group) and a microarray performed using Affymetrix GeneChip Mouse Gene 2.0 ST Array. The purity of glomeruli samples was evaluated via RT-PCR using podocyte (Nphs1) and tubular (Fxyd2) markers prior to the microarray analysis (Figure 3). The purity of the RNA glomeruli samples without any tubular contamination was also quality checked via Bioanalyzer and only those with RNA of greater 7 were utilized for the microarray study.

Figure 8 shows the heat map highlighting the marked differential expressions of genes in the glomeruli of 4-week-old WT and KO mice. It presents a very distinct pattern of genes that are upregulated or downregulated even at 4 weeks of age in the glomeruli of WT and KO mice, comprising of genes which are involved in a wide variety of biological processes. Table 1 shows the Ingenuity Pathway Analysis (IPA) predicted activated upstream regulators that may be responsible for the gene expression changes observed. It identified 55 unique transcription factors and proteins in the Col4a3 KO glomeruli, with the transcription factor, NF-κB, predicted by IPA to be the most activated of all upstream regulators. Figures 10, 11, and 12 show the heat maps of differentially expressed target genes of IL-1β, TLR4 and MYD88, and NF-κB in the glomeruli of 4-week-old WT and KO mice, respectively. They all highlight the distinct patterns of gene upregulation and downregulation even at 4 weeks of age. Figure 9 maps the activated signal transduction pathways in experimental AS as predicted by IPA in 4-week-old KO glomeruli. It was shown that there are changes in the collagen protein levels in the extracellular matrix, levels of cytokines, as well as a number of membrane receptors, such as TLR4, TNF-α,
and IL-1β receptors, which all converge on the transcription factor, NF-κB. NF-κB, thereafter, influences a number of inflammatory genes.
Figure 8. Levels of differential gene expression in kidney tissues in WT and KO mice. Heat map of marked differential gene expression levels in the glomeruli isolated from 4-week-old WT and KO mice (n = 8 for both groups). Columns represent kidney samples, rows present different genes, and each cell indicates the expression value of the gene in that sample. Red colour indicates increased expression relative to mean (white colour), whereas blue colour indicates decreased expression relative to mean. Courtesy of Dr. Xuewen Song and Dr. York Pei.
Table 1. Ingenuity Pathway Analysis predicted activated upstream regulators in 4-week-old KO glomeruli. Ingenuity Pathway Analysis (IPA) predicted activated upstream regulators in Col4a3−/− glomeruli. The upstream regulators are arranged by the z-score, which predicts the activation state of specific upstream regulators using expression patterns of downstream target genes. An upstream regulator is considered activated and significant if equal to or greater than 2. A z-score of equal to or less than -2 is considered an inhibited upstream regulator. Bolded upstream regulators suggest activation of Toll-like receptor (TLR) signalling. Regulators indicated with an asterisk (*) signal downstream of FREM1. Courtesy of Dr. Song and Dr. Pei.
Figure 9. Schematic summary of aberrant activation of signalling pathways in AS mice. Map of signalling pathways in AS as predicted by IPA. Upregulated genes are labelled in red and downregulated genes are labelled in green. Genes that were not differentially expressed at the mRNA level are labelled in black. Genes that are predicted to be activated by the IPA upstream regulator analysis is indicated by a red asterisk and genes that are predicted to be inhibited is marked by a green asterisk. Genes highlighted in blue (a-c) are genes whose altered levels of differentially expressed target genes were further examined and visualized via heat maps (presented in subsequent figures). Courtesy of Dr. Xuewen Song and Dr. York Pei.
Figure 10. Levels of differentially expressed IL-1β target genes. Heat map of differentially expressed target genes of IL-1β in the glomeruli of 4-week-old KO and WT mice (n = 8 for both groups). Columns represent kidney samples, rows present different genes, and each cell indicates the expression value of the gene in that sample. Upregulated genes are labelled in red and downregulated genes are labelled in blue. Courtesy of Dr. Xuewen Song and Dr. York Pei.
Figure 11. Levels of differentially expressed TLR4 and MYD88 target genes. Heat map of differentially expressed target genes of TLR4 and MYD88 in the glomeruli of 4-week-old KO and WT mice (n = 8 for both groups). Columns represent kidney samples, rows present different genes, and each cell indicates the expression value of the gene in that sample. Upregulated genes are labelled in red and downregulated genes are labelled in blue. Courtesy of Dr. Xuewen Song and Dr. York Pei.
Figure 12. Levels of differentially expressed NF-κB target genes in kidney tissues of WT and KO mice. Heat map of differentially expressed target genes of NF-κB in the glomeruli of 4-week-old KO and WT mice with red depicting upregulation and blue representing downregulation (n = 8 for both groups). Columns represent kidney samples, rows present different genes. Interestingly, the heat map shows that mRNA levels for several genes regulated by NF-κB actually decreased. The mechanism responsible for these changes were not addressed in this current study. It possibly represents secondary effects downstream of the original stimulus for the activation of NF-κB. Courtesy of Dr. Xuewen Song and Dr. York Pei.
3.3 Targeting NF-κB in vitro

In order to determine whether there was any biological effect of MS417 on NF-κB inactivation, the NF-κB luciferase activity was measured in human kidney-2 (HK-2) cells cultured in medium containing angiotensin II (Ang II), with or without the NF-κB-blocking compound, MS417. HK-2 cells were transfected with an NF-κB reporter construct and the NF-κB activation activity was determined by measuring the luciferase activity. As expected, Ang II caused a robust increase in NF-κB activity in HK-2 cells transfected with the NF-κB reporter construct. This effect was attenuated with MS417 treatment, which inhibited activation of Ang II-induced NF-κB (Figure 13).
Figure 14. **MS417-inhibited Ang II-induced NF-κB activation in HK-2 cells.** Human-kidney 2 cells were transfected with pNF-κB-Luc plasmid as described previously. After 24 hours of incubation with Ang II (10⁻⁷ mol/L) with or without MS417 (1 uM), cells were lysed and the lysates were used for luciferase activity assay. Ang II stimulated the luciferase activity and the treatment with MS417 attenuated the Ang II-induced rise in NF-κB activation in the HK-2 cells. n = 3 for all groups. *p < 0.001 vs. transfection control; † p < 0.001 vs. Ang II-treated group.
3.4 Analysis of NF-κB inhibition in vivo using whole kidney samples

3.4.1 Whole animal kidney examination

Compared to corresponding sham animals, there was no significant change in body weights after MS417 treatment in either WT mice or Col4a3 KO mice, indicating no apparent toxicity observed based on body weight measurements (Figure 14). No mortality in either untreated and treated groups were observed during the course of the experiment (data not shown). BUN and creatinine levels were measured to assess kidney function. Plasma BUN was increased in vehicle-treated WT kidneys (Figure 15). However, compared to control, the plasma BUN level was not significantly altered with MS417 treatment in the KO kidneys. Plasma creatinine values were changed to a similar extent in both WT and MS417-treated KO animals (Figure 16).

To determine if MS417 treatment would help alleviate kidney function impairment, the urinary albumin excretion rates were measured for all experimental groups (UAE, Figure 17). The vehicle-treated KO mice had elevated UAE, but despite a trend of a decrease in the change in albuminuria level, the UAE failed to reach statistical significance when compared to the WT mice (Figure 17).
Figure 14. Body weight change measurements in WT and KO mice. Body weight measurements were recorded from the start of the treatment at 4 weeks of age till sacrifice at 7 weeks of age for all experimental groups. There were no significant differences among the three experimental groups in their body weight measurements at the time of sacrifice. Results are shown as mean ± SE. n = 4 for vehicle-treated WT; n = 3 for vehicle-treated KO; n = 5 for MS417-treated KO.
Figure 15. Blood urea nitrogen (BUN) levels in 7-week-old WT and KO mice following vehicle or MS417 treatment. Plasma samples were obtained at sacrifice at 7 weeks of age after 3 weeks of daily treatment. BUN levels were measured with frozen plasma samples. MS417 treatment trended towards a reduction in the BUN levels in the KO kidneys compared to the vehicle-treated KO kidneys, but the difference failed to reach statistical significance. Results are presented as mean ± SE. n = 13 for WT sham; n = 9 in vehicle-treated KO group; n = 9 in MS417-treated KO group. *p < 0.05 compared to WT sham; p = 0.12 for MS417-treated KO group vs vehicle-treated KO group.
Figure 16. Serum creatinine levels assessed in 7-week-old animals following vehicle or MS417 treatment. Creatinine levels were measured with frozen plasma samples. There was a clear rise in the serum creatinine level in the vehicle-treated KO kidneys, which was attenuated with the administration of MS417; however, the difference failed to reach statistical significance. Results are presented as mean ± SE. For WT with vehicle treatment, n = 13; n = 9 for KO with vehicle treatment; n = 9 for KO with MS417 treatment. *p < 0.05 compared to WT sham; p = 0.21 for MS417-treated KO kidneys compared to vehicle-treated KO kidneys.
Figure 17. Urinary albumin excretion (UAE) assessed in 7-week-old animals. Body weight measurements were recorded from the start of the treatment at 4 weeks of age till sacrifice at 7 weeks of age for all experimental groups. The rise in albuminuria observed in the vehicle-treated KO group is decreased with MS417 treatment, though the difference failed to reach statistical difference. Results are shown as mean ± SE. n = 10 for all groups. *p < 0.05 compared to WT sham; p = 0.07 for MS417-treated KO vs vehicle-treated KO.
3.4.2 Proinflammatory cytokine expression

Renal inflammation and the accumulation of macrophages play an important role in kidney injury. Thus, the expression profiles of proinflammatory cytokines, interleukin-6 (IL-6) and interleukin-1β (IL-1β) were assessed in cortical kidney samples as indicators of inflammation and disease progression (Figure 18). The measurement of mRNA levels of proinflammatory cytokines, IL-6 and IL-1β, showed dramatic induction of all genes in vehicle-treated KO mice compared to the kidneys of WT mice. However, the mean mRNA levels of IL-6 and IL-1β were lowered in the kidneys of the MS417-treated KO mice compared to the kidneys of WT mice with a statistically significant difference for IL-1β (Figure 18). The differences were not statistically significance when the proinflammatory mRNA expression levels of MS417-treated kidneys were compared to vehicle-treated KO kidneys.
Figure 18. Proinflammatory cytokine levels in kidney tissue in WT and Col4a3 KO mice after sham or MS417 treatment. mRNA expression levels of IL-6 (A) IL-1β and (B) TNF-α in WT and KO mouse kidney after vehicle or MS417 treatment. GAPDH was used as internal control. Results are normalized to WT sham and presented as mean ± SE. n ≥ 10 in each experimental group for IL-6 and IL-1β. n values are varying per experimental group as during the performance of the experiments, amplifications failed in some of the wells and were omitted for the data analysis. *p < 0.05 compared to WT sham; p = 0.19 for MS417-treated KO vs vehicle-treated KO (A); p = 0.54 for MS417-treated KO kidney vs vehicle-treated KO kidney (B).
3.5 Analysis of NF-κB inhibition *in vivo* via microarray studies of isolated glomeruli

To determine whether there were any changes in the molecular pathways linked to the disease progression of Alport Syndrome with the administration of MS417, the total RNA was extracted from the glomeruli of 38-day-old male mice from three experimental groups: WT with vehicle treatment; KO with vehicle treatment; and KO with MS417 treatment (n = 7 for all experimental groups). As previously described, the purity of the glomerular RNA samples to ensure the absence of tubular contamination was evaluated via RT-PCR using podocyte and tubular markers (Figure 3). Table 2 shows the summary of the glomerular isolation perfusions performed, the number of glomeruli RNA samples with RIN higher than 6, the number of RNA samples sent for Bioanalyzer analysis, and the number of RNA samples used for the microarray analysis. Table 3 lists the RNA glomeruli samples that were selected for the microarray study. When the microarray analysis was performed, it became evident that the assignment of the genotypes to the experimental animals was incorrect. Table 4 then lists the newly assigned genotype and treatment grouping after an inaccuracy in the genotype assignment in the experimental group was discerned. A total of 21 glomeruli samples were utilized for the study.

A microarray was then performed using Affymetrix GeneChip Mouse Gene 2.0 ST Array on these samples. Figure 19 shows the heat map of the differential gene expression levels in the glomeruli of WT and KO mice after 10 days of either sham or MS417 treatment. The initial changes in gene expression that was previously observed in the glomeruli of 4-week-old mice in vehicle-treated WT and KO groups were sustained in this experiment. However, there was no significant marked differential expressions of genes in the MS417-treated KO group when compared to the vehicle-treated KO group.

Figures 20, 21, and 22 illustrate the heat maps of differentially expressed target genes of
IL-1β, TLR4 and MYD88, and NF-κB in the glomeruli of WT and KO mice treated for 10 days with sham or MS417, respectively. Once again, the distinct patterns of marked differential gene expression noted in the 4-week-old WT and KO glomeruli are conserved in the glomeruli after the 10-day treatment with sham or MS417. The 10-day treatment of MS417 in KO mice did not alter the gene expression patterns of NF-κB, IL-1β, and TLR4 and MYD88 targets compared to that of the vehicle-treated KO mice.
Table 2. Summary of glomerular isolation perfusions performed and RNA yield qualities for analyses. A total of 34 perfusions were completed on three experimental groups of WT vehicle (n = 15), KO vehicle (n = 8), and KO treatment (n = 11) mice, which yielded n = 11, n = 7, and n = 7 of acceptable-quality RNA samples (RIN > 9) for WT vehicle, KO vehicle, and KO treatment groups, respectively. RIN score of greater than or equal to 6 is considered a good quality RNA suitable for analysis. A total of 25 RNA samples were submitted for Bioanalyzer analysis and a total of 21 RNA samples were utilized for the microarray analysis. Courtesy of Dr. Xuewen Song and Dr. Pei York.
Table 3. RNA quality of glomeruli samples selected for microarray study. The RNA concentration and the sample purity levels (260/280 and 260/230) levels are shown for the individual RNA samples that were selected for the microarray study. In total, 21 RNA samples were submitted, which is comprised of n = 7 for each experimental group of WT vehicle, KO vehicle, and KO treatment. A 260/280 ratio of approximately 2.0 or higher is acceptable as pure for RNA and a 260/230 range of 2.0 to 2.2 is considered pure for RNA samples\textsuperscript{156}. Courtesy of Dr. Xuewen Song and Dr. Pei York.

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Table 4. Reassignment of experimental groups for microarray study. The genotypes of the three experimental groups, WT vehicle, KO vehicle, and KO treatment (n = 7 per group), were reanalyzed. The first column shows the RNA ID of the individual samples. The second column shows the treatment assignment to the WT and KO animals; however, when the microarray analysis was performed, it became evident that the assignment of the genotypes of the animals was incorrect. The corrected genotype and treatment is shown in the third column. The RNA samples were then reassigned experimental grouping as WT vehicle (n = 5), WT treatment (n = 5), KO vehicle (n = 9), and KO treatment (n = 2). In total, 21 individual samples of glomeruli were experimented on. Courtesy of Dr. Xuewen Song and Dr. Pei York.

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RNA for microarray (n=21)

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Figure 19. Levels of differential gene expression in glomeruli in WT and KO mice after 10-day treatment with sham or MS417. Heat map of marked differential gene expression levels in the glomeruli isolated from 38-day-old WT and KO mice treated with either sham or MS417 for 10 days after which the mice were sacrificed. n = 9 for vehicle-treated KO; n = 2 for MS417-treated KO; n = 5 for vehicle-treated WT; n = 5 for MS417-treated WT. Columns represent kidney samples, rows present different genes, and each cell indicates the expression value of the gene in that sample. Upregulated genes are shown in red and downregulated genes are shown in blue. Courtesy of Dr. Xuewen Song and Dr. York Pei.
After 10-day treatment with MS417

Figure 20. Marked differential gene expressions of IL-1β target genes in glomeruli of WT and KO mice. Heat map of marked differential gene expression levels of IL-1β targets in the glomeruli isolated from WT and KO mice treated with vehicle or MS417 for 10 days. n = 9 for vehicle-treated KO; n = 2 for MS417-treated KO; n = 5 for vehicle-treated WT; n = 5 for MS417-treated WT. Columns represent kidney samples, rows present different genes, and each cell indicates the expression value of the gene in that sample. Upregulated genes are labelled in red and downregulated genes are labelled in blue. Courtesy of Dr. Xuewen Song and Dr. York Pei.
After 10-day treatment with MS417

Figure 21. Levels of marked differential gene expression of TLR4 and MYD88 targets in glomeruli in WT and KO mice. Heat map of TLR4 and MYD88 target gene expression levels in the glomeruli isolated from WT and KO mice after a 10-day treatment with vehicle or MS417. n = 9 for vehicle-treated KO group; n = 2 for MS417-treated KO; n = 5 for vehicle-treated WT; n = 5 for MS417-treated WT. Upregulated genes are shown in red and downregulated genes are shown in blue. Courtesy of Dr. Xuewen Song and Dr. York Pei.
Figure 22. Marked differential gene expression levels of NF-κB target genes in glomeruli of WT and KO mice. Heat map of marked differentially expressed NF-κB target genes in the glomeruli isolated from WT and KO mice treated with vehicle or MS417 for 10 days before sacrificing. n = 9 for vehicle-treated KO; n = 2 for MS417-treated KO; n = 5 for vehicle-treated WT; n = 5 for MS417-treated WT. Columns represent kidney samples, rows present different genes, and each cell indicates the expression value of the gene in that sample. Upregulated genes are labelled in red and downregulated genes are labelled in blue. Courtesy of Dr. Xuewen Song and Dr. York Pei.
Chapter 4

Discussion

Alport Syndrome is a progressive nephropathy of the glomerular basement membrane, a major component of the glomerular filtration barrier. It is caused by mutations in the genes encoding the α3/α4/α5 chains of type IV collagen, which is one of the principal constituents of the GBM. Thus, the dysregulation of the collagen IV alpha chains manifests in a clinical course present with microscopic hematuria, which progresses to proteinuria, and eventually culminating in renal failure. Renal biopsies from patients also typically show segmental or diffuse glomerulosclerosis, tubular atrophy, interstitial fibrosis, as well as interstitial infiltration of lymphocytes and macrophages, correlating with progressive renal failure. Yet, the exact molecular mechanisms by which the mutations exert their pathogenic effects on the glomerulus remain elusive. A greater understanding of the mechanisms behind the gradual degeneration of the GBM in AS would lead to the discovery of renoprotective methods, which could potentially prevent glomerulopathies in the future. Therefore, using in vitro and in vivo models, this study examined the possibility of inflammation as a major driving force behind the glomerular injury in AS. Furthermore, the impact of NF-κB inhibition via competitive blockade of BRD4 on the renal disease progression in AS was investigated.

4.1 Major changes in gene expression are observed in the glomeruli prior to a decline in GFR in experimental AS

The molecular effects of COL4A3/A4/A5 mutations were investigated in the glomeruli of WT or mutant (Col4a3−/−) mice via microarray analysis. Microarray technology is a powerful tool that allows the analysis and quantification of thousands of gene expression levels
simultaneously\textsuperscript{159,160}. Thus, such high-throughput approach has been utilized in recent years to discern a global viewpoint of the disease at the molecular level, enabling the evaluation of the cellular and metabolic derangement associated with the diseased state\textsuperscript{161}. The gene expression profiles in this study give information with regards to both upregulated and downregulated genes in the early phase of disease progression in this experimental model. Figure 8 shows the heat map of differentially expressed genes with twofold greater expression in 4-week-old KO compared to WT kidneys, showcasing a prominent pattern of pathway activation and inactivation that is consonant with the \textit{COL4A3} mutation. The heat map revealed marked overexpression of genes involved in inflammation mediated by chemokine and cytokine signalling pathways as well as PDGF signalling pathway in 4-week-old KO glomeruli compared to the age- and sex-matched control glomeruli. Genes involved in a variety of non-immune pathways, such as angiogenesis, blood coagulation, gonadotropin-releasing hormone receptor, integrin signalling, PI3 kinase, as well as Wnt signalling, were downregulated in the early timepoint of \textit{COL4A3} deletion.

Inflammation has been well-established as an important driver of pathophysiology across a broad range of diseases in the literature, including in nephropathies\textsuperscript{162,163}. The expression levels of proinflammatory mediators, such as cytokines and chemokines, are upregulated in the diseased state in comparison to healthy controls, conveying their crucial role in the pathogenesis of CKD. For instance, TNFRSF12A (tumour necrosis factor receptor superfamily member 12A) is a receptor for tumour necrosis factor ligand superfamily member 12 (TNFSF12; also called TNF-related weak inducer of apoptosis (TWEAK)), a cytokine that is widely expressed with overlapping signalling function with tumour necrosis factor (TNF)\textsuperscript{164,165}. Excessive activation of
the TNF/TWEAK pathway has been observed in kidney injury to promote NF-κB-dependent inflammatory response, fibrosis, angiogenesis, as well as apoptosis\textsuperscript{165,166}.

Likewise, C-X-C motif chemokine 10 (CXCL10) is a cytokine belonging to the CXC chemokine family that has been implicated in inflammatory diseases, which is seen to be strongly expressed in 4-week-old KO mice\textsuperscript{167}. It was seen in the study by Petrovic-Djergovic \textit{et al.} (2015) that stimulation of cultured podocytes with IFN-γ and TNF-α markedly induced the expression of \textit{Cxcl10} mRNA and CXCL10 secretion, illustrating its role in the propagation of innate and adaptive immunity, as well as inflammation\textsuperscript{168}. Kaffe \textit{et al.} (2018) also investigated the pathogenetic role of CXCL10 in disease progression \textit{in vivo} by blocking the CXCR3-CXCL10 axis, inhibiting macrophage recruitment in congenital hepatic fibrosis in the presence of polycystic kidney disease\textsuperscript{169}. It was found that CXCL10 inhibition slowed the progression of the disease by reducing CD45\textsuperscript{+} immune cell infiltration, collagen deposition, the total number of macrophages, as well as proinflammatory T cells.

Adrenomedullin (ADM) is a vasodilatory peptide abundantly expressed in the kidney with antiproliferative effects on glomerular mesangial cells\textsuperscript{170}. Its production has been seen to be activated in several pathophysiological states, including cardiac, hepatic, respiratory, renal failure, tumour growth, as well as during inflammatory states\textsuperscript{171,172}. A body of evidence indicates that inflammatory cytokines, such as TNF-α and IL-1β, upregulates ADM mRNA expression, suggesting that inflammatory cytokines may trigger ADM gene expression during inflammation\textsuperscript{171}. A rise in ADM level was observed to significantly ameliorate proteinuria, glomerular and tubular injury, as well as renal dysfunction in salt-related renal hypertension\textsuperscript{173}. Thus the increase in delivery of ADM gene in the kidney suggests a renoprotective role,
producing a wide spectrum of beneficial effects in compensation for the impaired renal function in the diseased state\textsuperscript{174}.

Another gene that was overexpressed in the KO glomeruli is CYP1B1, which is a member of the cytochrome P450 enzyme family I, subfamily B, polypeptide \textsuperscript{175,176}. It is expressed in cardiovascular and renal tissues, with a major role in the mediation of Ang II-induced activation of NADPH oxidase, generation of reactive oxygen species (ROS), proliferation, hypertrophy and/or deactivation of a wide range of xenobiotics\textsuperscript{176–178}. CYP1B1 expression has been implicated in pathophysiology, particularly in tumours as well as cardiovascular and renal abnormalities\textsuperscript{179}. For instance, Zordoky and group reported that acute doxorubicin (DOX) toxicity, a chemotherapy drug that causes oxidative damage and induces inflammatory changes in the heart, kidney, and liver tissues, caused an induction of CYP1B1 expression in both the kidneys and liver in rats in a mechanism which has yet to be fully elucidated\textsuperscript{177}. Similarly, the deleterious pathophysiological effects of Ang II infusion in the kidneys were observed to be minimized by the inhibition of CYP1B1 activity by treatment with a selective CYP1B1 inhibitor, TMS, in rats, and by \textit{Cyp1b1} gene disruption in the mice\textsuperscript{175,180,181}.

Transgelin (TAGLN), also designated SM22\textalpha{}, is a cytoskeletal protein whose expression has been known to be induced in kidney injury\textsuperscript{182,183}. A differentiation marker of smooth muscle cells during embryogenesis, TAGLN is present transiently in the glomeruli during development and later absent in normal adult glomeruli\textsuperscript{184}. However, the reappearance of TAGLN within injured glomeruli is a major finding that links its expression to the initiation and/or progression of renal diseases characterized by podocyte injury and proteinuria, corresponding to the overexpression seen in the KO glomeruli in the microarray analysis\textsuperscript{185}. The literature has documented marked TAGLN upregulation in experimental models of nephropathies, as well as in
renal tissue of patients with proteinuric diseases, such as diabetic nephropathy and IgA nephropathy\(^{186}\). Furthermore, microarray and proteomic analyses in obstructive nephropathy model suggest TAGLN is upregulated during fibrosis development, an important pathological feature that eventually leads to renal failure\(^{187}\).

Genes downregulated in diseased animals compared to controls were mainly required for cell structure and localization, cell cycle, metabolic processes, and developmental processes (Figure 8). Among the 16 genes belonging to this group were, for example, secreted frizzled related protein 1 (SFRP1), kallikrein 1 (KLK1), and klotho. SFRP1, a secreted antagonist of Wnt signalling, is a known tumour suppressor that is involved in cell growth\(^{188}\). Though the role of SFRP1 in CKD has not yet been elucidated, the loss of its expression and the contribution its absence has on pathological events have been documented in a variety of cancers, including renal cell carcinomas (RCC), in which the loss of SFRP1 is often observed in human cancers\(^{189,190}\). This deregulation of SFRP1 expression level in RCC may allude to its role in aberrant Wnt signalling in the development of solid tumours\(^{190,191}\). In addition, study by Matsuyama and group found that kidneys from Sfrp1\(^{-/-}\) mice showed significant expression of myofibroblast markers, aSMA, as well as fibroblast genes, vimentin, after unilateral ureteral obstruction (UUO), giving rise to significant expansion of renal fibrosis\(^{192}\). The inactivation of SFRP1 also brought about an elevation of phosphorylated c-Jun and JNK levels, which are mediators of non-canonical Wnt signalling activation, corroborating SFRP1’s role as a modulator of renal damage through the non-canonical Wnt/PCP pathway. Thus, SFRP1 may be a key player in regulation and development of Wnt/PCP-mediated renal interstitial fibrosis in CKD\(^{192}\).

KLK1 deficiency has been documented in experimental models of hypertension, diabetic nephropathy, and kidney injury\(^{193}\). KLK1 is a highly-conserved serine protease expressed in the
kidney, which cleaves low molecular weight kininogen into kinin, which is then involved in kidney function and blood pressure regulation via vasodilatory and natriuretic effects\(^\text{194}\). In experimental acute kidney injury (AKI), renal KLK1 activity is markedly diminished, and the gene transfer of \(KLK1\) can abrogate renal injury by minimizing apoptosis and inflammatory cell recruitment\(^\text{193,195,196}\). Recent research has also identified the kidney-secreted hormone, klotho, as a central player in CKD-related inflammation with anti-aging and nephroprotective properties\(^\text{197}\).

A decrease in klotho level has been observed not only in chronic or acute animal models of renal injury characterized by renal inflammation, such as diabetic nephropathy, but also in patients with G1 category of CKD, in which normal GFR is accompanied by pathological albuminuria or other evidence of kidney injury\(^\text{198,199}\). Furthermore, interestingly, studies have found that inflammatory cytokines, including TWEAK and TNF-\(\alpha\), downregulate renal klotho gene transcription via NF-\(\kappa\)B both \textit{in vitro} and \textit{in vivo}\(^\text{200}\). Likewise, exogenous provision of klotho to the kidneys of \(db/db\) mice negatively regulated NF-\(\kappa\)B activation and the suppression of subsequent production of proinflammatory cytokines, such as RANTES, MCP-1, IL-6, and IL-8, in response to TNF-\(\alpha\) stimulation, showcasing a bidirectional relationship between klotho and inflammation\(^\text{197,201,202}\).

Downregulation of inward rectifier potassium channel 16 (\(KCNJ16; K_{\text{ir}}5.1\)) was also observed in Figure 8, which mirrors the findings reported in the literature, in which disruption of \(KCNJ16\) induced severe hypokalemia\(^\text{203}\). Human mutations in \(KCNJ16\) are associated with renal pathologies characterized by salt-wasting, renal tubulopathy, and hypokalemia, known as EAST syndrome, highlighting the critical role of the \(K_{\text{ir}}5.1\) potassium channel in the distal nephron\(^\text{203,204}\). Similarly, \(KCNJ1\) (\(K_{\text{ir}}1.1\)) loss brings about type II Bartter syndrome, which features polyuria, renal salt wasting, and hypotension; such clinical manifestations are largely
recapitulated in Kcnj1-null mice\textsuperscript{205}. Schmidt et al. (2007) further uncovered that proinflammatory cytokines, IL-1\(\beta\) and TNF-\(\alpha\), extensively suppressed the expression of KCNJ1, postulating the cytokines may mediate LPS-induced downregulation of tubular sodium transporters\textsuperscript{206}.

To fully delineate the molecular mechanisms behind the progressive deterioration of the GBM in the early stages of AS, the changes in the gene expression in the glomeruli were analyzed by microarrays in experimental model of AS immediately following the onset of albuminuria. Microarray analysis has been extensively applied in the field of experimental nephrology due to its efficiency in the analysis of changes in the transcriptome\textsuperscript{207}. The results in this study showed that there was a distinct pattern of differentially expressed genes between the Col4a3 KO and WT kidneys before the materialization of major structural and functional changes at 4 weeks of age (Figure 8). Many of the upregulated differentially expressed genes in the KO glomeruli were implicated in chemokine- and cytokine-induced inflammatory pathways, whereas those that were downregulated in diseased animals were involved in cell structure, cell cycling, as well as metabolic and developmental processes. The serial changes in glomerular gene expression in early experimental AS were in accordance with previous findings in other nephropathies. Thus, it was found that at the onset of proteinuria, the kidneys of experimental AS were marked with changes in gene expression prior to the development of structural and functional manifestations of the disease compared to WT controls.
4.2 Pathway analysis of the differential gene expression established that pathways converged on NF-κB

In this study, the gene expression profile of Col4a3−/− glomeruli was further investigated to better understand the biological mechanisms of AS. The microarray dataset, which established that the onset of proteinuria is marked with a change in gene expression with the identification of 771 differentially expressed genes in mice with experimental AS compared to controls, was further analyzed for potent activated upstream regulators. This was done with the application of the Ingenuity Pathway Analysis (IPA), which calculates the activation z-score that represents the bias in gene regulation, thus reducing the chance that random data will generate significant predictions\textsuperscript{208–210}. With the z-scores filtered at 2.0, the IPA is then able to identify the cascade of upstream transcriptional regulators that are predicted to be activated based on the expression pattern of the target genes\textsuperscript{211,212}. Table 1 shows the 55 predicted putative upstream regulators, with NF-κB revealed to be the most activated upstream regulator, along with a multitude of regulators that are implicated in Toll-like receptor (TLR) signalling\textsuperscript{213–216}. Figure 8 then schematically summarizes the signalling pathways in experimental AS as predicted by IPA, emphasizing the central role of NF-κB in the modulation of inflammatory pathways in AS; its function as a major downstream convergent point for a multitude of proinflammatory transducers, including TLR4, is clearly recapitulated.

Renal inflammation is a universal response to injury, in which the innate immune system is stimulated to bring about an array of inflammatory responses as the first line of defense\textsuperscript{215}. The innate immune system, composed of germlike-encoded receptors expressed by macrophages, dendritic cells, monocytes, neutrophils, and natural killer cells, collectively serves to monitor extracellular and intracellular compartments for signs of infection or tissue injury\textsuperscript{217}. 

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These sensors, appropriately called pattern recognition receptors (PRRs), recognize common molecular patterns and translate these diverse triggers of renal inflammation into induction of cell activation and secretion of proinflammatory cytokines and chemokines. PRRs may respond to various bacterial and viral pathogen-associated molecular patterns (PAMPs), as well as non-microbial host-derived stimuli, such as damage-associated molecular patterns (DAMPs), which are endogenous molecules released by injured tissues during inflammation\textsuperscript{218}. The activation of the PRRs mediates downstream pathways through transcription factors that modulate the expression of genes with proinflammatory phenotype\textsuperscript{219}.

Among the several distinct classes of PRRs is the membrane-bound TLR family, which effectuate inflammatory responses by being able to detect a wide array of PAMPs and DAMPs in the extracellular space\textsuperscript{163}. TLRs are expressed on a variety of immune cells, including macrophages, neutrophil granulocytes, mast cells, dendritic cells, and T and B lymphocytes, as well as on other cell types, such as parenchymal and epithelial cells\textsuperscript{194,220}. The binding of a ligand to a TLR activates numerous signalling pathways, in which TLR activates adaptor proteins, which in turn initiate NF-κB in the actuation of inflammatory responses. Two main TLR signalling pathways are myeloid differentiation factor 88 (MYD88)-dependent pathway and the TIR-domain-containing adaptor protein-inducing IFN-β (TRIF)-dependent pathway\textsuperscript{221}. The MYD88-dependent pathway is activated by all known TLRs except TLR3, and leads to the production of proinflammatory cytokines, such as TNF, IL-1β, IL-12, IL-6, IL-8, and CC-motif chemokine ligand 2 (CCL2), whereas TLR3 and TLR4 can trigger the TRIF-dependent pathway, which brings about the production of type I interferons (IFNs), IFN-α and IFN-β, against viral infection\textsuperscript{222,223}. Generally, all TLRs can induce the expression of NF-κB-dependent genes, including most proinflammatory cytokines and chemokines, in addition to being able to induce
the expression of procytokines, IL-1 and IL-18. Recent studies have suggested that TLRs have an important role in the pathogenesis of renal diseases, with their exaggerated activation documented in renal ischemia, acute kidney injury, ESRF, acute tubulointerstitial nephritis, renal transplant rejection, as well as delayed allograft function. Thus, although inflammation mediated by PRRs can help to resolve and repair an initial insult, it has been recognized that overactivation of DAMP signalling may trigger inappropriate sterile inflammation, thereby contributing to unnecessary organ damage and dysfunction. The data listed on Table 1 agrees with previous reports that TLR and NF-κB signalling are important in the regulation of inflammatory and repair processes in the kidneys of AS, with predicted upstream regulators implicated in the activation of TLR signalling indicated in bolded letters.

TLR4 is the most extensively studied amongst the numerous TLRs that have been described in the literature, with significant implications in renal diseases. TLR4 has been shown to have an active role in the development of tubulointerstitial injury, albuminuria, as well as glomerular damages. For instance, in patients with diabetic nephropathy, TLR4 was found to be highly expressed in the renal tubules, positively correlating with interstitial macrophage infiltration. Furthermore, in animal models of glomerulosclerosis and mesangioproliferative glomerulonephritis, it was shown that TLR4 activation in podocytes could induce proinflammatory cytokines and promote podocyte injury. Similarly, TLR4-deficient mice exhibited less albuminuria, mesangial expansion, infiltration of macrophages, and decreased proinflammatory and extracellular matrix-associated gene expression in the glomeruli compared to wildtype mice. Subsequently downstream of that, mice that lacked the adaptor protein, MYD88, which is required for TLR signal transduction, presented a marked reduction in inflammation as well. This finding then led to an examination of the role of IL-1 receptor 1
(IL-1R1), which is a MYD88-dependent transmembrane receptor\textsuperscript{232}. In IL-1R1 mutant mice, a significant reduction in neutrophil response was observed, emphasizing IL-1R1’s essential function in neutrophil recruitment in response to inflammation and injury\textsuperscript{221}.

One of the ligands of IL-1R1 that was seen to be upregulated in the microarray data of the \textit{Col4a3\textsuperscript{-/-}} mice (Table 1, Figure 10) is IL-1β, a potent multifunctional proinflammatory cytokine that has been implicated in the sterile inflammatory response\textsuperscript{233,234}. Unlike most inflammatory cytokines that are mainly transcriptionally regulated, IL-1β is produced in the cytosol as a biologically inactive pro-IL-1β, which is activated intracellularly via proteolytic cleavage by caspase-1\textsuperscript{233}. In turn, the activation and conversion of caspase-1 from its zymogen form, pro-caspase-1, is under the direction of Nod-like receptor (NLR) protein family members, which collectively forms an inflammasome complex\textsuperscript{235}. This stimulation of inflammasomes cleaves caspase-1, which then hydrolyzes pro-IL-1β into its mature and functional form, exciting a proinflammatory response through the cleavage and thus activation of IL-1β, IL-18, as well as pyroptosis, a programmed lytic cell death. Hence, IL-1β is yet another key mediator that drives acute neutrophilic inflammatory response, with the microarray datasets mirroring that of the observations documented in other studies in the literature.

Accordingly, heat maps representing the expression patterns of IL-1β, TLR4/MYD88, and NF-κB target genes were generated in order to ascertain the cellular manifestations of inflammation in the experimental model of AS (Figures 10-12). NF-κB target genes that are differentially expressed in the 4-week-old KO glomeruli compared to their age-matched WT controls confirmed the upregulation of genes involved in apoptosis signalling pathway, B and T cell activation, inflammatory pathways mediated by chemokine and cytokine signalling, p53 pathway, as well as Toll receptor signalling pathway to name a few (Figure 12). This pattern of
differentially expressed gene profiling of IL-1β target genes (Figure 10) and TLR4 and MYD88 target genes (Figure 11) in the glomeruli of 4-week-old KO mice are also reiterated in the heat maps generated by the microarray studies, illustrating the enhancement of pathways that are involved in the recruitment and development of sterile inflammation that initiates renal injury and dysfunction.

4.3 A novel approach to inhibiting NF-κB-mediated gene expression: BRD4 antagonism

These findings suggest that the TLR4 pathway is a prominent mediator of sterile inflammation in the pathophysiology of CKD and the inhibition of its signalling pathway could block the damage brought about by the acute inflammatory response. However, blocking TLR4, as a central endotoxin receptor that is the innate immune system’s primary responder to pathogens, could also have detrimental effects in infectious and immunological settings, compromising the host’s immunogenicity. Therefore, targeting the downstream mediator of TLR4 signalling, NF-κB, which is a point of convergence for a myriad of other signalling pathways relevant to inflammation, may limit the risks of disrupting the rapid innate immunity against microbial infections.

Numerous factors have been discovered to contribute to the transcriptional activation of NF-kB target genes and with it multitudinous methods in which NF-κB has been inhibited have been described in the literature, including genetic and pharmacologic maneuvers. However, due to its protective role in innate immune response to infection, a complete inhibition of NF-κB may bring about adverse effects; thus, a selective blockade of its activity would help maximize the beneficial effects. Recently, emerging evidence has suggested that posttranslational modifications of NF-κB may play a critical role in the transcriptional regulation
of NF-κB-mediated inflammatory response\textsuperscript{240,241}. In particular, the acetylation of NF-κB’s RelA subunit has emerged as a significant component of NF-κB signalling linked to the regulation of DNA binding, IκB binding, as well as transactivation potential\textsuperscript{241}. The acetylation at lysine-310 is recognized by two bromodomains of BRD4, which in turn recruits and activates CDK9 to phosphorylate RNA polymerase II for the transcription of NF-κB target genes\textsuperscript{140,142,242}. Hence, lysine-310 acetylation, through its capacity to create docking sites for the recruitment of NF-κB transcriptional cofactors, is required for the full transcriptional activity of NF-κB\textsuperscript{242}.

Correspondingly, in order to study the biological effects of NF-κB inhibition via pharmacological BRD4 blockade, an \textit{in vitro} NF-κB luciferase study was carried out. NF-κB stimulation was effected using Ang II in HK-2 cells and MS417/BRD4 activity was interfered with the administration of MS417. The NF-κB luciferase assay revealed that MS417 was able to efficiently inhibit NF-κB after induction with Ang II, supporting BRD4’s capability as an effective NF-κB inhibitor (Figure 13). Other studies have demonstrated the efficacy of MS417 in the potent suppression of NF-κB activity. For instance, Zhang \textit{et al.} showed that MS417 effectively attenuated the NF-κB transcriptional activation of proinflammatory genes in kidney cells treated with TNF or infected with HIV\textsuperscript{140}. Moreover, MS417 treatment was able to ameliorate inflammation and kidney injury in HIV-1 transgenic mice. Similarly, MS417 was shown to inhibit NF-κB acetylation and its transcriptional activation in a murine model of diabetic nephropathy\textsuperscript{142}. Consistent with these findings, this study also clearly delineates and exemplifies the role of acetylation of NF-κB and the effect of its inhibition via BRD4 blockade.
4.4 Treatment of BRD4 inhibitor led to a decrease in cytokine gene expression and a trend towards improved creatinine levels and albuminuria

BET bromodomain-specific inhibitor, MS417, and its role in the modulation of NF-κB transcriptional activity has been studied in other experimental models of nephropathies, such as HIVAN and diabetic nephropathy; however, bromodomain inhibition has not been previously examined in AS\textsuperscript{140,142}. Thus, in this study, the effect of BRD4 antagonism on NF-κB-moderated inflammatory gene expression was further investigated \textit{in vivo} using \textit{Col4a3} KO and WT mice. This pilot study was able to confirm that the drug, MS417, was first and foremost, well-tolerated in the KO mice compared to the respective control groups via body weight measurements—alterations in body weight is considered an indicator of \textit{in vivo} toxicity (Figure 14)\textsuperscript{243,244}. Moreover, MS417 promoted a modest but therapeutic trend towards recovery in BUN, serum creatinine, as well as UAE levels in the KO mice compared to the vehicle-treated WT mice (Figures 15-17). Taken together, these results suggest that MS417 does not cause drug toxicity, and is able to comparatively reduce proteinuria and improve renal function through its capacity to inhibit NF-κB acetylation and transcriptional activation in the kidneys of the AS mice.

MS417 was discovered to also attenuate the expression of NF-κB target genes. The analysis of the mRNA levels of proinflammatory cytokines, IL-6 and IL-1β suggested that BET bromodomain inhibition downregulates NF-κB-mediated transcription of proinflammatory cytokine and chemokine genes in \textit{Col4a3}\textsuperscript{−/−} mice (Figure 18). This finding corroborates previous literature positing that selective inhibition of acetylated NF-kB binding to the transcriptional cofactor, BRD4, does effectively attenuate NF-κB transcriptional activation of proinflammatory genes, which are markers of kidney injury severity\textsuperscript{245,246}. Elevated levels of IL-6 and IL-1β in CKD have been observed in other studies \textit{in vivo} and \textit{in vitro}, in which their increased expression
levels were positively correlated with the severity of the renal disease progression\textsuperscript{119,247–249}. One study in 2014 found that upon analyzing the glomerular mRNA from an Alport mouse model, there was a significant induction of proinflammatory cytokine, IL-6, that correlated with GBM destruction\textsuperscript{119}. Koga and group have examined the expression of proinflammatory cytokines, IL-6, IL-1\(\beta\), TNF-\(\alpha\), and TGF-\(\beta\) in a murine experimental model of AS, in which they were discovered to be markedly induced in Alport kidneys compared with WT\textsuperscript{250}. The elevation in cytokine levels seen in the kidney tissues of \textit{Col4a3} KO mice were found to be significantly preventable for IL-1\(\beta\), while moderately ameliorated for IL-6 with the usage of NF-kB inhibitor, MS417 (Figure 18).

To further investigate the mechanism of BRD4 inhibition in the pathophysiology of experimental AS, a microarray study was performed to discern the effects of MS417 treatment on the KO glomeruli compared to WT kidneys (Figures 19-22). However, perchance due to the fallacious dosage administered to the mice, conflicting results were yielded in which there was not a significant alteration in the gene expression levels in the treated mice compared to the KO.

4.5 Conclusions

Using a combination of \textit{in vitro} and \textit{in vivo} approaches, this thesis examined the potential for NF-kB inhibition via pharmacological approach to BRD4 antagonism in experimental models of AS, and the impact of that molecular obstruction on the pathologic manifestations. A microarray study of the differentially expressed gene levels in the isolated glomeruli from \textit{Col4a3} KO and WT mice revealed a defining genetic profile as early as 4 weeks of age in the diseased state in comparison to healthy controls. A bioinformatic analysis via IPA of the microarray dataset predicted activated upstream regulators, uncovering NF-kB as the most
activated transcription factor as well as its role as a key convergence centre for multiple affected pathways, indicating sterile inflammation as a potential driver of the renal pathogenesis in AS—an exaggerated reaction of the innate immune system gone awry. A novel BRD4 inhibitor, MS417, was demonstrated to be an effective inhibitor of NF-κB activation both in vitro and in vivo without affecting any biotoxicity in the HK-2 cells and Col4a3 mice. MS417 was demonstrated to trend towards an attenuation of BUN, plasma creatinine, and UAE levels compared to WT mice. Furthermore, MS417 administration attenuated the augmented proinflammatory cytokine expression levels of IL-6 and IL-1β, with statistical significance for the latter. Further microarray studies were accomplished with isolated glomeruli from 4-week-old KO and WT mice with vehicle or MS417 treatment, which did not cause a similar attenuation of inflammation previously observed, possibly due to the dosage error. However, overall, these findings demonstrate the role of NF-κB-mediated sterile inflammation on the disease progression in experimental AS, guiding the development of therapeutic strategies specifically targeting the signalling pathway responsible for the deleterious effects of NF-κB.

4.6 Pitfalls and future directions

Several other hypotheses may be derived from the IPA analysis (Figure 9), for instance, it is possible that early interaction between the podocytes and the abnormal basement membrane leads to the activation of MMPs, subsequently bringing about a degradation of the ECM and the generation of DAMPs that activate the innate immunity mediate in part by TLR4. However, this aspect of the IPA analysis was not examined in this study and future experiments will be necessary to address this hypothesis.
The study was based on Zhang et al. (2012) and Liu et al. (2014), both papers which used an in vivo dosage of 0.08 mg/kg when treating db/db and HIV-1 transgenic mice with the BRD4-antagonizing compound, MS417\textsuperscript{140,142}. However, post-treatment renal analyses revealed that the mice were dosed at a differing dosage of 0.0016 mg/kg due to a calculation error made. In addition, the genotypes of the mice samples were rechecked during the microarray study using the Col4a3 expression and it was discovered that there was an issue with the initial assignment of the mice genotypes. At the time of the glomeruli sample submission for the microarray study, it was presumed that n = 7 for each vehicle-treated WT, vehicle-treated KO, and MS417-treated KO experimental groups were originally submitted (Table 2). However, the repeated genotyping analysis reassigned vehicle-treated WT as n = 5, vehicle-treated KO n = 9, and MS417- treated KO n = 2 (Table 4). For future studies, the correct dosage of MS417 as well as accurate genotyping, resulting in a higher n value for each experimental group, would help in yielding more effectual and precise outcomes. Despite the experimental errors, these analyses and the yielded negative results still provided data that plays a part in the effort to uncover novel therapies for AS.
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