

# Inflammation as a Potent Therapeutic Target for Interventions in Diabetic Nephropathy

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## Abstract

Microvascular disease following diabetes-induced hyperglycemia results in abnormalities in blood flow and increased vascular permeability, which affects renal glomeruli. Diabetic nephropathy is the most common cause of end-stage renal disease, characterized by metabolic and hemodynamic alterations that precede fibrosis and proteinuria. Diabetic nephropathy is regarded to be initiated by damaging metabolic end products and changes in the vascular tone, therefore receiving the most therapeutic attention. However, growing evidence indicates that there is an inflammatory component in the pathological process. In the diabetic milieu, activation of renal cells and circulating leukocytes results in the production of cytokines and chemokines that commence and sustain an inflammatory response and eventually causes renal injury. Classically, most research is focused on monocyte/macrophage recruitment and activation in glomerular disease. Monocyte chemoattractant protein-1 (MCP-1) produced by renal cells attracts monocytes to glomeruli and tubulointerstitium. Subsequently, hallmark proinflammatory molecules (e.g. TNF- $\alpha$ , TGF- $\beta$  and IL-1) produced by recruited leukocytes augments chemokine production and fibrosis. These processes appear in a relatively small environment surrounding the glomerular basement membrane (GBM), mainly regulated by the glomerular endothelial cells and podocytes. Increasing knowledge indicates that more distant cell types participate in the progression of pathology as well. Tubular expression of Toll-like receptor 4 (TLR4) promotes inflammation in an NF- $\kappa$ B related manner. Several processes are involved in perspective of fibrosis, varying from epithelial- and endothelial-to-mesenchymal transition to deposition of distant fibrocytes into renal tissue and even dysregulation of mesenchymal stem cells. The increasing body of evidence on the inflammatory component provides new possible targets for interventions in diabetes-induced kidney injury. Targeting transcription factors like NF- $\kappa$ B or downstream mediators such as VEGF has shown beneficial effects in previous studies. Therefore, this review provides a broad overview of the major inflammatory mediators and cellular contributors in diabetic disease and their role in the development and progression of diabetic nephropathy.

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## Introduction

Patients suffering from either type 1 or type 2 diabetes mellitus are characterized by hyperglycemia and consequently micro- and macrovascular disease<sup>[1]</sup>. In the classical definition, microvascular disease is highly pathogenic to delicate vessel systems like the renal glomeruli due to abnormalities in blood flow and increased vascular permeability<sup>[2]</sup>. Dysfunction of the glomerular capillaries, resulting from multiple processes causing metabolic and hemodynamic changes, has been regarded as main origin of diabetic nephropathy<sup>[3][4]</sup>. Diabetic nephropathy is currently the most common cause of end-stage renal disease, characterized by morphological and functional alterations which lead to a progressive decline in renal function and glomerulosclerosis<sup>[5][6]</sup>. Even though metabolic and hemodynamic alterations accounting for the development of diabetic nephropathy are mostly treated by insulin treatment and blockade of the renin-angiotensin aldosterone system (RAS), 25% patients nevertheless progress to end-stage renal disease<sup>[7][8]</sup>. In diabetic conditions, the augmented metabolic behavior of renal cells causes oxidative stress. Excessive production of reactive oxygen species (ROS) has been linked to vasoconstriction and transforming growth factor (TGF)- $\beta$  induced tissue fibrosis and extracellular matrix (ECM) production<sup>[3][9]</sup>. Furthermore, due to its high toxicity to cells, ROS accounts for vascular smooth muscle cell growth and migration, and endothelial dysfunction. Therefore, ROS are regarded as a major contributor in the development of diabetic kidney disease<sup>[9]</sup>. On the other hand, hemodynamic changes include hyperperfusion and hyperfiltration in the glomeruli, which facilitates protein leakage out of the capillaries<sup>[10]</sup>. Abnormalities in blood pressure occur during the development of diabetic nephropathy and hypertension is a critical factor in the progression of the disease<sup>[11]</sup>. Elevation of the glomerular vascular tone further develops glomerulosclerosis via podocyte injury, mesangial cell ECM overproduction and glomerular basement membrane (GBM) thickening. The hemodynamics in the diabetic environment are under regulatory control of multiple factors including vascular endothelial growth factor (VEGF)-A, nitric oxide (NO) and even the cytokine TGF- $\beta$ <sup>[10]</sup>. The glomerular arteriolar tone is affected by multiple factors including NO and the RAS, of which its bioactivity is influenced by hyperglycemia<sup>[12]</sup>. Following hyperglycemia, a strong increase in intrarenal production of angiotensin II causes vasoconstriction of the efferent renal artery contributing to the progression of diabetic nephropathy<sup>[13][14]</sup>.

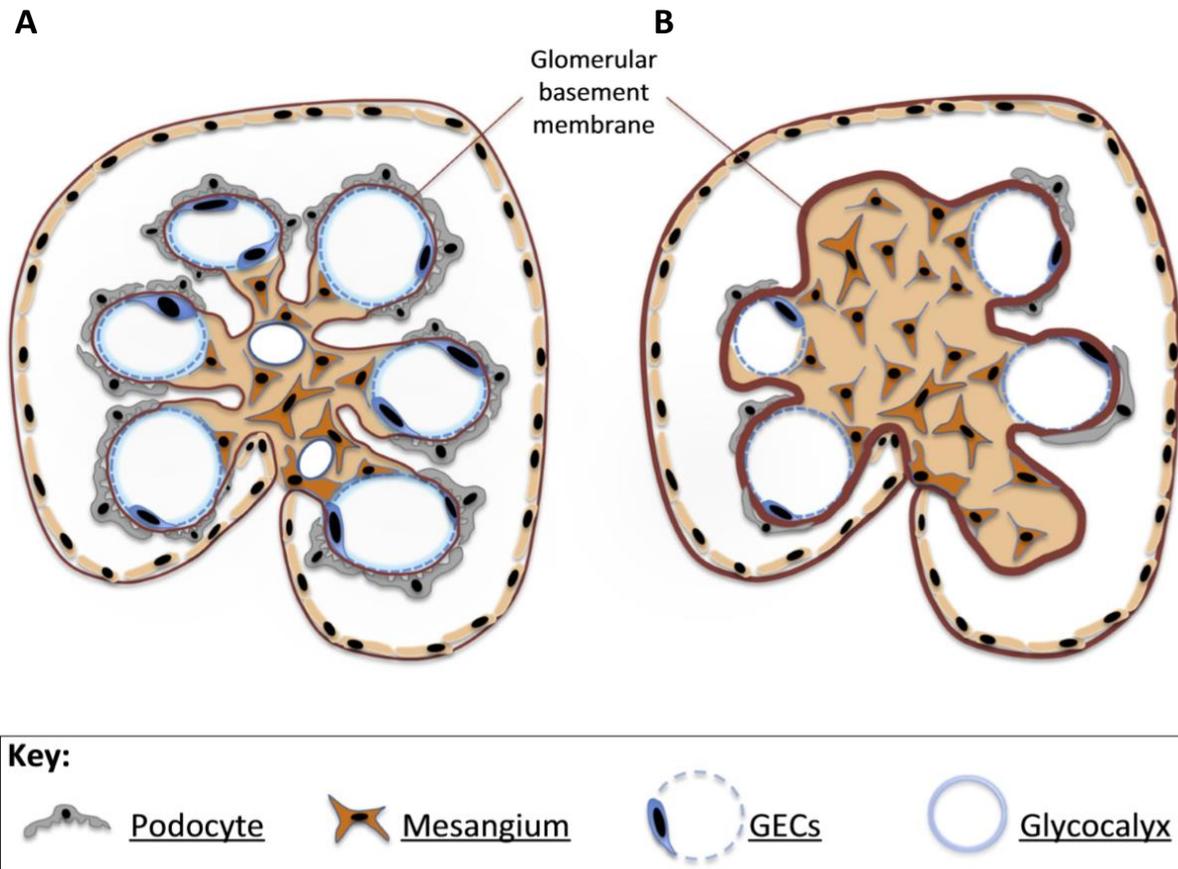
However, ROS overproduction fulfills a regulatory role as well, since it can initiate an inflammatory response by inflammasome activation<sup>[15]</sup>. The progression of diabetic nephropathy is under regulatory control of cytokines, which hints towards the involvement of an inflammatory component in the pathological process. Accordingly, research shifted its focus towards the inhibition of inflammation to slow the progression of diabetic kidney disease<sup>[16]</sup>. Nowadays, chronic inflammation is regarded as a key initiator of the development of diabetes-associated vascular pathology such as diabetic nephropathy<sup>[15]</sup>. Diabetic patients and animal models display an increased macrophage infiltration through overproduction of chemoattractants (e.g. monocyte chemoattractant protein-1 (MCP-1), also known as chemokine (C-C motif) ligand (CCL)2) and leukocyte adhesion molecules (i.e. integrins) in their kidneys<sup>[3]</sup>. It is thought that the macrophage influx and the successive fibrosis result from a combination of proinflammatory cytokines, chemokines and growth factors<sup>[16]</sup>. Especially the effect of tumor necrosis factor (TNF)- $\alpha$  and TGF- $\beta$  on macrophages, which both are upregulated by ROS, has extensively been studied. TNF- $\alpha$  produced by activated macrophages sustains the inflammatory response by stimulation of macrophage recruitment via MCP-1<sup>[17]</sup>. TGF- $\beta$  produced by activated renal cells recruits fibroblasts, augments their proliferation via c-Myc overexpression, and stimulates differentiation to myofibroblasts, leading to fibrosis<sup>[18]</sup>. However, there are many more components and cellular contributors in the inflammatory pathways to consider. Instead of solely

focusing on the role of the inflammatory cells, which mainly consists of monocytes/macrophages, cells located around the GMB (being glomerular endothelial cells, podocytes and mesangial cells) are of major importance in the initiation and propagation of the inflammatory response. In response to high glucose, advanced glycation end products (AGEs) and mitochondrial ROS, glomerular endothelial cells and podocytes exert proinflammatory behavior, whereas mesangial cells accumulate extracellular mesangial matrix<sup>[15][19]</sup>. Mesangial cells alter phenotypically to an embryonic state that contributes to an mesangial expansion and glomerulosclerosis<sup>[20]</sup>. Besides the glomerular cells, the interstitial tubules participate in and get affected by inflammation as well. Tubular toll-like receptor (TLR)4 expression correlates with interstitial macrophage infiltration and hemoglobin A1c level, a hallmark of diabetes<sup>[5]</sup>. Furthermore, mice suffering from diabetic nephropathy show a reduction of potent stem cell populations, implicating a deteriorated repair capacity<sup>[21]</sup>. Among these stem cells are the mesenchymal stem cells that have the potency to differentiate into endothelial cells and to regulate the immune response, offering a possible applicability as a treatment in diabetic nephropathy<sup>[22]</sup>. However, resident mesenchymal stem cells frequently contribute to fibrosis via differentiation to myofibroblasts, regulated by local signaling molecules<sup>[23]</sup>. Altogether, diabetic inflammation seems a promising but complex therapeutic target that deserves better understanding. This review attempts to offer a broad perspective of the entire diabetic inflammatory response, from its circulating molecules to the host cellular response and the subsequent signal transduction, in order to better understand all of its components and contribution to diabetic nephropathy.

### **Diabetes-induced inflammation in the glomerulus**

Traditionally, diabetic nephropathy is regarded as a glomerular disease with albuminuria as a prominent outcome measure preceding end-stage renal disease<sup>[24]</sup>. The classical classification of diabetic nephropathy describes several developmental stages, which are focused on characteristics of dysfunction of the glomerular filtration barrier (see Fig. 1)<sup>[26]</sup>. During the initiation of diabetic nephropathy, hyperglycemia is the biggest thread for the glomerulus, especially the endothelial cells. The endothelium of the microvasculature is the primary barrier between the blood and the subsequent tissue and serves as a modulator of the vascular tone, hemostasis, maintenance of smooth muscle cells and inflammation<sup>[24]</sup>. Endothelial dysfunction has always been described as the inability to initiate NO-mediated vasodilation to relieve vasoconstriction. It was believed that, due to hyperglycemia, the level of endothelial NO synthase (eNOS) is reduced in diabetic nephropathy, resulting in a lower availability of NO and inducing endothelial dysfunction<sup>[27]</sup>. Furthermore, hyperglycemia upregulates the expression of the cannabinoid 1 receptor, which is suggested to play a critical role in diabetic nephropathy<sup>[28]</sup>. Mainly in podocytes, stimulation of the cannabinoid 1 receptor aggravates various pathological processes during glomerulopathy, including oxidative stress. When blood glucose levels are therapeutically reduced in diabetic nephropathy to normoglycemic levels, RAS signaling is implied to induce cannabinoid 1 receptor activation as well<sup>[28]</sup>. However, the pathogenesis of endothelial malfunctioning consists of a more complex metabolic syndrome and the upregulation of inflammatory markers<sup>[24]</sup>.

In response to high glucose, endothelial cells elevate the expression of TLR and adhesion molecules and start producing cytokines, resulting in macrophage infiltration<sup>[15][29]</sup>. TLRs are a subset of innate immune receptors that are expressed on both immune cells (e.g. macrophages and T-cells) and renal cells including endothelial cells, podocytes and tubular epithelial cells<sup>[30]</sup>. Of these TLRs, TLR2

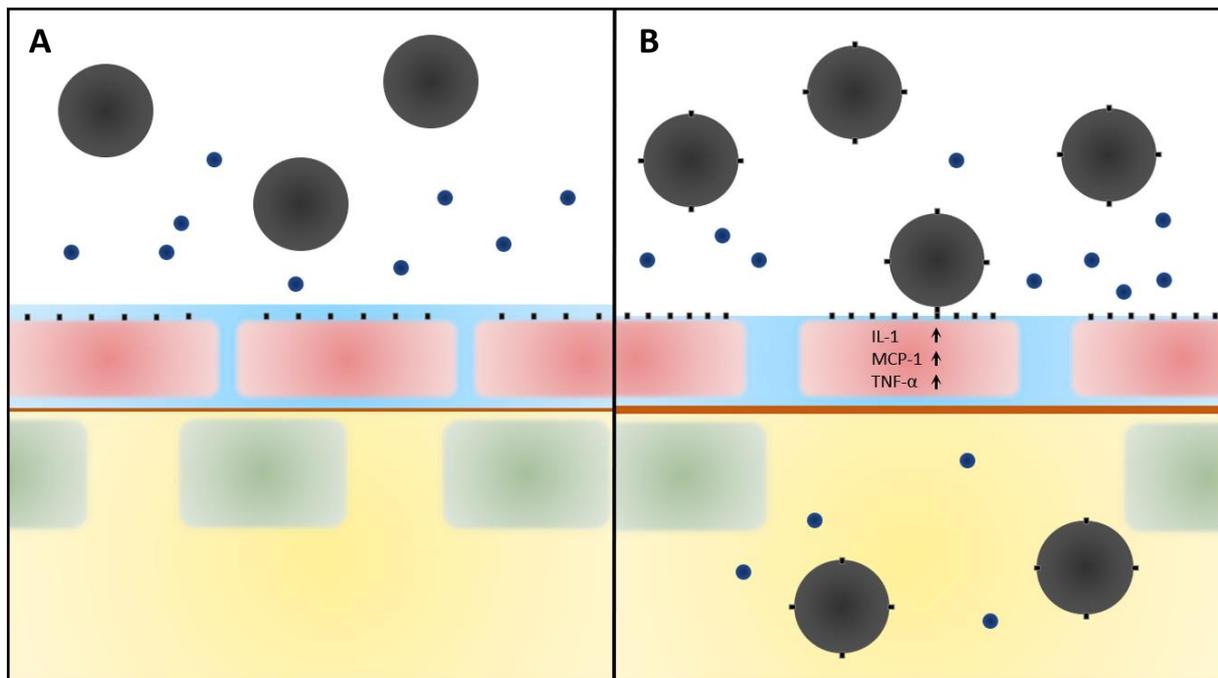


**Figure 1** The structure of the glomerulus in (A) healthy conditions and (B) diabetic nephropathy. During diabetic glomerulopathy, extensive mesangial expansion and glomerular basement membrane thickening could be noticed. Meanwhile, general loss of podocytes and glomerular endothelial cells (GECs), accompanied by the glomerular endothelial glycocalyx, occurs. Adopted from Ref. [25].

and TLR4 are overexpressed in glomerular cells suffering from diabetic nephropathy. Even though TLR2 is not directly upregulated by hyperglycemia, its expression is somehow elevated in diabetes-induced renal disease<sup>[15]</sup>. Stimulation of TLR2 enhances albuminuria and the production of IL-6 and TNF- $\alpha$ , while inhibition attenuates renal hypertrophy, podocyte loss and reduces pro-inflammatory behavior of macrophages. Similarly, TLR4 is associated with the upregulation of cytokines and chemokines and is regarded as a promising target to suppress inflammation in the glomeruli and loss of renal function<sup>[15]</sup>. Diabetic mice deficient for TLR4 are protected against inflammation, glomerular and tubular injury, interstitial fibrosis and albuminuria<sup>[30]</sup>. TLR activation, which generally occurs via bacterial or viral component recognition, can be induced in so-called sterile diseases such as diabetes via damage-associated molecular patterns (DAMPs)<sup>[30][31]</sup>. TLR2 activation in podocytes, combined with hyperglycemia-induced cellular hypertrophy and cell cycle arrest, results in increased apoptosis and eventually proteinuria and fibrosis<sup>[15][20]</sup>. Cellular debris resulting from delayed and incomplete removal of apoptotic bodies can initiate an inflammatory response via TLR activation in progressive renal diseases like diabetic nephropathy<sup>[31]</sup>.

TLR activation and TNF signaling induce nuclear factor-kappa B (NF- $\kappa$ B) signal transduction when stimulated<sup>[15][32]</sup>. NF- $\kappa$ B is a transcription factor closely related to many processes occurring in diabetic nephropathy and fulfills a key regulatory role in the pathogenic production of chemokines, adhesion molecules and cytokines<sup>[32]</sup>. Chemokines interact with and recruit inflammatory cell, mediating cell migration and infiltration. The most infamous chemokine is MCP-1 which, as its name

implies, predominantly interacts with monocytes and macrophages. In the glomerulus, MCP-1 is mainly produced by the mesangial cells and its production is increased by high levels of blood glucose, ROS and cytokines like IL-1, TNF- $\alpha$  and TGF- $\beta$ <sup>[33]</sup>. Beside molecular components, MCP-1 excretion by mesangial cells can directly be induced via mechanical stretching and the consequent angiotensin II expression, that fortifies when mesangial expansion is displayed<sup>[32]</sup>. MCP-1 is upregulated in the hypertensive kidney and circulation monocytes exacerbate the protein expression<sup>[34]</sup>. Simultaneously, NF- $\kappa$ B signaling upregulates intercellular adhesion molecule (ICAM)-1 and vascular cell adhesion molecule (VCAM)-1, which is strongly influenced by high glucose<sup>[35][36]</sup>. ICAM-1 and VCAM-1 are involved in the attachment of leukocytes to the vascular wall and infiltration into the subsequent tissue. Both adhesion molecules are overexpressed in the early development of nephropathy and the diabetic environment aggravates the adhesion of predominantly monocytes to the glomerular endothelial cells<sup>[35][36]</sup>. Animal models lacking ICAM-1 are resistant to diabetic nephropathy, while VCAM-1 expression correlates with the number of infiltrating leukocytes and severity of the disease<sup>[37]</sup>. Following tissue infiltration, leukocytes produce proteolytic enzymes which damage the renal tissue and cause organ damage via fibrosis. Figure 2 displays a simplified image of the processes involved in leukocyte infiltration in healthy conditions and diabetic nephropathy.



**Figure 2** The interactions of the glomerular filtration barrier with leukocytes in (A) healthy conditions and (B) diabetic nephropathy. In healthy circumstances, leukocytes (shown in grey) in the blood cannot attach to the glomerular endothelial cells (red) due to a thick glycocalyx (light blue), since the integrins (ICAM-1, VCAM-1; black) are covered by the proteoglycan layer. Neither leukocytes or albumin (dark blue) can surpass the glomerular basement membrane (GBM; orange), so these are not present in the pre-urine (yellow). In diabetic nephropathy, however, the glycocalyx is reduced, exposing the integrins and enabling adhesion. Simultaneously, cytokine production by endothelial cells activates leukocytes, upregulating integrin expression. Leukocytes and albumin are able to cross the thickened GBM and injure podocytes (green), and are abundant in the pre-urine.

Although most research focusses on macrophages when investigating infiltrating white blood cells in diabetic nephropathy, the consistence of the leukocyte subsets is more complex. Besides the proteolytic and pro-inflammatory cytokine producing M1 macrophages, the main subtype contributing to the renal pathology, anti-inflammatory M2 macrophages modulating tissue repair, remodeling and neovascularization are present as well<sup>[37]</sup>. M2 macrophages attempt to control the inflammatory

response via production of IL-10, the commonly known anti-inflammatory cytokine, and TGF- $\beta$ 1. Macrophage differentiation is an actively regulated and reversible process, in which VEGF seems to play an important role. In pregnancy research, it was uncovered that VEGF polarizes macrophages towards the M2 phenotype<sup>[38]</sup>. Even though it was initially believed that VEGF was pathogenic in diabetic nephropathy, upregulated by inflammation-associated factors (AGE, TGF- $\beta$ , ROS etc.), beneficial effects are frequently reported. Actually, anti-VEGF therapy in patients is deteriorating for kidney disease, contributing to hypertension and proteinuria<sup>[39]</sup>. Podocyte loss in diabetic nephropathy has a deteriorating effect on VEGF levels, since they are the predominant source of its production. On the other hand, CD4 and CD8 T lymphocyte accumulation in the glomerulus is associated with the development of proteinuria in diabetic nephropathy. Activated lymphocytes produce an endoglycosidase called heparanase, an enzyme that cleaves heparan sulphate, one of the major constituents of the GBM and the glomerular endothelial glycocalyx<sup>[32][40]</sup>. Enzymatic cleavage of heparan sulphate in the GBM does not directly lead to proteinuria<sup>[41]</sup>. Digestion of heparan sulphate in the glomerular endothelial glycocalyx, however, leads to reduction of glycocalyx thickness and the onset of albuminuria<sup>[42]</sup>. The glomerular endothelial glycocalyx is a complex surface layer consisting of polysaccharides and proteoglycans important for the modulation of vascular permeability, coagulation and inflammation<sup>[43]</sup>. A disrupted glycocalyx, which occurs in diabetic disease due to high glucose levels, results in leakage of macromolecules through the GBM and increased leukocyte adhesion to endothelial cells via enhanced exposure of adhesion molecules<sup>[43][44]</sup>. Sulfation of endothelial heparan sulfate facilitates binding to adhesion molecules<sup>[45]</sup>. Inhibition of heparan sulphate sulfation reduces macrophage accumulation despite high levels of MCP-1, preventing inflammation and fibrosis.

As previously stated, glomerular dysfunction and the consecutive NF- $\kappa$ B signaling aids to the production of cytokines. In combination with infiltrating leukocytes as a source of cytokine excretion, interleukins (e.g. IL-1, IL-6 and IL-18) and TNF- $\alpha$  are excreted, thereby sustaining inflammation. IL-1 is capable of inducing adhesion molecule expression and its presence is elevated in diabetic nephropathy<sup>[17]</sup>. It is believed that AGEs and mitochondrial ROS due to high glucose provoke inflammasome activity in endothelial cells and podocytes, thereby causing a rise in IL-1 production and initiating an innate immune response<sup>[15]</sup>. IL-1 is implicated to develop intraglomerular hemodynamic abnormalities<sup>[32]</sup>. IL-6, which is produced by both resident endothelial and mesangial cells as infiltrating leukocytes, accounts for glomerular endothelial cell abnormalities and mesangial proliferation and ECM production<sup>[32][46]</sup>. IL-6 serum levels are specifically upregulated in diabetic patients suffering from albuminuria and its mRNA expression correlates with the urinary albumin excretion, implicating that IL-6 plays a part in the later stages of diabetic nephropathy<sup>[3]</sup>. IL-18 is produced by infiltrating T-lymphocytes, macrophages and monocytes, and proximal tubular cells<sup>[37]</sup>. Its most prominent effect is the stimulation of mesangial cells to produce chemokines and intensify the levels of IL-1, IL-6 and TNF- $\alpha$ <sup>[3][37]</sup>. TNF- $\alpha$  is produced by monocytes and macrophages and initiates a local inflammatory response, recruiting macrophages and neutrophils to its site<sup>[46]</sup>. TNF- $\alpha$  is regarded as a pivotal contributor to diabetic nephropathy, since it has cytotoxic effects on renal cells itself and is involved in many regulatory processes of renal pathology<sup>[32]</sup>. Most of its execution function via NF- $\kappa$ B activation, which contributes cytokine production, the inflammatory response, leukocyte adhesion and apoptosis<sup>[46]</sup>. Thereby TNF- $\alpha$  sustains and exacerbates inflammation, resulting in a deterioration of the kidney damage in diabetic nephropathy<sup>[32]</sup>.

Inflammation in diabetic nephropathy is not limited to cellular activity and production of inflammatory mediators. There are several pathways directly affected by the diabetic milieu, contributing to cell

dysregulation. The abundance of glucose availability causes a higher metabolic turnover and enhanced glycolysis in renal cells, resulting in cell injury<sup>[1]</sup>. Mitochondria of high glucose exposed cells produce excessive amounts of ROS, causing DNA damage. Indirectly, via the glycolysis intermediate glucose-6-phosphate, glucose is converted into fructose by activation the polyol pathway<sup>[47]</sup>. Activation of the polyol pathway upregulates ROS and NF-κB activation and is recently uncovered as a pathogenic contributor in diabetic proximal tubule cells<sup>[48]</sup>. Further downstream, enhanced glucose catabolism elevates the levels of glycolysis intermediate protein kinase C, resulting in upregulation of NF-κB as well<sup>[1]</sup>.

Substantial evidence dedicates a considerable role to the complement system in diabetic nephropathy. Activation of the complement system has extensive regulatory effects regarding inflammation, affecting apoptotic cell clearance, T-cell immunity and TLRs<sup>[49]</sup>. Hyperglycemia can directly and indirectly activate complement deposition, which functions pro-inflammatory via upregulation of protein kinase C and NF-κB<sup>[50]</sup>. Overactivation of complement is pathogenic, which predominantly occurs via the lectin pathway. Due to high glucose, mannose-binding lectin binds to advanced glycosylated proteins on the cell surface of renal cells<sup>[1]</sup>. Consequently, the complement cascade is activated which via cleavage of hallmark complement factors C3 and C5 triggers both inflammation and the formation of the membrane attack complex (MAC)<sup>[51]</sup>. In diabetic nephropathy, augmented glomerular levels of C3 and raised mRNA expression of C3 and C5 in the kidneys has been detected, along with the consecutive complement factors<sup>[1]</sup>. Activation of complement factors C5 to C9, which ultimately forms the MAC, are correlated with the severity of disease, as well as the abundance of deposition<sup>[50]</sup>. Furthermore, MAC deposition is increased in diabetic glomeruli. MAC insertion in the podocyte membrane results in cell injury and therefore is labeled as a major contributor to proteinuria<sup>[52]</sup>.

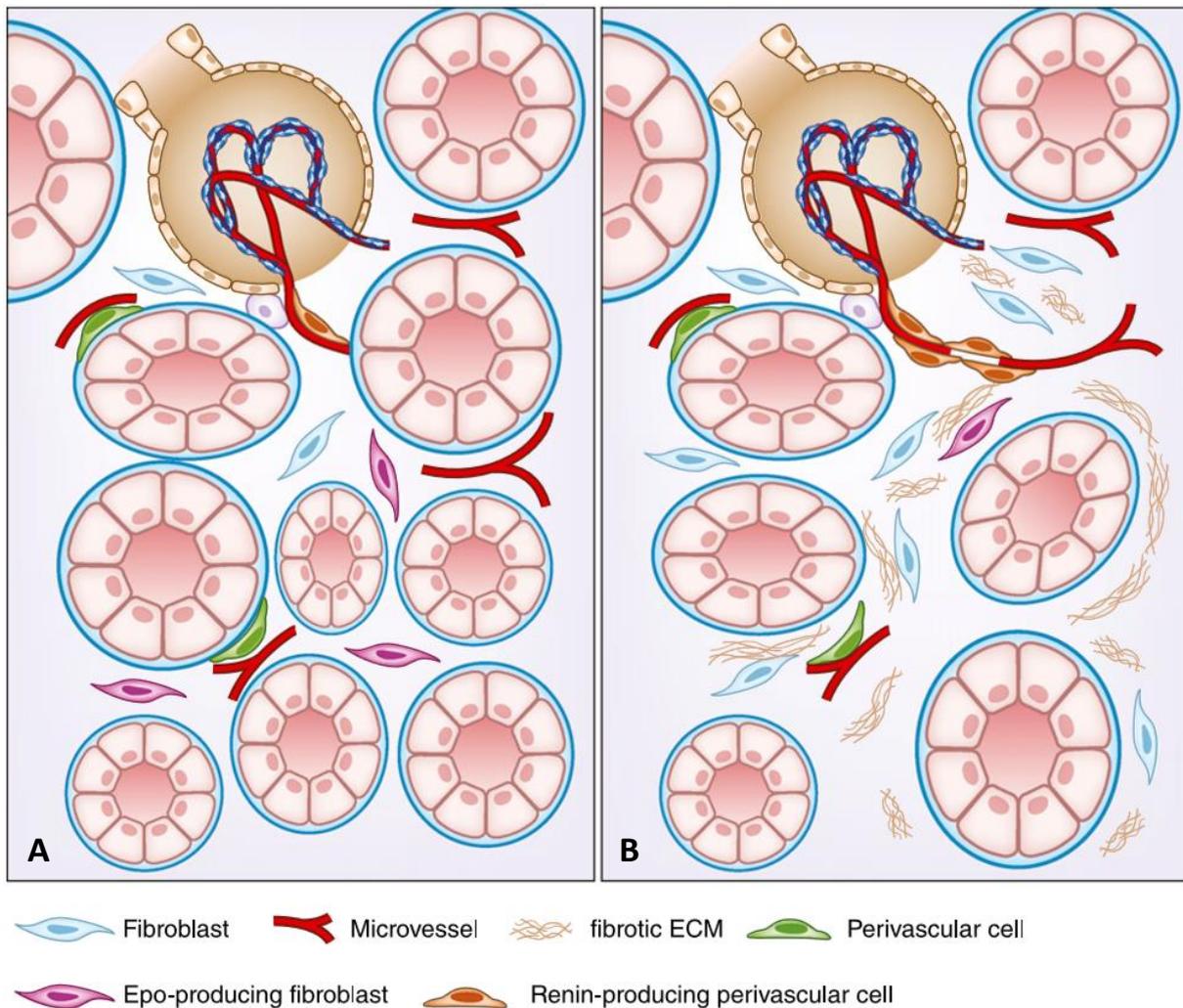
### **Diabetes-induced inflammation in the tubulointerstitium**

As previously stated, the renal glomeruli have been the priority in diabetic nephropathy. However glomerular disease is present in only one-third of type 2 diabetes patients with albuminuria<sup>[53]</sup>. Several type 1 and type 2 diabetes patients, however, display more advanced interstitial lesions, in which the deterioration of kidney disease correlates better with the tubular and interstitial alterations than the glomerular pathology<sup>[53][54]</sup>. Even stronger, tubulointerstitial injury eventually determines the rate of regression of renal function in diabetic nephropathy<sup>[55]</sup>. Tubulointerstitial fibrosis and inflammation are considered hallmarks of the pathogenesis of diabetic nephropathy, and both associated with hyperglycemia<sup>[1]</sup>. Tubular cells are directly affected by hyperglycemia due to alterations in metabolic behavior<sup>[53]</sup>. The consequent production of vasoactive mediators induces systemic and intraglomerular hypertension, leading to tubular and interstitial dysfunction in a non-glomerular manner. Hyperglycemia worsens by upregulation of the sodium/glucose cotransporter 2 (SGLT2), which mediates glucose reabsorption in the proximal tubules<sup>[25]</sup>. Besides hyperglycemia, protein leakage out of the glomerular capillaries accounts for tubulointerstitial injury<sup>[56]</sup>. Albumin initiates an immune response via activation of TLR2 and TLR4, also upregulated by hyperglycemia in the tubulointerstitium, associated with renal injury and kidney disease. The tubulointerstitium, accounting for over 90% of the volume, encompasses several interacting compartments surrounded by ECM, being the tubular epithelium of different nephron segments, interstitium and peritubular capillaries (see Fig. 3A)<sup>[58][59]</sup>.

Similar to the pathology in the renal glomeruli, the tubular epithelial cells display dysfunction in response to hyperglycemia in diabetic nephropathy. TLR activation is of major importance in the dysregulation of tubular epithelial cells as well. TLR2 may not be upregulated in renal tubules of diabetic nephropathy patients, inhibition of TLR2 significantly reduces NF- $\kappa$ B activation, implying an important mediatory role in the pathology<sup>[15][30]</sup>. TLR4, however, is upregulated in diabetic tubular epithelial cells, supporting the promotion of inflammation by mediation of inflammatory factors and kidney fibrosis<sup>[30]</sup>. Besides TLRs, Notch signaling is upregulated in the renal diabetic milieu. Notch signaling is essential during the development of tubular epithelial cells, however, causes pathogenicity when reactivated in adult tissue following high glucose, TGF- $\beta$ , VEGF or ischemia<sup>[60]</sup>. In proximal tubular epithelial cells, Notch signaling promotes the development tubulointerstitial fibrosis via increased peritubular inflammatory cell infiltration and proliferation of fibroblasts. Besides, Notch signaling mediates TGF- $\beta$  induced epithelial-to-mesenchymal transition (EMT) and gene regulatory processes, which accumulate ECM contributing to fibrosis<sup>[60][61]</sup>. Furthermore, hyperglycemia induces cellular senescence in tubular cells via p21-related cell cycle arrest<sup>[62]</sup>. These senescent tubular cells in the interstitium produce cytokines such as TNF- $\alpha$  that induce apoptosis and inflammation, thereby resulting in a reduced tubular function<sup>[63]</sup>. Expression of the TNF superfamily cytokines is the greatest in renal tubular epithelial cells and to some extent podocytes, and is linked to tubular atrophy and interstitial fibrosis accompanying the interstitial inflammation in diabetic nephropathy<sup>[32]</sup>.

Activation of the NF- $\kappa$ B pathway in tubular epithelial cells by members of the TNF superfamily, which are implied in glomerular and tubulointerstitial inflammation, promotes the release of MCP-1 and CCL5, also known as RANTES<sup>[64]</sup>. NF- $\kappa$ B signaling is also elevated in peripheral blood mononuclear cells of diabetic nephropathy patients, suggesting involvement of systemic inflammation in pathological process<sup>[65]</sup>. TNF-induced NF- $\kappa$ B signaling however could be inhibited via  $\alpha$ 7 nicotinic acetylcholine receptor (nAChR) stimulation, which reduces production of TNF- $\alpha$  by macrophages<sup>[66]</sup>. Agonists targeting the  $\alpha$ 7 nAChR have shown anti-inflammatory effects in proximal tubular cells<sup>[67]</sup>. MCP-1 overexpression, however, induces pathology in the tubulointerstitium in a similar way as in the glomerulus, resulting in macrophage infiltration<sup>[3]</sup>. Pro-inflammatory M1 macrophages amplify MCP-1 abundance, worsening tubulointerstitial damage<sup>[34]</sup>. Whereas MCP-1 is of major importance in the glomeruli, CCL5 has a similar activation and macrophage recruitment activity in the tubulointerstitium suffering from diabetic nephropathy<sup>[65][68]</sup>. However, CCL5, presented on lymphocytes, fibroblasts, mesangial cells and renal tubular cells, has the potency to attract T lymphocytes as well, predominantly the CD4-positive subset<sup>[69]</sup>. However, the contribution of CCL5 to renal pathology remains unclear, since genetic deletion of CCL5 leads to increased glomerular and interstitial injury, and albuminuria<sup>[34]</sup>. How CCL5-directed T lymphocyte infiltration functions is still not completely understood, however, CCL5 is strongly upregulated in diabetic nephropathy patients and the increase correlates with disease severity<sup>[32]</sup>. In the tubulointerstitium, CXCL10, better known as interferon  $\gamma$ -induced protein-10 (IP-10), is upregulated by diabetes-induced endothelial cells damage and this leads to a stronger influx of T cells<sup>[69]</sup>. Reduction of IP-10 expression results in less T cell infiltration and improved renal function, without affecting macrophage behavior.

Recruitment of macrophages and T lymphocytes combined with activation and injury of tubular epithelial cells, affects the constitution of the interstitium. The interstitium is described as intertubular space, with the exception of the glomeruli and the interstitial vasculature<sup>[57]</sup>. The interstitium harbors cells (mainly various types of fibroblasts), leukocytes, ECM and interstitial fluid. In physiological conditions, renal fibroblasts are relatively inactive cells, aiding to the structural support



**Figure 3** The composition of the tubulointerstitium in (A) healthy conditions and (B) diabetes-induced fibrosis. In healthy circumstances, the interstitium consists of a balanced number of supportive fibroblasts and antifibrotic Epo-producing fibroblasts in ECM, surrounding the tubules and the peritubular capillaries. Perivascular cells, better known as pericytes, and renin-producing perivascular cells are located on the interstitial microvasculature. During fibrosis, fibroblasts proliferate and get activated, producing pathological amounts of ECM, and Epo-producing fibroblasts are lost. Renin-producing perivascular cells number go up, upregulating RAS signaling and deteriorating microvascular complications, without affecting normal pericyte abundance. Adopted from Ref. [57].

through ECM deposition and direct cell-cell interactions. During pathophysiology, however, the interstitium significantly expands to over 60% of the kidney tissue, through accumulation of fibroblasts and their deposited ECM, and infiltrating mononuclear cells<sup>[57]</sup>. Furthermore, many resident cell types (e.g. endothelial cells and epithelial cells) dedifferentiate into ECM-producing fibroblasts, while there develops a disbalance between profibrotic and antifibrotic fibroblasts. EMT and endothelial-to-mesenchymal transition (EndMT) account for the transition into fibroblasts<sup>[70]</sup>. Antifibrotic fibroblasts, like Epo-producing fibroblasts, are progressively lost during fibrosis (See Fig. 3B)<sup>[57]</sup>.

Besides attraction of leukocytes, the NF- $\kappa$ B pathway also stimulates the production of CCL21, a chemokine generally known for its role in the migratory behavior of dendritic cells towards the lymphatics<sup>[71]</sup>. Through endotoxins and cytokines such as TNF- $\alpha$  and IL-1, CCL21 guides dendritic cells to the appropriate location in draining lymph nodes, simultaneously influencing adhesion molecule expression. In renal injury, however, CCL21 also recruits fibrocytes, thereby contributing to the pathogenesis of interstitial fibrosis<sup>[64][72]</sup>. Similar to fibroblasts, infiltrated leukocytes stimulate fibrocytes to produce and deposit an excess of ECM<sup>[31]</sup>. Fibrocytes are a unique kind of circulating cell

population capable of extensive collagen 1 synthesis, which display influenced migratory behavior due to CCR2, CCR5 and CCR7 expression, the complementary receptors for MCP-1, CCL5 and CCL21, respectively<sup>[31]</sup>. Basically, fibrocytes are a subset of circulating bone marrow-derived monocytes that display fibroblast-like characteristics, able to differentiate into fibroblasts<sup>[73]</sup>. When differentiated into fibroblasts, fibrocytes contribute to fibrotic process, in which fibroblasts become activated and display yet another phenotype. The subset of activated fibroblasts, referred to as myofibroblasts, are characterized by de novo expression of mesenchymal markers like alpha smooth muscle actin ( $\alpha$ -SMA)<sup>[70]</sup>. In general, myofibroblasts in the interstitium originate from activated resident fibroblasts and pericytes, expansion of perivascular fibroblasts, differentiation of fibrocytes and both EMT and EndMT<sup>[74]</sup>. Although EMT and EndMT do contribute to the overpopulation of myofibroblasts, these cells do not display migratory behavior which is typical for mesenchymal cells, in contrast to pericytes<sup>[75]</sup>. In diabetic nephropathy, myofibroblasts are regarded as principal effector cells of pathogenic fibrosis of which its number correlates with the deposition of interstitial ECM<sup>[61]</sup>.

Just like any other tissue, the tubulointerstitium requires provision of nutrients and oxygen. Peritubular capillaries are crucial for this supply and sustain a normal morphology and functionality of the renal tubules<sup>[53]</sup>. Therefore, chronic tubulointerstitial injury might be caused by the loss of these capillaries, resulting in ischemia. Ischemia can either result from excessive production of angiotensin II or a reduced bioactivity of NO<sup>[53]</sup>. Peritubular capillaries consist of two cell types, being peritubular endothelial cells and pericytes. In recent years, peritubular endothelial cells have been implied to fulfill a central role in renal fibrosis, since their repair process is required for fibrosis prevention<sup>[76]</sup>. Dysfunction or loss of peritubular endothelial cells increases endothelial permeability. In line, IL-1 production by proximal tubular epithelial cells through diabetes-induced NF- $\kappa$ B signaling interferes with hyaluronic acid production, another main constituent of the endothelial glycocalyx, directly increasing endothelial permeability<sup>[32]</sup>. Furthermore, proximal tubular epithelial cells might regulate peritubular capillaries during the development of diabetic nephropathy by decreasing the vasodilating properties<sup>[27]</sup>. Also located in the peritubular capillaries are contractile pericytes, also called perivascular cells, that harbor both fibroblast and smooth muscle cell features<sup>[75]</sup>. Pericytes affect microvascular blood flow and the systemic blood pressure, and stabilize the endothelium via TGF- $\beta$  signaling and ECM production. Some specialized pericytes, called renin-producing perivascular cells, accumulate in the interstitium during fibrosis and thereby overstimulate RAS signaling<sup>[57]</sup>. Due to their fibroblast-like properties, pericytes can differentiate into myofibroblasts in response to hyperglycemia-induced TGF- $\beta$  signaling<sup>[75]</sup>. In kidney fibrosis, approximately 20% of the myofibroblasts originate from pericytes.

### **Possible therapeutic approaches**

In recent years, extensive studies regarding the inflammatory response during the pathology of diabetic nephropathy created a much clearer image of all contributing components. This also provides many new possible therapeutic targets to ameliorate the development of the disease. In terms of improvement of vasoreactivity, targeting NO signaling with antioxidants might be beneficial since they maintain eNOS activity<sup>[77]</sup>. Intake of antioxidants like taurine, which is the most abundant sulfonic acid in humans, protects against glomerular hypertrophy, interstitial fibrosis and proteinuria in human renal tubuli and diabetic nephropathy animal models<sup>[77][78]</sup>. Furthermore, therapeutic provision of taurine seems to have systemic effects due to reduction of blood glucose levels, thereby improving

several metabolic processes and kidney function<sup>[78]</sup>. Taurine reduces inflammation by repression of TNF- $\alpha$ , MCP-1 and NF- $\kappa$ B activity and taurine-reactive byproducts exert anti-inflammatory properties as well<sup>[79][80]</sup>.

Since hyperglycemia is the initial driver of diabetic nephropathy, therapeutic glyemic control can slow its progression and reduce loss of renal function<sup>[28]</sup>. As described earlier, TLR2 and TLR4 expression is glucose-regulated and responsible for the induction of NF- $\kappa$ B signaling and the consequent pathological processes. These receptors might be promising targets for interventions in diabetic nephropathy. Although there are clinical trials with TLR2 and TLR4 antagonists in diabetes, there are no trials focusing on nephropathy upcoming<sup>[15]</sup>. Nevertheless, silencing of TLR4 shows reduction of hyperglycemia-induced NF- $\kappa$ B activation and thereby inhibits IL-6 and MCP-1 synthesis of proximal tubular cells, making it a possible anti-inflammatory target<sup>[5]</sup>. Another possible target under hyperglycemic control is the cannabinoid 1 receptor, which is suggested to play a critical role in diabetic nephropathy<sup>[28]</sup>. Antagonistic repression of cannabinoid 1 receptor activation attenuates glomerulopathy due to the reversal of podocyte loss. Chronic blockade of the cannabinoid 1 receptor represses renal macrophage infiltration and the consequent release of IL-1 and IL-18. Insulin resistance induced by hyperglycemia of podocytes elevates VEGF-A production<sup>[25]</sup>. Even though the deteriorating effects of anti-VEGF therapy has been mentioned earlier, specifically targeting VEGF-A production in podocytes seems to be beneficial in diabetic nephropathy. VEGF-A disturbs the maintenance of the glomerular endothelial glycocalyx, resulting in renal complications<sup>[25]</sup>. Administration of soluble VEGF receptor-1, also known as sFLT-1, interferes with VEGF-A activity, thereby reversing previous kidney injury via reduced albuminuria, mesangial matrix and glomerular TNF- $\alpha$  levels<sup>[81]</sup>. Distribution of the inactive isoform of VEGF-A restores the glycocalyx, highlighting the pathogenic capability of dysfunctional VEGF signaling. Besides VEGF, endothelin-1 production by endothelial cells triggered by high glucose sheds the endothelial glycocalyx as well<sup>[25]</sup>. Triggering the endothelin receptor contributes to renal cell injury, inflammation and fibrosis and its inhibition has shown renoprotection in trials regarding diabetic nephropathy<sup>[82]</sup>. Blood glucose levels, however, can be relieved using SGLT2 inhibitors, oral hypoglycemic agents that block sodium coupled glucose reabsorption, thereby improving diabetes control and blood pressure<sup>[25]</sup>. Although the underlying mechanisms are currently unknown, SGLT2 inhibitors show strong cardiovascular and renoprotective effects. SGLT2 inhibition functions anti-inflammatory as well, preventing macrophage accumulation and reducing expression of TLR4 and MCP-1 amongst others in diabetic nephropathy patients<sup>[83]</sup>.

When blood glucose is reduced in diabetic nephropathy to normoglycemic levels, RAS signaling is implied to induce glomerular and interstitial inflammation. Angiotensin II and its NF- $\kappa$ B activation and ROS production induces IL-6 and IL-18 levels as well as complement activity, which makes it a promising therapeutic target. Indeed, diabetic patients treated with angiotensin II receptor blockers show reduced MCP-1 excretion and improved renal function<sup>[32]</sup>. In line, direct repression of MCP-1 via CCR2 inhibition reduces macrophage infiltration. Inhibition of CCR5, which is also upregulated by RAS and TNF signaling, affects the onset and progression of diabetic nephropathy by disturbance of monocyte/macrophage infiltration and activation<sup>[84]</sup>. Previously, the fundamental role of TNF signaling in the pathology of diabetic nephropathy has been highlighted. The expression of TNF receptor 1 is implied in the pathogenesis and serves as a tool of disease prognosis<sup>[85]</sup>. Administration of soluble TNF- $\alpha$  antagonist ameliorates renal hypertrophy through reduction of TGF- $\beta$ <sup>[86]</sup>. Intake of apigenin, which is a flavonoid present in fruits and vegetables, has antioxidant and anti-inflammatory properties, via reduction of TNF- $\alpha$ , NF- $\kappa$ B and IL-6, and attenuates renal function in diabetic nephropathy<sup>[87]</sup>. As mentioned earlier, using a proximal tubular cell-specific  $\alpha$ 7 nAChR agonist, TNF-induced NF- $\kappa$ B

signaling is blocked, protecting cells against renal injury in ischemia-induced acute kidney injury<sup>[67]</sup>. In diabetes, treatment with  $\alpha 7$  nAChR agonist lowers TNF- $\alpha$  levels and normalizes blood glucose and levels<sup>[66]</sup>.

Targeting NF- $\kappa$ B directly or one of the subsequent inflammatory components (e.g. chemokines) could also be a therapeutic approach. Blockade of NF- $\kappa$ B inhibits ICAM-1, VCAM-1 and MCP-1 expression in renal epithelial cells exposed to high glucose and renal tissues of diabetic mice<sup>[35]</sup>. Although no studies regarding diabetic nephropathy exist, anti-ICAM-1 antibodies are able to prevent macrophage infiltration and thereby be beneficial in patients with diabetic vascular complications<sup>[15]</sup>. AGE-induced VCAM-1 inhibition in mesangial cells represses RAS activity and thereby enlightens the vascular complications in diabetic nephropathy as well<sup>[88]</sup>. While blocking of leukocytes has beneficial effects, attraction of potent regenerative cell types might stimulate the recovery of injured tissues. As hinted earlier, recruitment of mesenchymal stem cells to sites of tissue injury has been implicated as a possible regenerative treatment. This even though resident mesenchymal stem cells contribute considerably to the dramatic increased abundance of myofibroblasts<sup>[23]</sup>. Upon renal injury, resident mesenchymal stem cells located in the perivascular areas easily differentiate into fibroblasts, leading to local perivascular fibrosis<sup>[89]</sup>. However, admission of bone marrow-derived mesenchymal stem cells has proven to recover kidney function and glucose control, and downregulate MCP-1 and interleukin expression in diabetic nephropathy<sup>[22]</sup>. These mesenchymal stem cells reduce the influx of leukocytes into the kidney via downregulation of ICAM-1 and the consequent cytokine production of TNF- $\alpha$  and TGF- $\beta$ <sup>[90]</sup>. Furthermore, mesenchymal stem cells inhibit EMT of tubular epithelial cells and have the potency to regenerate the epithelial and endothelial lining, making them a very potent therapeutic approach in the treatment of diabetic nephropathy.

## Discussion

Diabetic nephropathy is a complication that many type 1 and type 2 diabetes mellitus patients face and the single most common cause of end-stage renal disease. Although initially diabetes-related renal disease has been dedicated to metabolic and hemodynamic alterations due to hyperglycemia, growing evidence indicates that there is a strong inflammatory component in the initiation and progression of kidney complications. Mainly high glucose-induced NF- $\kappa$ B and ROS signaling are hallmark regulators of leukocyte migration and activation, as well as vascular complication and fibrosis, resulting in proteinuria. This, however, does not suggest that the primary believe that regulation of cell behavior and the vascular tone was the origin of the microvascular pathology in diabetic nephropathy. On the contrary, many pathways involved in these processes are under regulatory control of both hemodynamic and inflammatory mediators. For example, hyperglycemia-mediated expression of angiotensin II, which has been a prime target for interventions in RAS signaling, induces both vasoconstriction in the renal arteries and production of interleukins and complement factors upon NF- $\kappa$ B and ROS activation. Due to glomerulosclerosis, angiotensin II production rises through mesangial cells expansion-induced mechanical stretch, aggravating its pro-inflammatory effects. Moreover, oxidative stress following mitochondrial hyperactivity causes overexpression of ICAM and VCAM, as well as NF- $\kappa$ B and cytokine activity<sup>[91]</sup>. Altogether, it seems more likely that vascular and inflammatory complication accompany each other in diabetic nephropathy rather than there is one general initiator of pathogenesis. This might explain why the vast majority of the diabetes patients receiving insulin or RAS treatment still progress to end-stage renal disease. In line, combined therapy with endothelin-1

receptor antagonists, as an anti-inflammatory agent that protects the glycocalyx and prevents proteinuria, and RAS blockade, for hemodynamic control, has shown beneficial results in clinical trials<sup>[82]</sup>.

In this review, the pathology in diabetic nephropathy in the glomerulus and the tubulointerstitium have been discussed separately. Since diabetic nephropathy historically has been regarded as glomerular disease, primarily initiated by podocyte injury, abundant evidence on glomerular pathology is gathered over time. Recent research highlights the fundamental role of the tubulointerstitium in diabetic renal diseases, which contributes unquestionably to the glomerular and tubulointerstitial pathology. Therefore, therapeutic interventions in diabetic nephropathy should target both the glomerulus and the tubulointerstitium. TLR4 inhibition ameliorates the pathophysiology via glomerular cells as well as in the tubular epithelial cells, serving as a general target against kidney disease. Through its glomerulus conserving effects, TLR4 inhibition lowers proteinuria and thereby protects the tubulointerstitium from albumin-induced inflammation<sup>[30][56]</sup>. On the other hand, TLR4 repression in the tubular epithelial cells dramatically suppressed the excretion of cytokines, chemokines and adhesion molecules, which contributes to the alleviation of podocyte injury amongst others<sup>[5][92]</sup>. Inhibition of TLR4 in mice suffering from advanced diabetic nephropathy therefore shows significant renoprotective effects in both the glomerulus and the tubulointerstitium, even in eNOS knockout mice with the complementary vasoactive restraints<sup>[5]</sup>.

Presence and maintenance of anti-inflammatory cells and the interference with migratory behavior of leukocytes and fibrocytes seems of major importance. Proteolytic enzyme and the consecutive proinflammatory cytokine production by infiltrated leukocytes exacerbates glomerular and interstitial injury, while fibrocytes aggravate kidney fibrosis. However, administration of bone marrow-derived mesenchymal stem cells suppresses inflammation in the appropriate conditions, thereby alleviating renal pathology. This implicates that local anti-inflammatory cell signaling through these stem cells transcends the proinflammatory behavior of renal cells. Mesenchymal stem cells can lose their anti-inflammatory characteristics and contribute to the pathological processes, so maintaining anti-inflammatory cell signaling is of major importance. The postulated disbalance between proinflammatory and fibroblasts that exert anti-inflammatory properties raises the question whether pathological cell types, like the in diabetes overabundant renin-producing perivascular cells, could be converted into beneficial cells<sup>[57]</sup>. Hypothetically, transformation into Epo-producing fibroblasts might attenuate hemodynamic control and restore the composition of the interstitium.

Altogether, the growing knowledge on the inflammatory component resulted in an exponential rise in possible therapeutic approaches in the treatment of diabetic nephropathy. It is essential, nevertheless, that the main origin of the disease is rectified as well. Chronic hyperglycemia could be relieved by administration of SGLT2 inhibitors via prevention of glucose reabsorption, lowering blood glucose levels. Also, natural supplements such as taurine and apigenin, which are respectively abundant in mammals and fruits and vegetables, have antioxidant, anti-inflammatory and renoprotective properties, providing simply accessible agents. Therapeutic usage of such antioxidants, however, remains rather hypothetical, since the dosage in interventions strongly exceeds natural levels. They do imply that supplementary intake of antioxidants from natural or chemical origin might be an addition to the beneficial effects of diabetes-related interventions. Although there are increasing numbers of possible treatments, prevention of diabetes-induced renal disease still requires extra effort. Preclusion of diabetes incidence would result in a drastic decrease in kidney injury and relieve the health burden significantly.

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