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Innate immunity in diabetic kidney disease

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Abstract | Increasing evidence suggests that renal inflammation contributes to the pathogenesis and progression of diabetic kidney disease (DKD) and that anti-inflammatory therapies might have renoprotective effects in DKD. Immune cells and resident renal cells that activate innate immunity have critical roles in triggering and sustaining inflammation in this setting. Evidence from clinical and experimental studies suggest that several innate immune pathways have potential roles in the pathogenesis and progression of DKD. Toll-like receptors detect endogenous danger-associated molecular patterns generated during diabetes and induce a sterile tubulointerstitial inflammatory response via the NF-κB signaling pathway. The NLRP3 inflammasome links sensing of metabolic stress in the diabetic kidney to activation of proinflammatory cascades via the induction of IL-1β and IL-18. The kallikrein-kinin system promotes inflammatory processes via the generation of bradykinins and the activation of bradykinin receptors and activation of protease-activated receptors on kidney cells by coagulation enzymes contributes to renal inflammation and fibrosis in DKD. In addition, hyperglycaemia leads to protein

glycation and activation of the complement cascade via recognition of glycated proteins by mannan-binding lectin and/or dysfunction of glycated complement regulatory proteins. Data from preclinical studies suggests that targeting these innate immune pathways could lead to novel therapies for DKD.

Key points

- Renal inflammation involving the upregulation of inflammatory signaling pathways, release of cytokines and chemokines and infiltration of immune cells, contributes to the pathogenesis and progression of diabetic kidney disease (DKD).
- In the diabetic kidney, recognition of endogenous danger-associated molecular patterns by Toll-like receptors (TLRs), particularly TLR2 and TLR4, induces inflammatory responses
- Inflammasome activation not only amplifies renal inflammation but also has a role in the development of fibrosis; pharmacological agents that target inflammasome components may have therapeutic potential in DKD
- The kallikrein-kinin system and protease-activated receptor (PAR) signaling have been implicated in the progression of DKD; inhibition of kallikrein using kallistatin is renoprotective in diabetic mice
- The complement system is activated in human DKD; C5a and C3a receptor antagonists improve kidney fibrosis in rats with DKD, supporting a pathogenetic role of complement in this disease
- Targeting inflammatory signalling pathways is a promising novel therapeutic approach for DKD; further studies of the roles of innate immunity pathways in DKD may lead to the identification of novel drug targets

[H1] Introduction

Chronic kidney disease (CKD) is a public health problem that causes substantial morbidity and mortality.[1] Diabetic kidney disease (DKD) is a major cause of chronic kidney disease (CKD) in the USA and many other developed and developing regions, and accounts for approximately half of the end-stage renal disease (ESRD) burden in the developed world. In 2015, an estimated 415 million people worldwide had diabetes mellitus and 5 million deaths were attributable to this disease, which incurred a total global health expenditure of US$673 billion dollars[2]. By 2040, the prevalence of diabetes is expected to increase to 642 million people of whom 30-40% will develop DKD.

The pathogenesis of DKD is complex and involves a multitude of different pathways. Although traditionally regarded to be a noninflammatory glomerular disease that is induced by metabolic and haemodynamic changes, increasing evidence from clinical and experimental studies indicates that both systemic and local renal inflammation have crucial roles in the development and progression of DKD. Infiltration of immune cells, predominantly macrophages, is commonly observed in the glomeruli and interstitium of renal biopsy samples at all stages of DKD.[3] Moreover, macrophage accumulation correlates with progression to ESRD in patients with diabetes.[4] Infiltration of other immune cells, such as activated T cells, was also increased in the kidneys of patients with type 2 diabetes mellitus (T2DM) compared with those from patients with non-diabetic conditions such as renal cell cancer, huge angiomyolipoma and kidney injury, and the number of T cells correlated with the degree of proteinuria in these patients.[5]

Increased expression of inflammatory cytokines, chemokines, adhesion molecules, and growth factors including IL-6, ICAM-1, CCL-2, TNF, TGF-β and

VEGF has also been observed in renal biopsy samples from patients with DKD.[6] A proteomics analysis of three independent cohorts of patients with T1DM or T2DM identified a kidney risk inflammatory signature (KRIS) comprising 17 circulating inflammatory proteins that was associated with progression to ESRD during a follow-up period of 8-13 years[7]. The identification of these KRIS proteins, which include 6 members of the TNF receptor superfamily, could potentially lead to new therapeutic targets for DKD and new prognostic tests for risk of ESRD. A genome-wide transcriptome analysis of human kidney biopsy samples also identified the presence of inflammatory and fibrotic molecules in the context of DKD, including significant upregulation of complement C3, C-X-C motif chemokine 6 (CXCL6) and collagen alpha-2(I) chain (COL1A2).[8]

A large body of evidence supports a central role of a tubulointerstitial inflammatory component, and in particular the proximal tubular epithelial cell, in the pathogenesis of DKD.[9,10] Chronic non-resolving inflammation in the kidney drives the fibrotic response that occurs in this disease.[11] Renal inflammation and the development of fibrosis is a complex process with a large number of interacting pathways that lead to a chronic inflammatory infiltrate, which includes macrophages and other immune cells that release cytokines and profibrotic factors[12,13] and interact with intrinsic kidney cells[14] to create a profibrotic microenvironment. Persistent inflammation therefore triggers a profibrotic cascade in the kidney (Fig. 1).[15] Mitochondrial dysfunction in kidney tubular cells also contributes to renal inflammation. Leakage of mitochondrial DNA into the cytoplasm of these cells has been reported to lead to NF-κB-driven cytokine expression and immune cell recruitment via the stimulator of interferon genes (STING) pathway, indicating a link between a metabolic defect and inflammation.[16] Finally, accumulating evidence

indicates that activation of innate immunity orchestrates renal inflammation in DKD.

In this Review, we discuss the current status of anti-inflammatory therapies for DKD. We then highlight advances in the understanding of inflammatory mechanisms that underlie DKD with a focus on five immune-related pathways that utilize pattern recognition receptors or cell surface receptors to trigger inflammation — Toll-like receptor (TLR) signalling, the NLRP3 inflammasome, nucleotide-binding oligomerization domain (NOD)-like receptor (NLR) signalling, the kallikrein-kinin system; protease-activated receptor (PAR) signalling and the complement cascade. We also comment on the therapeutic potential of targeting each of these pathways for anti-inflammatory therapy in DKD.

[H1] Anti-inflammatory effects of DKD therapies

During the past two decades, the only treatment that has been approved for renoprotection in DKD is renin-angiotensin system (RAS) blockade, which has anti-hypertensive and anti-albuminuric effects. Angiotensin-converting enzyme (ACE) inhibitors and angiotensin II receptor blockers (ARBs) reduce intraglomerular pressure, water and sodium reabsorption and albuminuria; these effects slow the progression of renal disease. **[Au:OK?]**[Yes] However, even in the setting of maximal RAS blockade, the residual risk of progression to ESRD remains high[17] and a subgroup of patients may develop advanced CKD or ESRD in the absence of substantial albuminuria. In addition, several clinical studies of RAS blockade, including ONTARGET, VA-NEPHRON-D and LIRICO, showed no long-term renal benefits or an increased risk of adverse events, such as acute kidney injury and hyperkalemia, when patients received dual RAS blockade (combined treatment with

an ACE inhibitor and an ARB).[18-20] Thus, a gap exists for the development of new drugs that target other pathogenic pathways in DKD.

Novel antidiabetic drugs such as SGLT2 inhibitors, GLP1 agonists and endothelin receptor antagonists have shown promising renoprotective effects when used in combination with adequate RAS blockade in clinical trials. **[Au: OK?]** **[Yes]**In addition several anti-inflammatory molecules targeting chemokines, transcription factors, adhesion molecules and kinases have been developed and shown renoprotection in pre-clinical and early-phase clinical studies.[21]

[H2] SGLT2 inhibitors

Sodium-glucose cotransporter-2 (SGLT2) inhibitors are a class of anti-diabetic drugs that target SGLT2 proteins, which are primarily expressed in the proximal tubule of the kidney and have an important role in the reabsorption of glucose from the glomerular ultrafiltrate. Treatment with SGLT2 inhibitors therefore results in increased urinary secretion of glucose and sodium and loss of intravascular volume. Clinical trials have shown that the SGLT2 inhibitors empagliflozin, canagliflozin and dapagliflozin improve glycaemic control and reduce body weight, blood pressure and major adverse cardiovascular events (MACE) in patients with T2DM at risk of cardiovascular morbidity.[22-24] Secondary analyses of these trials[22,25,26] and exploratory analyses in animal models[27-29] suggested improvements in renal outcomes with these therapies. CREDENCE is the first renal outcome trial to show that a SGLT2 inhibitor, canagliflozin, lowers the risk of kidney failure and cardiovascular events in patients with established nephropathy.[30] This result was consistent with the findings of a *post hoc* analysis that suggested that empagliflozin might slow the progression of CKD in patients with T2DM regardless of their heart failure history.[31] In a pooled analysis of the EMPA-REG trials, the beneficial effects of empagliflozin on body weight and

blood pressure were preserved with decreasing eGFR,[32] making this drug an attractive therapeutic agent for patients with diabetes even at the latter stages of CKD.[33]

The mechanisms that underlie the beneficial effects of SGLT2 inhibitors on renal and cardiovascular outcomes are diverse and extend beyond their haemodynamic and metabolic effects. The interplay between diabetes, MACE and heart failure is complex and multifactorial, and in addition to inducing intravascular volume loss, SGLT2 inhibitor treatment might reduce blood pressure and enhance muscle insulin sensitivity and pancreatic β cell function[34] Experimental studies have shown that SGLT2 inhibitors also have anti-inflammatory effects on renal tissues. For example, empagliflozin treatment reduced the renal expression of IL-6, CCL-2 and NF-κB in diabetic Akita mice.[27] Similarly, in cultured kidney tubular cells, empagliflozin reduced the high-glucose-induced overexpression of Toll-like receptor 4 (TLR4), NF-κB and IL-6.[28] Dapagliflozin not only ameliorated albuminuria, but also reduced the levels of markers of renal inflammation (CCL-2 and NF-κB) and oxidative stress (Nox2 and Nox4) in kidney tissue in *db/db* mice.[29] SGLT2 inhibitors **[Au: Edit OK or did you mean that there is currently no evidence that SGLT2 inhibitors have direct effects on the immune system?]** **[There is currently no evidence that SGLT2 inhibitors have direct effects on the immune system]** may exert their anti-inflammatory effects via modulation of the redox state, RAS activity and haemodynamic changes.

[H2] GLP1 receptor agonists

Glucagon-like peptide 1 (GLP1) receptor agonists are a class of promising renoprotective antidiabetic drugs. These agonist have high homology to endogenous GLP1, which is an intestinal incretin hormone that augments insulin secretion from pancreatic β cells, inhibits glucagon secretion in response to glucose, decreases gastric

emptying and suppresses appetite. Several clinical trials (LEADER, SUSTAIN-6 and REWIND) have reported that GLP1 receptor agonists (liraglutide, semaglutide and dulaglutide) not only reduced the incidence of adverse cardiovascular outcomes, but also lowered the rate of new-onset macroalbuminuria and GFR loss in patients with T2DM.[35-39] A renoprotective effect of the GLP1 receptor agonist extendin-4 that was independent of its glucose-lowering effect has been demonstrated in rats with STZ-induced DKD[40]. In this model, administration of exendin-4 reduced ICAM-1 expression and NF-κB activation in kidney tissue. Similarly, treatment with extendin-4 reduced the levels of infiltrating inflammatory cells in the glomeruli of diabetic *db/db* mice. The improvement in diabetic nephropathy with extendin-4 treatment was associated with an increase in the renal expression of peroxisome proliferator–activated receptor-α (PPARα), which accounts for the anti-inflammatory effects of GLP1 receptor agonists as PPARα is a transcription factor that regulates expression of genes (NF-κB, PAI-1, ICAM-1) involved in inflammation.[41,42] **[Au: Please can you explain how PPARα accounts for the anti-inflammatory effects - what is the role of PPARα in modulation of renal inflammation?]**[Revised]

[H2] Endothelin receptor antagonists

Increasing evidence from experimental and human studies suggests a role of endothelin type A receptor antagonists as potential therapies for DKD. Increased renal endothelin expression is associated with vasoconstriction, mesangial proliferation, podocyte injury, inflammation and fibrosis in DKD.[43] These effects are mediated by binding of endothelin to endothelin type A (ETA) receptors, whereas binding of endothelin to ETB receptors leads to vasodilation.[44] Numerous endothelin receptor antagonists, including bosentan, atrasentan and avosentan, have been shown to reduce albuminuria and improve renal morphology and function in experimental models of

diabetes, and to prevent the progression of DKD in phase 2 and 3 clinical trials. Some of these trials were terminated, however, owing to high mortality that was associated with fluid retention.[45] The phase 3 SONAR clinical trial of atrasentan in patients with DKD was designed to exclude those at risk of fluid retention and enrich for those who had satisfactory anti-proteinuric responses to this treatment. In 2019, this trial reported long term beneficial effects of atrasentan on progression of DKD.[46] Independent of its effects on blood pressure, chronic endothelin infusion directly enhanced renal inflammation in rats via increased glomerular permeability, ICAM-1 and CCL-2 overexpression and infiltration of immune cells into the renal cortex. [47] These effects were attenuated by treatment with an ETA antagonist.

[H2] Chemokine-targeting therapies

In DKD, C-C motif chemokine 2 (CCL2)–C-C chemokine receptor type 2 (CCR2) signaling has an important role in the recruitment of T cells and macrophages to the kidney.[48] Beneficial effects of various CCR2 antagonists (RS504393, RS102895 and CCX140-B) have been reported in models of diabetes. In *db/db* mice, treatment with RS504393 markedly improved insulin resistance and reduced infiltration of macrophages and urinary albumin excretion.[49] Similar beneficial effects were observed when *db/db* mice were treated with RS102895.[50] In transgenic mice that expressed human CCR2 and were diabetic owing to deletion of the leptin receptor (*db/db*) or diet-induced obesity, oral administration of CCX140-B decreased albuminuria and glomerular hypertrophy, increased podocyte numbers and improved glycemic control.[51]

In a phase II clinical trial, treatment with CCX140-B compared with placebo significantly reduced proteinuria in patients with diabetes who were receiving treatment with RAS blockade.[52] Another phase II clinical trial, showed that 12 weeks

of treatment with emapticap pegol (NOX-E36), which inhibits CCL2, in addition to RAS blockade, improved urinary albumin to creatinine ratio (UACR) and HbA1c levels in patients with T2DM[53]. Importantly, the beneficial effects of this treatment on albuminuria and glycemic control were maintained for 8 weeks and 4 weeks after cessation of treatment, respectively, suggesting a sustained benefit of blockade of CCL2–CCR2 signalling. Conversely, a phase II clinical trial that investigated the effect of a dual CCR2 and CCR5 antagonist (PF-04634817) on albuminuria in patients with diabetic nephropathy reported only modest efficacy; hence the study was prematurely discontinued. The discrepant results may be explained by a sicker cohort with a higher baseline UACR and lower eGFR in the dual blockade study.[54] Other cytokine-targeting therapies such as anti-TNF and anti-IL-1β have been shown to improve glycemic control and metabolic profile in patients with diabetes,[55] but their efficacy has not been tested in DKD.

[H2] Transcription factor-targeting therapies

Targeting transcription factors that activate inflammatory pathways is another novel anti-inflammatory approach for DKD. Nuclear factor erythroid 2-related factor 2 (Nrf2) regulates the expression of several antioxidant enzymes such as glutathione S-transferase, glutathione peroxidase and heme oxygenase-1. Among mice with streptozotocin (STZ)-induced DKD, those that were deficient in Nrf2 ($Nrf2^{-/-}$) had increased ROS production, more severe glomerular lesions and significantly higher urine albumin-to-creatinine ratios (UACR) than their wild type littermates, indicating that Nrf2 protects against STZ-induced renal injury via anti-oxidative effects.[56]

The BEAM phase II clinical trial of bardoxolone methyl, a potent NF-κB inhibitor and Nrf2 activator, in patients with DKD showed promising results with a persistent increase in eGFR in the treatment group compared to the placebo group at

52 weeks.[57] However, the follow-up BEACON phase III trial in patients with T2DM and stage 4 CKD was prematurely terminated owing to higher rates of cardiovascular death in the bardoxyline methyl group.[58] Separate *post hoc* analyses of the BEACON trial data reported that treatment with bardoxylone methyl increased the risk of fluid overload and heart failure in participants who had elevated baseline levels of B-type natriuretic peptide[59] but that this treatment preserved kidney function and could potentially delay the onset of ESRD.[60] Thus, another bardoxylone methyl trial (TSUBAKI) in patients with DKD was restarted in Japan with a dose escalation design to exclude those at high risk of fluid overload (defined as patients with B type natriuretic peptide levels >200 pg/ml). Preliminary results from TSUBAKI published in abstract form demonstrate a significant improvement in GFR in the bardoxylone methyl group compared to the placebo group with no effect of the intervention on blood pressure, sodium retention or risk of heart failure.[61] No data on inflammation was reported.

[H2] Adhesion molecule inhibitors

The expression of endothelial adhesion molecules such as intercellular adhesion molecule 1 (ICAM-1), vascular cell adhesion protein 1 (VCAM-1) and vascular adhesion protein 1 (VAP-1) is markedly increased and associated with disease severity in patients with DKD.[62] [63] As these molecules have an important role in leukocyte adhesion to the endothelium, their inhibition might interfere with leukocyte trafficking and attenuate inflammation in DKD.

Expression of VAP-1 is induced during inflammation.[64] In addition to facilitating the interaction between endothelial cells and leukocytes, VAP-1 has semicarbazide sensitive amine oxidase (SSAO) activity, which triggers the synthesis of other endothelial adhesion molecules, such as ICAM-1 and E-selectin, and activates

inflammatory signaling pathways, including MAPK and NF-κB, via the generation of H_2O_2.[65] The ALBUM clinical trial showed a beneficial effect of a VAP-1 inhibitor (ASP8232) on DKD as evidenced by albuminuria reduction and slowing of eGFR decline in patients with T2DM and CKD.[66]

[H2] JAK–STAT blockade

Activation of the Janus Kinase (JAK)–Signal Transducer and Activators of Transcription (STAT) pathway has been implicated in the pathogenesis of DKD.[67] An analysis of human renal biopsy samples showed that increased JAK and STAT expression in the glomeruli and tubulointerstitium correlated with the progression of DKD.[68] Preclinical studies demonstrated that Akita diabetic mice with podocyte-specific overexpression of JAK2 had increased albuminuria, mesangial expansion, glomerular basement membrane thickening and podocyte loss compared to Akita diabetic mice without overexpression of JAK2 These features were reversed by treatment with a JAK1 and JAK2 inhibitor, suggesting a therapeutic potential of JAK–STAT blockade in DKD.[69] A phase II clinical trial in patients with T2DM and eGFR 25–70 ml/min/1.73 m^2 showed that an oral, selective inhibitor of JAK1 and JAK2, baricitinib, reduced albuminuria during a 24-week study period, suggesting a potential therapeutic role of JAK1/JAK2 inhibition for the treatment of DKD, even at more advanced stages of the disease[70]. In addition, the levels of inflammatory biomarkers such as urinary CXCL-10 and CCL-2 and plasma TNFR1 and TNFR2 were also decreased with baricitinib treatment, suggesting a renoprotective effect via an anti-inflammatory action of baricitinib.

The positive results of clinical trials of anti-inflammatory therapies together with advances in understanding of the pathophysiology of DKD, suggest that further studies are warranted for the identification and development of new drugs that target

inflammation in DKD. The emerging data discussed below suggests that innate immunity contributes to the initiation and progression of renal inflammation in DKD. An in-depth understanding of the contribution of innate immunity to the pathogenesis of DKD could pave the way for development of novel, renoprotective anti-inflammatory therapies for this disease.

[H1] Toll-like receptors

TLRs are germline-encoded pattern recognition receptors (PRRs) that have an integral role in innate immunity. The human genome encodes 10 TLRs (TLR 1–10). These type 1 transmembrane receptors belong to the interleukin-1 receptor (IL-1R) superfamily, share significant homology in their cytoplasmic regions (e.g. the Toll/IL-1R (TIR) domain)[71] and contain a leucine-rich repeat motif in their extracellular domain for pathogen recognition.[72]

TLRs are expressed by a variety of immune cell, including macrophages, dendritic cells, T cells, B cells and NK cells as well as by nonimmune cells, including kidney tubular epithelial cells, endothelial cells, podocytes and mesangial cells.[73] They are synthesized in the endoplasmic reticulum and either localize to the cell surface or remain in intracellular endosomes. Cell surface TLRs (TLR1, 2, 4, 5 and 6) detect the presence of pathogen-associated molecular patterns (PAMPs), such as lipopolysaccharide (LPS) and bacterial flagella, as well as damage-associated molecular patterns (DAMPs), such as high mobility group protein B1 (HMGB1) protein and heat shock proteins (HSPs), that are released from stressed or injured cells.[74] TLRs that localize to endosomes (TLR3, 7, 8, 9 and 10) recognize microbe-derived nucleic acids.

During infection, pathogens such as bacteria may enter the host through a breached epithelial barrier, leading to activation of macrophage TLRs by PAMPs.

TLR signaling results in the production of inflammatory cytokines that act near the site of infection to recruit neutrophils and induce neutrophil production of antimicrobial molecules, including peptides, reactive oxygen species (e.g. H_2O_2 and superoxide anion) and leukotrienes. Locally produced cytokines can spill over to the circulation and induce systemic effects. Both macrophages and neutrophils act to limit infection by phagocytosing pathogens.[75]

The release of DAMPs by injured or necrotic cells acts as a danger signal that activates the immune system to distinguish between dangerous and safe conditions.[76] Activation of TLRs in response to DAMPs can therefore induce sterile inflammation **[G]**. By contrast, cells that undergo normal physiological death processes are normally scavenged without further consequence. Increasing evidence supports a putative role of TLRs in various noninfectious inflammatory conditions such as diabetes, atherosclerosis and chronic kidney disease.[77-81]

DAMPs that are released in response to cell stress and injury such as HSPs, fibrinogen, heparin sulfate and biglycan, promote inflammatory responses by binding to TLR2 and/or TLR4. This binding results in TLR dimerization and conformational changes that trigger a downstream inflammatory cascade through recruitment of adapter proteins including myeloid differentiation factor 88 (MyD88), TIR-domain-containing adaptor protein (TIRAP), TIR-domain-containing adaptor inducing interferon (TRIF) and TRIF-related adaptor molecule (TRAM).[82] This TLR signalling cascade leads to translocation of NF-κB into the nucleus and transcription of proinflammatory genes, including those that encode IL-6, IL-1β and TNF (Fig. 2).[83,84]

[H2] TLR4 signalling in the kidney

TLR4 has a major role in renal inflammation and progressive fibrosis in kidney

disease.[85] Activation of TLR4 signaling in macrophages, endothelial cells and tubular cells during renal ischemia–reperfusion injury (IRI) synergistically mediates a NF-κB-driven pro-inflammatory response[86,87] that leads to amplification of tubulointerstitial inflammation and exacerbation of kidney injury.[88,89] In addition, the pro-inflammatory cytokine IL-8 induces profibrotic effects (upregulation of alpha-smooth muscle actin expression and downregulation of E-Cadherin) in tubular cells via increased TLR4 signaling.[90]

[H2] TLR signaling in DKD

The available data suggest that TLR2 and TLR4 have roles in the pathogenesis of DKD. TLR2 and TLR4 are overexpressed in the circulating monocytes of patients with diabetes[68]. Moreover, the levels of these TLRs correlate positively with HbA1c levels and homeostasis model assessment–insulin resistance (HOMA-IR) **[G]**, suggesting enhanced TLR activity in hyperglycemic conditions.[77-79] Overexpression of tubular TLR4 in kidney biopsy samples from patients with T2DM positively correlated with interstitial macrophage infiltration and HbA1C levels and negatively with renal function.[91] TLR4 mRNA and protein were overexpressed 4-10 fold in the glomeruli and tubules of kidney biopsy samples from patients with T2DM and microalbuminuria or overt DKD compared to those from patients with minimal change disease or healthy kidney tissue[92]. In addition, glomerular TLR4 gene expression was associated with loss of kidney function at 6-year follow-up in patients who had DKD and microalbuminuria at baseline.

The roles of TLR2 and TLR4 in DKD have been explored in experimental studies. TLR4-knockout mice were protected from STZ-induced albuminuria, renal dysfunction, inflammation and fibrosis; deletion of TLR4 not only suppressed tubular injury, but also reduced podocyte loss in the diabetic kidney.[91,93,94] TLR2 deficiency

also protected mice against the development of STZ-induced diabetic nephropathy.[95] In cultured podocytes, upregulation of TLR4 expression by high glucose was mediated through ROS generation and NF-κB activation.[96] The increase in TLR2 and TLR4 expression induced by high glucose in monocytes was mediated via activation of the PKC pathway and NADPH oxidase activity.[97] Both TLR2 and TLR4 expression was induced by high glucose in cultured tubular epithelial cells but only upregulation of TLR2 was sustained after prolonged exposure (7 days),[98] suggesting that TLR2 may be the predominant long-term mediator of renal inflammation in DKD. By contrast, another study showed that high glucose induced the expression of TLR4, but not of TLR2, via PKC activation in tubular epithelial cells[91]. In this study, silencing of TLR4 inhibited high glucose-induced upregulation of IL-6 and CCL-2, whereas no significant reduction in cytokine production was observed in TLR2-deficient tubular cells under the same high glucose conditions. High glucose also induced the expression of TLR4 but not of TLR2 in mesangial cells,[99] further supporting a predominant role of TLR4 in orchestrating renal inflammation in diabetes.

The levels of endogenous ligands of TLR2 and TLR4, such as HMGB1, biglycan, HSPs and hyaluronan, are increased in the sera and renal tissue of patients with diabetes in a high glucose, dyslipidemic and hypoxia milieu.[100-103] Increasing evidence indicates that HMGB1 has an important pathological role in DKD by activating TLR2 and TLR4 signaling. HMGB1 is a nuclear protein that is released into extracellular fluid from dendritic cells, macrophages and necrotic cells during inflammation (Fig. 2).[104] *In vitro* studies showed that high glucose conditions induced HMGB1 expression in mesangial and proximal tubular cells,[98,105] whereas treatment with a HMGB1 inhibitor, glycyrrhizin, mitigated high glucose-induced NF-κB

activation, oxidative stress and inflammation in these renal cells.[106,107] Moreover, serum from db/db mice induced upregulation of HMG1b, apoptosis and epithelial-to-mesenchymal transition (EMT) of cultured podocytes, whereas short-interfering RNA (siRNA) knockdown of HMGB1 suppressed these serum-mediated effects.[108] Treatment with HMGB1 short hairpin RNA had similar protective effects on podocytes in *db/db* mice.

HSP70 also has a role in induction of inflammation in DKD. Albumin increased the expression of HSP70 in proximal tubular cells in vitro and inhibition of HSP70 using cell-permeable inhibitors (VER-155008 and pifithrin-μ), a transcriptional inhibitor (KNK437) or a neutralizing anti-HSP70 antibody attenuated albumin-induced proinflammatory cytokine production via TLR4 signaling in mice with STZ-induced diabetes[102]. Knockout of TLR4 but not of TLR2 was renoprotective in these mice,[102] suggesting a contribution of albuminuria to renal inflammation and injury via activation of the HSP70-TLR4 axis.

[H2] Targeting TLR signalling in DKD

To date, no clinical studies have examined the effect of blockade of TLR signaling in DKD; however, increasing experimental data suggest that inhibition of TLR signaling, particularly that of TLR4 and/or TLR2, may confer renal protection. In the STZ-induced diabetic model, mice with knockout of endothelial nitric oxide synthase (eNOS) develop endothelial dysfunction and exhibit advanced nephropathic changes with severe albuminuria, glomerulosclerosis and arteriolar hyalinosis.[109] In diabetic eNOS knockout mice, treatment with the TLR4 antagonist, CRX-526, reduced STZ-induced renal inflammation, macrophage infiltration and tubulointerstitial fibrosis via suppression of NF-κB activation and TGF-β overexpression.[110] In *db/db* mice, administration of GIT27, a potent immunomodulator that targets macrophages

through blockade of TLR4, TLR2 and TLR6 signaling, had an anti-proteinuric effect[111]. Treatment with GIT27 also abolished high glucose and free fatty acid-induced proinflammatory cytokine production in cultured podocytes.

An alternative approach to inhibiting TLR4 signalling is targeting non-coding RNAs. MiR-203 binds to the 3'UTR region of TLR4 and suppresses its transcription[102], whereas the long non-coding RNA Gm6135 acts as a molecular sponge that binds to miR-203 and thereby upregulates TLR4 expression in mesangial cells exposed to high glucose[103]. These data suggest that the Gm6135–miR-203 axis has an important role in the regulation of TLR4 signaling in DKD. Targeting this axis may be a potential novel intervention to modulate TLR4 signaling in DKD.[112]

Although the available data suggest an important role of renal tubular TLR4 in orchestrating intrarenal inflammation in DKD, it is imperative that this hypothesis be further tested before clinical trials of interventions that target TLR signaling are considered. As nonspecific inhibition of TLR signaling may increase susceptibility to infection, targeting cell type-specific TLR4-mediated inflammatory pathways is a more desirable and attractive approach to prevent systemic immunosuppression. A logical tool for testing this hypothesis would be an inducible conditional tubular TLR4-knockout DKD model, as tubular epithelial cells have a crucial role in orchestrating renal inflammation. A better understanding of the pathophysiological mechanisms of tubular cell-specific TLR4 signaling may provide new insights into the development of specific treatment for tubulointerstitial inflammation and fibrosis in DKD.

[H1] Inflammasomes

Nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs) are a subgroup of PRRs that detect intracellular PAMPs and DAMPs. Similar to TLRs,

NLRs have an important role in innate immunity by linking sensing of microbial and metabolic stress to the activation of proinflammatory cascades. The human NLR family consists of 23 proteins. NOD1 and NOD2 are well characterized members of the NOD signalosome that mediate NF-κB-dependent cytokine production.[113] Other NLRs, including NLRP1, NLRP3, NLRP6, NLRP12 and NLRC4, oligomerize to form inflammasome complexes, which initiate inflammatory cascades that lead to activation of caspase-1 and the production of IL-1β and IL-18.[114]

The NLRP3 inflammasome is the most extensively studied inflammasome complex owing to its involvement in many human diseases including cancer, liver and kidney disease.[115-117] This inflammasome consists of a sensor molecule NLRP3, an adaptor protein ASC (apoptosis-associated speck-like protein containing a caspase activating recruitment domain) and an effector protein, pro-caspase-1.[118] NLRP3 activation is a two-step process. The first step, also known as priming, involves PAMP or DAMP-induced activation of TLR signaling, which leads to NF-κB-dependent expression of NLRP3, pro-IL-1β and pro-IL-18. The second step is triggered by various cellular mechanisms such as potassium efflux, pore-forming toxins, calcium influx, mitochondrial dysfunction and intracellular ROS production that promote the formation of the NLRP3 complex and activation of caspase-1 (Fig. 3).[73,118] Knockdown of TLR4 inhibited NLRP3 inflammasome activation and reduced ROS production and apoptosis in high glucose-induced podocyte injury.[119]

[H2] The NLRP3 inflammasome in DKD

Inflammasome activation has a crucial role in the pathogenesis of DKD. In patients with CKD, the expression of markers of inflammasome activation, such as CASP1, IL-1β and IL-18, in renal biopsy samples positively correlated with the severity of albuminuria.[120] Similarly, the glomerular expression of inflammasome markers such

as NLRP3, ASC, CASP1 and IL-18 was significantly increased in patients with DKD compared with nondiabetic healthy individuals.[121] These inflammasome-associated proteins were also upregulated in the kidneys of *db/db* mice.[121] The findings from a chimeric bone marrow transplantation study suggested that NLRP3 in the non-hematopoietic cellular compartment of the kidney has a more important role in mediating inflammatory processes in DKD than NLRP3 in infiltrating innate immune cells.[122]

Inflammasome activation was detected in podocytes and endothelial cells at an early stage of nephropathy in *db/db* mice.[123] High glucose levels induced NLRP3 inflammasome activation in cultured human podocytes, whereas shRNA inhibition of NLRP3 and ASC suppressed high glucose-induced activation of caspase-1 (CASP1) and IL-1β expression and attenuated podocyte injury.[124] In the kidneys of STZ-induced diabetic mice, hyperglycemia induced the expression of thioredoxin-interacting protein (TXNIP), which activated NADPH oxidase (Nox) to produce reactive oxygen species (ROS) and subsequently triggered inflammasome activation in podocytes,[125] resulting in podocyte loss and the onset of albuminuria.[126] Administration of angiotensin II also activated the NLRP3 inflammasome and induced loss of the podocyte proteins nephrin and podocin, podocyte mitochondrial dysfunction and albuminuria in mice.[127] These effects were attenuated by NLRP3 knockout, suggesting that NLRP3 inflammasome activation contributes to angiotensin II-induced podocyte apoptosis and mitochondrial dysfunction, which promotes proteinuria in DKD. Similarly, NLRP3 knockout improved renal function, glomerulosclerosis, tubulointerstitial inflammation and kidney fibrosis in mice with STZ-induced DKD.[128]

Tubular injury is one of the key determinants in the progression of DKD.[129,130] The role of the NLRP3 inflammasome in tubular injury was demonstrated by attenuation of high glucose-induced EMT and inhibition of the phosphorylation of SMAD3, MAPK p38, ERK1 and ERK2 (key signaling molecules with roles in proinflammatory and profibrotic responses in tubular cells) in a NLRP3-silenced proximal tubule cell line (human kidney 2 (HK-2) cells).[131] Activation of the NLRP3 inflammasome by high glucose was also suppressed by inhibition of tyrosine protein kinase SYK (also known as spleen tyrosine kinase) in HK-2 cells, indicating a role of SYK–JNK–NLRP3 signaling in the pathogenesis of DKD.[132] Expression of optineurin (OPTN), which acts as an autophagy receptor for damaged mitochondria during the process of mitophagy, was reduced in tubular epithelial cells from patients with DKD compared with nondiabetic healthy individuals and negatively correlated with renal interstitial inflammation.[133] In murine renal tubular epithelial cells, overexpression of OPTN enhanced mitophagy and inhibited high glucose-induced NLRP3 expression, cleavage of caspase-1 and release of IL-1β and IL-18.

Mitochondrial dysfunction is a characteristic of DKD and renal tubular cells have the highest mitochondrial content of all kidney cells.[134] Overproduction of mitochondrial ROS (mtROS) was associated with NLRP3 inflammasome activation in kidneys from patients with DKD and in those from *db/db* mice[135]. Administration of the mitochondrial-targeted antioxidant, MitoQ, reduced albuminuria and attenuated upregulation of inflammasome molecules such as NLRP3, caspase-1 and IL-1β and fibrotic markers including fibronectin, collagen 1 & collagen 4 in the kidneys of *db/db* mice. These results suggest that mtROS has an important role in NLRP3 inflammasome activation in DKD.

[H2] Targeting inflammasomes in DKD

Inhibition of the NLRP inflammasome is a promising novel therapeutic strategy for many inflammatory diseases.[136] Over the past decade, several pharmacological agents that directly target individual components of the inflammasome have been shown to inhibit inflammasome formation in clinical and experimental studies.[137] These agents include NLRP3 ATPase inhibitors, ASC inhibitors and caspase-1 inhibitors.

In *db/db* mice, caspase inhibition using M-920 reduced renal caspase 1, IL-1β, IL-18 and NLRP3 inflammasome activation and ameliorated albuminuria and renal extracellular matrix accumulation.[121] A small molecule NLRP3 inhibitor, MCC950, also reduced the production of active caspase-1 and IL-β in *db/db* mice and in mesangial cells exposed to high glucose levels. In addition, MCC950 ameliorated podocyte injury, renal fibrosis and kidney dysfunction in *db/db* mice.[138] Hence, inflammasome inhibition holds promise for the treatment of DKD. The potential renoprotective effects of directly targeting NLRP3 inflammasome assembly in DKD have not yet been investigated in clinical trials.

Activation of inflammasomes other than NLRP3 might also have a role in inflammation in DKD. For example, the NLRP1 inflammasome has been shown to induce IL-18 production in an experimental model of high fat diet-induced obesity and metabolic syndrome.[139] Moreover, NLRP3 has inflammasome-independent (non-canonical) effects on TGF-β signaling that contribute to renal fibrosis in DKD.[140] Thus, further research should focus on the various molecular mechanisms whereby inflammasome complexes mediate DKD in different renal cell types with the aim of identifying specific therapeutic targets.

[H1] The kallikrein-kinin system

The kallikrein-kinin system (KKS) has been implicated in the pathogenesis of inflammatory processes in ischemic stroke, skin wound healing, cardiovascular

disease and renal diseases.[141,142] Kininogen is converted into pro-inflammatory peptides known as kinins (bradykinin and kallidin) by serum or tissue kallikrein. These kinins in turn activate various intracellular signaling pathways through binding to their respective receptors (B2 bradykinin receptor (B2R) and B1 bradykinin receptor (B1R)) to induce increased vascular permeability, vasodilation and inflammatory responses. Under physiological conditions, bradykinin and kallidin bind mostly to the constitutively expressed B2Rs, whereas their metabolites des-Arg^{9}-bradykinin and des-Arg^{10}-kallidin bind to B1Rs, which are expressed at low levels in normal tissue and induced during tissue injury.[143] In addition to these cascades, kinins are degraded by angiotensin-converting enzyme (ACE), whereas kallikrein activity, which regulates the generation of kinins, is inhibited by an endogenous tissue inhibitor, kallistatin (Fig. 4).[144]

[H2] The KKS in DKD

As mentioned above, clinical studies have demonstrated that ACE inhibitors have beneficial effects in DKD that are independent of their effects on blood pressure and plasma angiotensin II levels.[145] As ACE inhibitors can prevent ACE-mediated degradation of bradykinin,[146] the KKS might have a role in these renoprotective effects. Treatment with the B2R antagonist HOE-140 reduced the beneficial effects of ACE inhibitors on albuminuria in STZ-treated rats and *db/db* mice, suggesting that B2R activation is one of the underlying renoprotective mechanisms of ACE inhibitors.[147,148]

A protective role of the KKS in DKD was further supported by the finding of downregulation of KKS components such as B2R, B1R, kallikrein and bradykinin and increased apoptosis in high glucose-treated podocytes and in the glomeruli of rats with STZ-induced diabetes.[149] Similarly, among mice with STZ-induced diabetes,

those with knockout of tissue kallikrein had increased albuminuria compared to wild type controls.[150] Patients with diabetes and microalbuminuria showed reduced urinary kininogen-1 levels compared to patients without microalbuminuria.[151] Conversely, plasma and renal bradykinin levels were increased in rats with STZ-induced diabetes[152] and plasma tissue kallikrein was upregulated in patients with T2DM.[153]

In contrast to the B2R activation, some studies have shown that B1R activation is associated with inflammatory and fibrotic responses in various kidney diseases including DKD, IRI and focal segmental glomerulosclerosis (FSGS).[154-156] Moreover, upregulation of B1R expression was detected in rats with STZ-induced diabetes.[157] In Akita diabetic mice, however, deletion of both B1R and B2R induced oxidative stress and mitochondrial damage and increased proteinuria, glomerulosclerosis and tubulointerstitial fibrosis to a greater extent than deletion of B2R alone,[158] suggesting that activation of B1R may be beneficial in preventing DKD progression.

[H2] Targeting the KKS in DKD

Exogenous treatment with kallikrein reduced albuminuria, renal inflammation and fibrosis in mouse models of T1DM and T2DM, suggesting a therapeutic potential of stimulating B2R activation in DKD.[159] However, the use of kallikrein as a potential therapy needs to be carefully evaluated as the role of the KKS in DKD is controversial. Tissue kallikrein induced pro-inflammatory responses such as the production of IL-6 and CCL-2 via activation of protease-activated receptor 4 (PAR4) in cultured proximal tubular epithelial cells[160] suggesting that KKS activation might have a deleterious effect in DKD. In addition, bradykinin induced the expression of IL-6, CCL2 and TGF-β in tubular epithelial cells[6] and upregulated CTGF, TGF-βRII and collagen 1 expression in mesangial cells via activation of MAPK signaling, suggesting proinflammatory and profibrotic effects of KKS activation.[161] In

uninephrectomized *db/db* mice, an accelerated model of murine DN, blockade of the B2R using icatibant attenuated both glomerular and tubulointerstitial injury with a reduction in renal inflammation and fibrosis. The renoprotective effect of icatibant in these mice was enhanced when combined with rosiglitazone, a specific ligand of PPAR γ ,[89] suggesting that B2R blockade in combination with thiazolidinedione treatment could have synergistic anti-inflammatory effects in advanced DKD.

Most studies of pharmacological blockade of B1R signaling show beneficial effects in animal models of renal disease, suggesting that the B1R might be a promising therapeutic target for DKD.[162] The pro-inflammatory transcription factor NF-κB modulates B1R expression both *in vitro* and *in vivo*,[163] and activation of B1R further enhances NF-κB-mediated cytokine synthesis. Several B1R antagonists have been tested in preclinical studies for the treatment of inflammatory diseases; however, none of these agents have been developed for clinical use.

Kallistatin not only regulates the activity of tissue kallikrein, but also exerts multiple protective effects by ameliorating oxidative stress, inflammation, apoptosis and fibrosis in various organs including the kidney. For example, endogenous kallistatin protected DOCA-salt hypertensive rats from renal injury.[164] In mice with IRI, adenoviral-mediated kallistatin transfer reduced cytokine production, tubular necrosis and apoptosis.[165] Kallistatin administration also reduced the expression of renal and circulating HMGB1, attenuated renal damage and reduced blood urea nitrogen and serum creatinine levels in a mouse model of polymicrobial-induced sepsis[166]. In *db/db* mice, overexpression of kallistatin suppressed renal inflammation and fibrosis in part by inhibiting NF-κB and TGF-β signaling pathways and also attenuated oxidative stress in the kidney.[167] Taken together, these findings suggest that enhancing kallistatin levels is a potential novel therapeutic strategy for DKD.

Overall, the available data from experimental studies indicate that exogenous kallistatin administration is consistently renoprotective in different renal diseases, whereas the effects of B1R and B2R blockade are inconsistent and vary between different experimental models. Further dissection of the molecular mechanisms by which KKS components impact upon the pathogenesis of DKD may lead to the identification of novel therapeutic approaches.

[H1] Proteinase-activated receptors

The PARs, PAR1 (also known as thrombin receptor), PAR2, PAR3 and PAR4, are transmembrane G protein-coupled receptors (GPCRs) that have important roles in the coagulation system and regulate hemostasis. PARs are activated by coagulation enzymes such as thrombin (which acts on PAR1, PAR3 and PAR4), factor Xa-VIIa complex (which acts on PAR2) and trypsin (which also acts on PAR2). These enzymes cleave the N terminus of the PAR receptor to expose a tethered ligand for induction of intracellular signal transduction[168,169], which results in aggregation of platelets and deposition of fibrin. Increasing evidence suggests that activation of the coagulation cascade orchestrates wound healing processes following tissue injury, whereas uncontrolled coagulation results in dysregulated inflammatory and fibrotic responses[170] and contributes to the progression of vascular disease and the metastatic processes of various cancers such as colon, breast and lung cancers.[171,172]

[H2] PARs in the kidney

PARs are expressed by various cell types in the kidney. For example, PAR1 is expressed in glomerular and tubular cells,[173,174] whereas PAR2 is expressed in mesangial cells, tubular cells, infiltrating immune cells and collecting duct principal cells.[175] PAR signaling has been shown to regulate renal hemodynamics under normal conditions. PAR1 activation induced renal vasoconstriction and a reduction in GFR in

an isolated perfused rat kidney, whereas PAR2 activation induced vasodilation in rat kidneys via upregulation of renal renin secretion.[176,177] In podocytes, PAR3 expression mediated the cytoprotective signaling of activated protein C and prevented puromycin aminonucleoside (PAN)-induced apoptosis through dimerization with PAR2.[178]

Accumulating data suggest that activation of PARs is associated with glomerular, microvascular and inflammatory dysfunction in progressive kidney disease.[179] PARs also have a role in platelet activation and inflammation in acute kidney injury.[180] In mice, PAR-1 is constitutively expressed in mesangial cells and tubular cells, and its expression is upregulated in experimental models of DKD or crescentic glomerulonephritis.[181] Mice with crescentic glomerulonephritis that were deficient in PAR1 or treated with a PAR1 inhibitor showed reduced kidney inflammatory cell infiltration, fibrin deposition and injury compared with controls.[182] In patients with IgA nephropathy, upregulation of renal PAR2 expression in the kidney tubules and interstitial cells correlated with the degree of interstitial fibrosis.[183] In an experimental model of FSGS, adriamycin (ADR)-induced inflammatory cytokine production was attenuated by inhibition of PAR2 signaling.[184] In human kidney tubular cells, overexpression of PAR2 led to a reduction in autophagy via activation of the PI3K–Akt–mTOR signaling pathway[185]. Consistent with this finding, mice that were treated with a PAR2 antagonist showed enhanced autophagy, reduced renal injury and lower levels of TNF-α, IL-1β and CCL-2 expression following unilateral ureteral obstruction (UUO) compared with controls.

[H2] PAR activation in DKD

Clinical studies have shown that long-term activation of the coagulation pathway might contribute to renal dysfunction in patients with diabetes.[186,187] Plasma levels of

coagulation factors such as factor VII, factor XII and fibrinogen strongly correlate with the development of vascular injury in patients with DKD.[188] Both PAR2 and PAR4 were upregulated in the renal tubules in biopsy samples from patients with DKD.[160] In proximal tubular epithelial cells, expression of these PARs was induced by tissue kallikrein, which promoted proinflammatory and profibrotic effects (Fig. 4).[160]

In *db/db* mice, glomerular PAR2 expression was increased and administration of a factor Xa inhibitor ameliorated glomerular hypertrophy and renal fibrin deposition.[189] Another study found upregulation of PAR1 in mesangial cells and interstitial peritubular cells from *db/db* mice, whereas PAR4 expression was not changed compared to age-matched *db/m* mice.[190] This increase in renal PAR1 expression may have a role in thrombin-induced extracellular matrix production in mesangial cells, which contributes to the progression of glomerulosclerosis.[191]

In human podocytes, high concentrations of thrombin induced F-actin cytoskeletal rearrangement and apoptosis via thrombin-mediated interactions between PAR3 and PAR4.[192] By contrast, sustained low level activation of thrombin owing to the factor V Leiden (FVL) mutation **[G]** protected diabetic mice from albuminuria and podocyte injury[193] Low doses of thrombin inhibited high-glucose-induced podocyte apoptosis in vitro, whereas high-dose thrombin had the opposite effect, suggesting a dual role of thrombin in chronic disease.

[H3] Targeting PARs in DKD

Anticoagulants are widely used to prevent and treat thromboembolic disorders. In particular, novel direct oral anticoagulants (DOACs), including the thrombin inhibitor dabigatran and the factor Xa inhibitors rivaroxaban, apixaban and edoxaban, have been shown to reduce bleeding risk, drug interactions and the need for coagulation

monitoring in patients with venous thromboembolism.[194] A systematic review of studies of DOAC use in patients with CKD or ESRD and atrial fibrillation showed that compared with warfarin, dabigatran and apixaban significantly reduced the risk of stroke, whereas apixaban and edoxaban reduced the incidence of major bleeding events in patients with moderate CKD (eGFR <60 ml/min/1.73 m^2)[195]. Among patients on haemodialysis with atrial fibrillation, DOACs did not reduce the risk of stroke and rivaroxaban and dabigatran, but not apixiban, increased the risk of major bleeding events compared with warfarin.

As diabetes mellitus induces intrarenal coagulation, which further triggers inflammatory and fibrotic processes, anti-coagulants might be beneficial for combating DKD. In *db/db* mice, treatment with a factor Xa inhibitor (fondaparinux) reduced albuminuria, ECM protein deposition and glomerular hypertrophy.[189] Similar outcomes were obtained with edoxaban in diabetic eNOS knockout mice[196]. In this model, edoxaban ameliorated diabetic glomerulosclerosis by reducing pro-inflammatory and pro-fibrotic responses and the expression of PAR1 and PAR2. By contrast, PAR2-deficient mice with STZ-induced DKD showed increased PAR-1 expression and mesangial expansion, which was probably due to upregulation of plasminogen activator inhibitor 1 (PAI-1) in the kidneys[197]. This finding suggests that targeting PAR2 for the treatment of DKD should be carefully evaluated before clinical application.

PAR1 expression was upregulated in the glomeruli of mice with STZ-induced diabetes and PAR1-knockout mice were protected from STZ-induced DKD suggesting that PAR1 inhibition also has potential for treating DKD.[181] Daily oral administration of the clinically approved PAR1 inhibitor vorapaxar can prevent atherosclerotic events in patients with history of myocardial infarction.[198] In mice

with STZ-induced diabetes, vorapaxar reduced kidney injury as evidenced by reduced albuminuria, glomerular fibronectin expression and mesangial expansion.[199] However, this study did not identify the renoprotective mechanisms of vorapaxar and a subsequent study reported that vorapaxar did not improve kidney function in an experimental model of T2DN.[200] Thus, further investigation is needed to understand the mechanisms of pharmacological inhibition of PAR1 in DKD. In terms of clinical application, extra monitoring is required for patients with advanced DKD or on antiplatelet or anti-coagulation therapy as long-term vorapaxar treatment may increase the risk of bleeding.[201]

[H1] The complement cascade

The complement cascade has a key role in innate immunity and is involved in the pathogenesis of several immune-mediated, inflammatory and age-related diseases such as osteoarthritis, Alzheimer's disease and age-related macular degeneration **[Au: OK? Such as?]**[Yes].[202] **[Au: I've moved the discussion of complement activation specifically in DKD to the "complement activation in DKD" subsection below to improve flow. Similar to the sections for the other immune-related pathways, please can you provide a brief general introduction to the complement system. I suggest that you briefly describe the function of the complement system and how it is activated (the classical and alternative pathways should be mentioned as well as the lectin pathway) before explaining the roles of the MAC complex and anaphylatoxins.]**[Ok, an introduction was added] The complement system is composed of a large number of plasma proteins, mostly proteases that react with each other by proteolytic cleavage to induce inflammatory responses. There are three distinct pathways (classical, alternative and lectin pathways) that activate the complement cascade through binding to different pattern recognition molecules. The

classical pathway is initiated by binding of C1q to an antigen-antibody complex, whereas the alternative and lectin pathways provide an antibody-independent activation mechanism. The alternative pathway is in a constant state of low-level activation (known as tickover) in which most of the C3b generated by a C3 convertase is hydrolysed (inactivated). When C3b comes into contact with an invading micro-organism it binds and amplification of this pathway is promoted by the binding of C3b to Factor B. The lectin pathway is triggered by binding of lectins **[G]** such as mannose-binding lectin (MBL) and collectin-11 to aberrant carbohydrate moieties expressed on the surface of **[Au: are these damaged or necrotic cells?]**[Yes] pathogens, infected and/or damaged cells, and the activation of MBL-associated serine proteases (MASPs), which cleave C4 and C2 and the subsequent events continue as the classical pathway.[203] **[Au: Is the lectin pathway also activated by carbohydrate stuructures on the surface of pathogens?]** [Yes]All three complement pathways converge at the formation of complement C3, which in turn initiates an enzymatic cascade that leads to the generation of effector molecules including C3a, C5a, C3b, and ultimately C5b-9, the membrane attack complex (MAC). The anaphylatoxins C3a and C5a are potent pro-inflammatory mediators of the innate immune response. C3b also acts as an opsonin that binds to pathogens and promotes phagocytosis. Finally, the MAC inserts into the cell membrane to form a pore that leads to ion flux, osmotic lysis and cell death.[204] .

Complement inhibitors have been tested in clinical trials in various complement-mediated conditions including autoimmune diseases and ischemic stroke.[205] Eculizumab, a humanized antibody against C5, has been approved by the FDA for use in paroxysmal nocturnal hemoglobinuria[206] and atypical hemolytic uremic syndrome (aHUS).[207]

[H2] Complement in the kidney

[Au: I'm not really sure where the highlighted text should go as there isn't much context. Has this been shown in a specific disease? If it relates to DKD it should be moved to the DKD section.][**It is not specific to DKD, but for complement system in the kidney in general. How about moving to the paragraph below?]**

Activation of the complement system has been implicated in many immune-mediated kidney diseases, including aHUS, C3 glomerulopathy, IgA nephropathy, lupus nephritis and kidney transplant rejection. In the kidney, proximal tubular cells can synthesize C3[208] and express a membrane-bound C3-convertase, which can activate the complement cascade leading to the formation of the MAC.[209] Thus, complement-targeting therapies have been investigated as potential treatments for these diseases.[210]

Complement C5a has a role in the progression of aHUS and eculizumab has been shown to improve renal function[207] and prevent progression to ESRD in patients with this disease[211]. Eculizumab has achieved variable treatment responses in C3 glomerulopathy, which is characterized by accumulation of C3 in renal tissue. **[Au: Addition OK?]** **[Ok]**Studies are now underway to explore the therapeutic potential of targeting other components of the complement system in C3 glomerulopathy, for example using inhibitors of complement C3 and factor B and an antibody against MASP2.[212] In IgA nephropathy, clinical studies are investigating the efficacy of therapies that target the lectin pathway (using an antibody against MASP2) and the alternative pathway (using a factor B inhibitor) **[Au: Rather than including the trial numbers, please add the trials to the ref list. References to the trial pages on clincaltrials.gov will be sufficient if no data have been published.]** **[reference**

added]of the complement system.[213]

Complement-induced inflammation has an important role in antibody-mediated rejection (AMR) in kidney transplantation. Binding of donor-specific antibodies (DSA) to mismatched human leukocyte antigens on the graft activates the classical complement pathway.[214] DSA depletion using plasmapheresis combined with intravenous immunoglobulin and increased systemic immunosuppression remains the mainstay of treatment for ABMR. However, the potential role of novel complement-targeting therapies, such as a C1 inhibitor, in this setting is now being investigated.[215]

[H2] Complement activation in DKD

Two main mechanisms are thought to implicate complement in the development of DKD.[216] The first is activation of the lectin pathway in response to glycated proteins that are expressed on the surface of cells as a result of over-exposure to glucose. The second is hyperglycemia-induced glycation of complement regulatory proteins, which leads to dysfunction of their regulatory capacity. For example, CD59, a membrane protein that specifically inhibits MAC formation, is inactivated by glycation of its lysine-41 residue, resulting in increased MAC deposition, which in turn might facilitate auto-activation of complement pathways (Fig. 5).

Complement deposition has been observed in the kidneys of patients with DKD. Increased levels of C5a were detected in the renal tubules and the intensity of C5a staining positively correlated with kidney disease progression in these patients.[217] Differential transcriptome analysis of kidneys from patients with early-stage DKD and matched control samples from nondiabetic individuals showed that complement was the most significantly altered pathway identified by Ingenuity Pathway Analysis

[G][218]. In particular, C7 gene expression was upregulated 2-fold in the serum and kidney tissue of patients with DKD. Complement deposition and clinical data suggest that deposition of C1q and C3c detected by renal immunohistochemistry is associated with more severe renal damage in DKD.[219]

Targeted mass spectrometry enables the quantification of urinary complement proteins. Higher levels of urine factor H-related protein 2, a positive regulator of the alternative complement pathway, were associated with increased mortality risk, whereas urine CD59, an inhibitor of MAC assembly, and urine C4 and C8 were associated with a lower risk of ESRD and death in patients with T2DM and proteinuria.[220] Another study showed that the level of urinary complement activation products correlated closely with tubulointerstitial volume and injury and that elevated levels of these products were independent risk factors for tubular injury in patients with DKD.[221]

In a long-term (55 weeks) diabetic model in rats, C5 inhibition resulted in reductions in C3 staining, urine protein excretion and mesangial expansion.[222] In addition to inhibiting thrombin activity, the cell surface glycoprotein thrombomodulin has an anti-inflammatory function via a lectin-like domain that prevented glucose-induced complement activation in podocytes and endothelial cells *in vitro*[223]. Diabetic mice that expressed thrombomodulin that lacked the lectin-like domain had greater albuminuria, glomerular complement (C3, C5b-9) deposition, mesangial expansion, and renal TGF-β levels than controls that had intact thrombomodulin. Together, these studies in animal models confirm a contribution of complement activation to renal injury in DKD.

[H2] Targeting the complement system in DKD

The potential role of complement inhibitors in the treatment of DKD has been

investigated in experimental studies. In *db/db* mice, inhibition of C5a using a novel aptamer (NOX-D21) improved renal function, reduced tubulointerstitial fibrosis and attenuated upregulation of diacylglycerolacyltransferase-1 and sterol-regulatory element binding protein-1 expression as well as lipid accumulation in the kidney.[217] C5a and C3a receptor antagonists ameliorated endothelial-myofibroblast transition (EndMT) and glomerulosclerosis in the kidneys of rats with STZ-induced diabetes and in human glomerular endothelial cells cultured in high glucose conditions.[224] Similarly, another study showed that treatment with a C3a antagonist improved renal morphology and function in rats with STZ-induced diabetes by suppressing IκBα phosphorylation and TGF-β–Smad3 signaling pathways.[225]

These findings together with clinical and experimental evidence supporting a role of complement in the pathogenesis of DKD, suggest that use of complement-targeting therapies could be a novel approach to the treatment of this disease. However, further research is needed to elucidate the role of C5a and C5aR signaling in the development of inflammation and fibrosis in DKD and, in particular, the role of this signalling in recruiting and activating infiltrating immune cells as well as shifting various intrinsic kidney cells to inflammatory and fibrotic phenotypes. The roles of other components of the complement system such as the upstream C3a receptor and complement regulatory molecules in the development of DKD must also be investigated. The potential adverse consequences of long-term blockade of the complement system in chronic disease, particularly increased susceptibility to infections, remains a concern that must be addressed during the planning of clinical trials.

[H1] Conclusions

Inflammation has a crucial role in the pathogenesis of DKD and data from experimental studies and clinical trials suggest that anti-inflammatory effects might contribute to the renoprotective effects of therapies including SGLT2 inhibitors, GLP1 receptor agonists and endothelin receptor antagonists. In the setting of hyperglycaemia, tissue injury triggers activation of innate immunity via recognition of DAMPs by various PRRs, resulting in a cascade of inflammatory responses. Unresolved inflammation resulting from prolonged renal injury may lead to the development of DKD. Data from experimental and clinical studies suggest an association between various pro-inflammatory pathways including TLR signalling, inflammasomes, the KKS, PAR signalling and the complement system, and progression of DKD. Crosstalk between these cellular stress response pathways might underlie renal inflammation in this disease. Numerous preclinical studies have shown promising results with therapeutic targeting of innate immune pathways in DKD (Table 1), but clinical trials of anti-inflammatory treatments with favorable short-term outcomes are only just beginning to be published.[46,52,70] Importantly, nonspecific anti-inflammatory treatment for DKD may increase susceptibility to infection and therefore be inappropriate for use in this disease, as both diabetes[226] and advanced CKD[227] are associated with immune dysfunction. In the future, improved understanding of the integration and regulation of innate immunity pathways may provide novel, more specific and hence less toxic therapeutic targets for the treatment of DKD.

[Au: As I have moved some text around (mainly in the complement section) please use your software to update the reference list.][Updated]

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Table 1: Effects of modulating innate immunity pathways in experimental DKD

Innate immune pathway	Intervention (agent)	DKD model	Effect	Refs
TLR signalling	TLR4 agonist (CRX-526)	STZ-induced diabetes in eNOS-knockout mice	Reduced renal inflammation, macrophage infiltration and tubulointerstitial fibrosis via suppression of NF-κB activation and TGF-β overexpression	110
	Inhibitor of TLR4, TLR2 and TLR6 signalling (GIT27)	*db/db* mice	Reduced proteinuria	111
NLRP3 Inflammasome	Caspase inhibitor (M-920)	*db/db* mice	Reduced caspase 1, IL-1β, IL-18 and NLRP3 expression; ameliorated albuminuria and renal ECM accumulation	121
	NLRP3 inhibitor (MCC950)	*db/db* mice	Reduced active caspase -1 and IL-β production; ameliorated podocyte injury, renal fibrosis and kidney dysfunction	138
The kallikrein-kinin system	B2K antagonist (icatibant)	Unx-*db/db* mice	Attenuated glomerular and tubulointerstitial injury; reduced renal inflammation and fibrosis	89
	Tissue kallikrein inhibitor (kallistatin)	Unx-*db/db* mice	Reduced renal inflammation and fibrosis via inhibition of NF-κB and TGF-β pathway; attenuated oxidative stress	167
Protease-activated receptor signalling	Factor Xa inhibitor (fondaparinux)	*db/db* mice	Reduced albuminuria, ECM protein deposition and glomerular hypertrophy	189
	Factor Xa inhibitor (edoxaban)	eNOS-knockout diabetic Akita mice	Reduced PAR1 and PAR2 expression; reduced pro-inflammatory and pro-fibrotic responses; ameliorated diabetic glomerulosclerosis	196
	PAR1 inhibitor (vorapaxar)	Mice with STZ-induced diabetes	Reduced albuminuria, glomerular fibronectin deposition and mesangial expansion	199
Complement system	C5a inhibitor (NOX-D21)	Unx-*db/db* mice	Reduced BUN and serum creatinine levels and tubulointerstitial fibrosis; attenuated renal lipid accumulation	217
	C5aR and C3aR antagonist	Rats with STZ-induced diabetes	Ameliorated EndMT and reduced glomerulosclerosis	224
	C3aR antagonist	Rats with STZ-induced diabetes on high fat diet	Ameliorated renal injury; reduced albuminuria and serum creatinine levels; attenuated cytokine production via inhibition of IκBα phosphorylation; reduced ECM deposition via inactivation of TGF-β–Smad3 signaling	225

B2K, B2 bradykinin receptor; BUN, blood urea nitrogen; C3aR, complement C3a receptor; C5a, complement C5a; C5aR, complement C5a receptor; ECM, extracellular

matrix; EndMT; endothelial-myofibroblast transition; eNOS, endothelial nitric oxide synthase; PAR, protease-activated receptor; STZ, streptozotocin; TLR, Toll-like receptor; Unx, uninephrectomized

Figure 1. The role of inflammation in the pathogenesis of diabetic kidney disease. **[Au: Title and edit of this legend OK?]**[Ok] Chronic exposure to diabetic substrates such as high glucose levels and advanced glycation end products damages renal cells, resulting in injury or cell death and the release of intracellular damage-associated molecular patterns (DAMPs) into the extracellular space. This 'danger signal' is recognized by pattern recognition receptors (PRRs) such as Toll-like receptors and nucleotide-binding oligomerization domain (NOD)-like receptors as well as by protease-activated receptors and other cell surface receptors with roles in the kallikrein-kinin system and the complement cascade. Activation of these receptors leads to inflammatory responses in renal cells. Kidney injury also induces the recruitment of bone marrow-derived monocytes into the circulation, the differentiation of monocytes into inflammatory macrophages and the upregulation of adhesion molecules in the vascular endothelium. These processes result in macrophage infiltration into the injured kidney and amplification of the inflammatory response. Chronic, unresolved renal inflammation owing to the activation of various signalling pathways (such as NF-κB, MAPK and TGF-β) eventually results in progressive kidney fibrosis.

Figure 2: Activation of TLR4 signaling in DKD. In response to diabetic stimuli, such as hyperglycaemia, dyslipidaemia and hypoxia, dendritic cells, macrophages and

necrotic cells release high mobility group protein B1 (HMGB1) into the extracellular fluid. The cell surface expression of toll-like receptor 4 (TLR4) is also upregulated in response to high glucose levels. Binding of HMGB1 to TLR4 expressed on tubular epithelial cells promotes TLR4 dimerization, which triggers a downstream inflammatory cascade and the production of reactive oxygen species (ROS). Recruitment of adapter proteins, including myeloid differentiation factor 88 (MyD88), TIR-domain containing adaptor protein (TIRAP), TIR-domain-containing adaptor inducing interferon (TRIF) and TRIF-related adaptor molecule (TRAM), to the intracellular domain of TLR4 leads to translocation of NF-κB to the nucleus and the transcription of genes that encode proinflammatory cytokines, including IL-6, IL-1β and TNF. The transcription of *TLR4* is also regulated by the TLR4–NF-κB pathway via recruitment of TIRAP, activation of PKC and the generation of reactive oxygen species (ROS). The long non-coding RNA Gm6135 upregulates and miR203 suppresses transcription of *TLR4* via binding to the TLR4 promoter.

Figure 3: NLRP3 inflammasome activation in DKD. Activation of the NLRP3 inflammasome in tubular epithelial cells is a two-step process. The priming step involves the binding of PAMPs or DAMPs to TLRs, which leads to MyD88 and NFκB-dependent induction of the transcription of NLRP3, pro-IL-1β and pro-IL-18. In DKD, DAMPs such as HMGB1 are released by dendritic cells, macrophages and necrotic cells in response to hyperglycemia, dyslipidaemia and/or hypoxia. The activation step involves various molecular mechanisms, including increased K^+ efflux, Ca^{2+} influx, the action of pore-forming toxins, mitochondrial dysfunction and reactive oyxgen species (ROS) production. These mechanisms trigger recruitment of the adaptor protein ASC to bind to NLRP3. Interaction of the resulting complex with

pro-caspase 1 (pro-CASP1) forms the inflammasome complex, which cleaves pso-CASP1 to form caspase 1. In turn, caspase 1 cleaves pro-IL-1β and pro-IL-18 to form the pro-inflammatory cytokines IL-1β and IL-18, which are secreted from the cell and induce inflammatory responses.

Figure 4: The roles of the KKS and PARs in DKD. Activation of the kallikrein-kinin system (KKS) is primarily mediated by kinins, including bradykinin and kallidin. These kinins are generated from high molecular weight (HMW) and low molecular weight (LMW) kininogens by plasma kallikrein and tissue kallikrein, respectively. Under physiological conditions, binding of bradykinin and kallidin to the B2 bradykinin receptor (B2R) expressed on the surface of tubular epithelial cells protects the kidney against oxidative stress, inflammation and fibrosis via the production of nitric oxide (NO) and prostaglandins (PGs). In response to hyperglycaemic conditions in DKD, the levels of plasma and tissue kallikrein, the coagulation enzymes thrombin, trypsin and factor Xa and the bradykinin B1 receptor (B1R) are increased. Binding of the bradykinin and kallidin metabolites, des-Arg^{9}-bradykinin and des-Arg^{10}-kallidin, to B1R induces a NFκB-dependent pro-inflammatory response. In addition, stimulation of B2R leads to a pro-inflammatory pathway via activation of MAPK signalling. Tissue kallikrein and coagulation enzymes such as thrombin, trypsin and factor Xa, can also stimulate pro-inflammatory and pro-fibrotic pathways via activation of protease-activated receptors (PARs). These enzymes directly cleave the N-terminus of the PAR to expose a tethered ligand that induces intracellular signaling pathways such as calcium (Ca^{2+}) flux and MAPK signaling. During high glucose-induced tissue injury, coagulation enzymes are upregulated and activate PARs, leading to cell proliferation,

inflammation and fibrosis. Kallistatin is an endogeneous tissue kallikrein inhibitor that regulates the activity of kallikrein and the production of kinins.

Figure 5: Potential mechanisms of complement activation in DKD. During diabetes, hyperglycaemia leads to increased glycation of proteins. Glycated proteins are recognized by mannan-binding lectin (MBL) and increase its autoreactivity, resulting in activation of the lectin pathway of complement activation. Activated MBL-associated serine proteases (MASPs) initiate an enzyme cascade that cleaves C4 and C2, leading to the generation of C3 convertase (C4bC2a) and ultimately the membrane-attack complex (MAC, also known as C5b-9). Hyperglycemia also induces glycation of complement regulatory proteins leading to dysfunction of their regulatory capacity. For example, glycation of CD59 abrogates its inhibitory effect on MAC formation, resulting in increased MAC deposition and potentially autoactivation of the complement cascade.

Glossary

Sterile inflammation

Pathogen-free inflammation triggered by damage-associated molecular patterns (DAMPs) that are released by cells in response to stress.

Homeostasis model assessment–insulin resistance (HOMA-IR)

A method for evaluation of insulin sensitivity from the basal (fasting) blood glucose and insulin levels.

Factor V Leiden (FVL) mutation

A genetic point mutation (R506Q) in the gene that encodes human coagulation factor V that results in resistance of factor V to inactivation by activated protein C and an

increase in blood clotting. Carriers of the FVL mutation have an increased risk of venous thrombosis.

Ingenuity Pathway Analysis (IPA)

A web-based software application for analysis, integration and interpretation of data from high-throughput experiments such as next generation sequencing and microarray. IPA aids in the identification of key regulators and activities of biological systems.

Lectins

Pattern recognition molecules that contain a C-type lectin domain (also known as a carbohydrate recognition domain).

ToC blurb

Increasing evidence suggests that inflammation contributes to the development and progression of diabetic kidney disease (DKD). Here the authors discuss the mechanisms by which innate immune pathways might contribute to DKD as well as the therapeutic potential of targeting these pathways.