The podocyte as a direct target of immunosuppressive agents

Eva Schönenberger¹, Jochen H. Ehrich², Hermann Haller¹ and Mario Schiffer¹

¹Department of Nephrology and ²Department of Pediatric Nephrology, Hepatology and Metabolic Diseases, Medical School Hannover, 30625 Hannover, Germany

Correspondence and offprint requests to: Eva Schönenberger; E-mail: schoenenberger.eva@mh-hannover.de

Abstract
Podocytes play a key role in maintaining the blood–urine barrier for high-molecular-weight proteins. They are considered to be terminally differentiated, and podocyte loss cannot be compensated by regenerative proliferation. Various diseases leading to podocyte damage and loss result in proteinuria and cause nephrotic syndrome. Therefore, direct therapeutic strategies to protect podocytes in disease situations are a logical concept to prevent disease or to delay disease progression. Acquired podocytopathies like idiopathic focal segmental glomerulosclerosis and minimal change disease are historically considered as immunological diseases. Therefore, immunosuppressive agents such as steroids and calcineurin inhibitors are the commonly used treatment strategies. However, the causative disease mechanisms behind these treatment strategies remain elusive. Recent evidence shows that immunosuppressive agents, in addition to the effect on the immune system, directly influence the unique structure and function of podocytes. In this context, the actin cytoskeleton of the podocyte and cytokines such as vascular endothelial growth factor play a pivotal role. In this review, we summarize the direct effects on podocytes obtained in vivo and in vitro after treatment with calcineurin inhibitors, mTOR inhibitors and glucocorticoids. These direct effects could play a key role in the treatment concepts of podocytopathies with an important impact on the long-term renal function in patients with pharmacological immunosuppression.

Keywords: cyclosporin A; FSGS; glucocorticoids; proteinuria; rapamycin

Introduction
Nephrotic syndrome is common in adults and is one of the most common kidney diseases in children [1]. The majority of non-genetic nephrotic syndromes are caused by membranous nephropathy or focal segmental glomerulosclerosis (FSGS) in adults and minimal change disease (MCD) in children [1,2]. In all these diseases, the podocyte, which is considered to be terminally differentiated, is the primary target of injury [3]. Recently, a possible autoantigen of idiopathic membranous nephropathy (MN) was identified, and the presence of autoantibodies was documented in 70% of patients with idiopathic MN [4]. However, the different pathophysologies of idiopathic FSGS and MCD are still ongoing subjects of debate and not fully understood. Podocyte foot process effacement and disruption of the glomerular slit diaphragm are common phenotypes observed in almost all glomerular diseases associated with nephrotic-range proteinuria. The common concepts of podocyte foot process effacement involve dedifferentiation, direct injury of the slit diaphragm or the actin cytoskeleton, and changes in the glomerular basement membrane and podocyte interaction [5]. The resulting loss of glomerular barrier function leads to proteinuria. However, sclerosis and adhesion of the glomerular tuft to the Bowman’s capsule are restricted to FSGS and absent in MCD. Persistent proteinuria is a prognostic marker for the progression to end-stage renal disease [6]. Patients presenting with long-term nephrotic-range proteinuria and without partial or complete remission progress to end-stage renal disease over the course of 3–6 years [7].

Acquired podocytopathies like idiopathic FSGS and MCD are historically considered as immunological diseases [8]. Therefore, immunosuppressive agents such as steroids and calcineurin inhibitors are the commonly used treatment strategies. More than 50% of nephrotic adults and ~80% of children respond to an induction therapy with glucocorticoids within a range of a few days to several months, and maintenance treatment with glucocorticoids will prevent relapses [9,10]. The response to corticosteroids is still the best prognostic factor for maintaining renal function in idiopathic nephrotic syndrome, irrespective of the histopathology. In steroid-resistant nephrotic syndromes, several other immunosuppressive agents were successfully used as rescue therapy.

After kidney transplantation, proteinuria is highly prevalent and associated with decreased patient and allograft survival irrespectively of the underlying primary renal disease. Depending on the definition, up to 45% of patients develop pathological proteinuria mostly due to recurrent glomerulonephritis, chronic allograft nephropathy, de novo transplant glomerulopathy or acute rejection [11]. More-
over, there is an ongoing debate about several immunosuppressive agents causing allograft proteinuria as it was shown for rapamycin and cyclosporine A [12,13]. This review discusses the current concepts of podocytes as the primary target of immunosuppressive agents.

**Glucocorticoids**

Glucocorticoids bind to the glucocorticoid receptor in the cytoplasm, form dimers, and translocate to the nucleus where they bind to glucocorticoid response elements on the DNA or interact with other transcription factors [14]. Glucocorticoid receptors have been described to be expressed in human podocytes and translocate to the nucleus upon treatment with dexamethasone [15]. Therefore, a direct effect of glucocorticoids on podocytes in the course of nephrotic syndrome seems likely.

The proteome of differentiated, cultured podocytes is particularly rich in actin cytoskeletal proteins, annexins, and stress-associated proteins such as heat shock proteins and antioxidant enzymes. Ransom et al. demonstrated that dexamethasone treatment of podocytes leads to increased expression of ciliary neurotrophic factor (CNTF), an interleukin-6 (IL-6)-type cytokine [16], increased expression of αB-crystallin and increased expression of heat shock protein 27 (hsp27) [17]. Both are molecular chaperones inducing thermotolerance [18,19]. Furthermore, Smoyer and colleagues observed the importance of hsp27 for the regulation of the morphological and actin cytoskeletal response of podocytes by regulating actin polymerization in a podocyte injury model, using puromycin aminonucleosid (PAN) [20]. This goes along with findings that dexamethasone enhances the stability of actin filaments against disruption by cytochalasin D, latrunculin A, or PAN by increasing the total amount of cellular polymerized actin and an increased activity of the actin-regulating GTPase RhoA. It was also previously demonstrated that these effects are specific to glucocorticoids compared with other classes of steroid hormones [21]. However, treatment with spironolactone, an aldosterone antagonist, reduced albuminuria, renal tissue renin-angiotensin activity, and increased AKT phosphorylation, thereby improving podocyte structural integrity in the transgenic Ren2 rat model with increased tissue renin-angiotensin activity [22].

Treatment with PAN induces podocyte apoptosis via p53-dependent apoptosis-inducing factor (AIF) translocation to the nucleus. This caspase-3-independent effect on podocyte apoptosis can be abolished by treatment with dexamethasone. Wada et al. demonstrated rescued podocyte viability in a PAN cell culture model upon treatment with dexamethasone by blocking p53 expression, lowering the proapoptotic Bax expression and increasing the expression of the antiapoptotic Bel-XL [23,24]. Bax belongs to the Bcl2 family and is known to mediate podocyte apoptosis induced by TGF-β [25]. Interestingly, dexamethasone failed to prevent podocyte apoptosis induced by UV light or TGF-β, which is primarily caused by caspase-3 activation [24]. Moreover, Wada and colleagues have shown that dexamethasone prevents the reduced ERK phosphorylation in PAN-treated podocytes. Interestingly, when ERK was directly inhibited in this cell model, dexamethasone exerted a proapoptotic effect which was associated with translocation of AIF [24], indicating that the ERK pathway itself has important impact in podocyte survival. However, the precise interaction of ERK signalling and dexamethasone remains to be determined.

In normal glomeruli, vascular endothelial growth factor (VEGF) is exclusively expressed by podocytes and is upregulated in minimal change nephropathy [26,27]. VEGF plays an important role in vasculogenesis and angiogenesis and induces vascular leakage and vasodilation. Treatment with dexamethasone led to a down-regulated VEGF expression in an immortalized human podocyte cell line [28]. However, these changes in VEGF expression affect different VEGF isoforms, and how this contributes to a clinical remission of disease remains controversial, since we recently could demonstrate that expression of VEGF-A and VEGF-C is important for podocyte survival [29] and that VEGF ablation therapy in patients leads to proteinuria and podocyte loss [30]. The ability of cytokine production links the podocyte to the immune system. Next to VEGF and TGF-β, podocytes produce the interleukins IL-6 and IL-8 [28]. Similarly, they express a variety of functional CC and CXC receptors [31]. Since cytokine expression can be suppressed by dexamethasone treatment [28], this effect may contribute to the treatment effects of dexamethasone in patients having nephrotic syndrome.

Previously, it was shown that dexamethasone also has effects on the intracellular quality control of protein synthesis and post-translational maturation of nephrin [32]. Rapid endoplasmic reticulum (ER) stress leads to formation of under-glycosylated nephrin, which remains in the ER as a complex with the chaperones calreticulin/calnexin. Treatment with dexamethasone restores the synthesis of fully glycosylated nephrin via stimulation of adenosine triphosphate production (ATP) in an energy-depleted cell model, suggesting reversibility of slit diaphragm injuries and foot process effacement [32]. There are a few more data linking the ER stress of podocytes to proteinuria and development of glomerulosclerosis as Heymann nephritis (membranous nephropathy) or PAN nephrosis (FSGS/MCD) [33]. Both diseases are known to respond usually to glucocorticoid treatment in patients. Furthermore, apoptosis of tubular cells in chronic calcineurin inhibitor-associated nephropathy is closely connected with depletion of molecular chaperones by prolonged ER stress [34], underlining that calcineurin plays an essential role in response to ER stress [35]. However, there are no data so far evaluating the effect of steroids on calcineurin inhibitor-associated nephropathy.

Treatment of rats with adriamycin, a rodent model of non-immune initiated FSGS, leads to proteinuria, kidney enlargement and glomerulosclerosis. This was significantly attenuated by prednisone [36]. Adriamycin rats treated with prednisone showed reduced proteinuria and less severe glomerular lesions, achieved most likely due to stabilized distribution of nephrin, podocin and CD2AP within the slit diaphragm complex [36]. Prednisone also restored VEGF expression and nephrin phosphorylation, supporting the data obtained in cell culture models [37].

Glucocorticoid therapy remains the primary treatment option for nephrotic syndrome, despite the lack of
Calcineurin inhibitors

Calcineurin is a serine/threonine phosphatase that is ubiquitously expressed in all mammalian tissues and tightly regulated by Ca\(^{2+}\)/calmodulin [38]. Calcineurin dephosphorylates the nuclear factor of activated T-cell (NFAT) family members, leading to nuclear translocation and activation of early genes of the T-cell-driven immune response, e.g., cytokines as IL-2 and IL-4. The immunosuppressive action of calcineurin inhibitors such as cyclosporin A (CsA) or tacrolimus (FK506) is due to the inhibition of the NFAT signalling in T cells by binding to the cytosolic cyclophilins or FK-binding proteins and subsequently inhibiting the phosphatase activity of calcineurin.

Recent evidence supports that the podocyte itself is a target of CsA. Faul et al. analysed the consequence of CsA treatment on the actin cytoskeleton of podocytes [39,40]. Treatment of podocytes with CsA leads to a stabilization of the actin cytoskeleton and stress fibres, while calcineurin mediates dephosphorylation of synaptopodin, an actin-organizing protein in podocytes. By blocking calcineurin, the phosphorylation of synaptopodin promotes binding to the chaperone-like protein 14-3-3. Subsequently, synaptopodin is protected against cathepsin L-mediated cleavage and degradation. Thereby, CsA has a stabilizing effect on the actin cytoskeleton. Moreover, calcineurin is tightly regulated by intracellular calcium levels. The podocyte cell membrane-associated transient potential cation channel 6 (TRPC 6) mediates calcium influx, and gain-of-function mutations are known to be causal for genetic forms of FSGS [41]. High levels of intracellular calcium would lead to an activation of calcineurin, and thereby loss of synaptopodin and stress fibres, and to an activation of the NFAT signalling pathway as a further potential mediator of FSGS [42]. Both pathways can be inhibited by treatment with CsA or FK506 [41,43,44]. Furthermore, CsA and steroids were reported to treat effectively early-onset nephrotic syndrome due to a mutation in the gene coding for phospholipase C epsilon [45]. Phospholipase C is an important intracellular mediator of TRPC 6 activity [41], and mutations are known to interfere with glomerular development and probably with glomerular repair processes as well [45].

Another cell membrane protein of the podocyte, the zona occludens-1 (ZO-1) protein, has been shown to be important for cellular integrity. Changes of the distribution or expression of ZO-1, which is present at the slit diaphragm and associates with nephrin, are linked to proteinuria [46,47]. PAN-treated rats, as a model for FSGS, show an increase in glomerular ZO-1 expression as a first promoting step to foot process effacement [48]. CsA treatment of these rats could partially reverse the proteinuria by inhibition of the increased ZO-1 expression. However, decreased ZO-1 expression also associates with proteinuria and can be normalized by overexpression of synaptopodin in a mouse model of transient proteinuria induced by lipopolysaccharides [39], indicating that ZO-1 is most likely downstream of synaptopodin in terms of preventing proteinuria by stabilizing the slit diaphragm.

Apoptosis has been shown in tubular and interstitial cells of CsA-treated animals and in tubular cells in vitro. Furthermore, CsA induces apoptosis in glioma cells and hepatocytes but protects endothelial cells and myeloid leukaemia cells from cell death [49]. The immortalized murine podocyte cell line used by Faul et al. showed an increased amount of stress fibres without inducing apoptosis after treatment with CsA [39]. This is in contrast to evidence published that CsA treatment of another permanent podocyte cell line was associated with altered expression of apoptosis regulatory genes such as Bcl2, Bax and FasL. Apoptosis of podocytes induced by CsA has been demonstrated to be dose- and time-dependent, and leads to decreased Bcl-XL levels. Pre-treatment of cultured murine podocytes with hepatocyte growth factor (HGF) could prevent these effects mediated via activation of the PI3 kinase pathway and restored Bcl-XL expression [49]. These data are discussed controversially in the field, questioning the origin of the cell lines used. Thus far, we have also failed to reproduce the proapoptotic effect of CsA on various murine podocyte cell lines we have generated over the years (M. Schiffer, unpublished observations).

CsA and FK506 can induce remission of proteinuria caused by MCD or FSGS [50,51]. CsA-treated rats did not increase their glomerular albumin permeability when treated with serum of patients with FSGS in comparison with non-CsA-treated animals with an increased proteinuria [52]. This supports the hypothesis of a circulating factor and an immunological pathophysiology of FSGS. But CsA was also shown to reduce proteinuria in Alport syndrome—a non-immunological disease, caused by mutations of the type IV collagen, which is an important structural component of the glomerular basement membrane [53,54]. Furthermore, CsA has important stabilizing effects on the podocyte actin cytoskeleton, implying that calcineurin inhibitors not only have immunosuppressive effects but also have direct non-immunological effects on the glomeruli. However, the clinical use of calcineurin inhibitors is often limited by acute and chronic nephropathy, and whether reduced glomerular perfusion as a direct effect of calcineurin inhibitor toxicity contributed to the non-immunological effects on the glomerulus remains unclear [55]. In order to clarify some of the controversies, recommendations made by an international workshop regarding the use of CsA in most histological variants of idiopathic nephrotic syndrome in both children and adults were published [56].
**mTOR inhibitors**

Mammalian target of rapamycin (mTOR) is a highly conserved serine/threonine kinase, which controls cell growth and metabolism in response to nutrients, growth factors, cellular energy, and stress. There are two functionally distinct multiprotein complexes. First, mTORC1 is sensitive to rapamycin (RN) and mediates temporal control of cellular growth by regulation of transcription, translation, ribosome biogenesis and nutrient transport. Second, mTORC2 is classically insensitive to RN, but long-term treatment was more recently shown to disrupt mTORC2 assembly [57]. The mTORC2 complex controls phosphorylation and stability of AKT, thereby playing a role in cell survival [58].

Via mTORC2, rapamycin gains influence on the PI3 kinase pathway, which may lead to a change in podocyte phenotype and cytoskeleton reorganization. Prolonged RN treatment of immortalized podocytes reduces the level of mTORC2 below those needed to maintain AKT signalling leading to a decrease in AKT phosphorylation and VEGF synthesis [59,60]. The PI3 kinase/AKT pathway promotes VEGF synthesis and conversely is activated by VEGF. There are complex interactions between mTOR, VEGF and AKT signalling affecting podocyte adhesion and survival. Next to the direct anti-proliferative effects, RN and everolimus are known to decrease VEGF synthesis, making them promising drugs for transplant patients developing Kaposi sarcoma or other malignancies [61,62]. FSGS lesions show a focal decrease in VEGF and loss of differentiation markers of podocytes [59,63], which supports the data about podocytes having an autocrine VEGF system that promotes survival and differentiation [64]. Furthermore, reduced phosphorylation of AKT upon podocyte treatment with RN decreased the expression level of nephrin, TRPC6, and the cytoskeletal adaptor protein Nck [60]. Nephrin directly interacts with Nck and PI3 kinase, thereby regulating rearrangement of the actin cytoskeleton at the slit diaphragm.

In the clinical setting, the podocyte as a direct target cell of RN came into focus after several studies showed develop-
opment or increasing proteinuria in patients treated with RN. In 2006, this was followed by the report of acquired FSGS with nephrotic syndrome in three renal transplant patients who received RN de novo [63]. At the same time, RN had been reported to achieve complete or partial remission in 12 out of 21 patients with steroid-resistant FSGS after 6 months of therapy [65]. In contrast, a phase 2 trial for FSGS treatment with RN after failed remission with at least one immunosuppressive agent was stopped prematurely because of a decrease in glomerular filtration rate and an increase in proteinuria [66]. It is noteworthy that only some of the patients treated with rapamycin develop proteinuria and nephrotic syndrome. Other factors may contribute to the onset of rapamycin-associated proteinuria, such as rapamycin serum level, the patient’s genetic background or pre-existing renal damages [67]. This is supported by the data obtained from an FSGS animal model using PAN treatment and from a renal mass reduction model [68,69]. Rapamycin led to a further increase in proteinuria by reducing podocin expression and damaging podocyte foot processes in PAN-treated rats. In contrast, in the model of chronic tubulointerstitial inflammation (renal mass reduction), treatment with RN led to higher podocin and nephrin expression. Thereby, the damage to podocyte foot processes was ameliorated leading to a lowered level of proteinuria. At the same time, the inflammatory and profibrotic damage was reduced as it was often reported before [70,71]. Wittmann and co-workers could confirm this in a streptozotocin-induced diabetes model in rats [72]. After induction of diabetes, treatment with RN reduced renal fibrosis and inflammation, while it also ameliorated glomerular hypertrophy and podocyte loss, and lowered the expression and activation of TGF-β and VEGF. The reduction of inflammatory cell infiltration reflects a part of the immunosuppressive effects of RN. These data suggest opposing effects of RN treatment depending on the underlying kidney lesion, and there are similar controversies for the treatment effects of everolimus, a derivate of RN. In an immunologically animal model causing FSGS, mesangial cell proliferation, and mesangiogenesis using anti-Thy1 antibodies, treatment with high doses of everolimus led to increased proteinuria and severe glomerular damage. When using a lower dose of everolimus, some beneficial effects were seen [73]. Without any further treatment, acute anti-Thy1 nephritis causes damage to the glomerular architecture and subsequently proteinuria, which is substantially restored 2 weeks after disease induction. When everolimus treatment started early after induction of anti-Thy1 nephritis, maximal glomerular damage was seen with mesangiolysis, inhibition of VEGF expression, marked proinflammatory effects and resulting increase of proteinuria [73]. Similar to RN, the development of PAN nephrosis was ameliorated by everolimus pre-treatment [74]. But contrary to RN, Vogelbacher et al. reported in a renal mass reduction model that treatment with everolimus worsened chronic disease progression, increased proteinuria and reduced renal function [61]. These data indicate that everolimus, similar to rapamycin, can lead to deleterious effects on previously damaged podocyte slit diaphragms, aggravating the injury. In addition, increased apoptosis of podocytes and renal tubular cells was shown in patients with delayed graft function and de novo RN treatment [75]. Type and time of renal lesions as well as repairation processes and probably not the general toxic effects are critical for the adverse effects of mTOR inhibitors, especially while treating during the proliferative phase of glomerular disease. This goes along with clinical findings that pre-existing proteinuria at levels >800 mg/day and a glomerular filtration rate <40 mL/min are predictors for a negative outcome of conversion from a calcineurin inhibitor to RN due to chronic allograft dysfunction [76].

The reduction of actin cytoskeleton adaptors and/or core components of the slit diaphragm upon treatment with RN might be responsible for rapamycin-associated proteinuria. Therefore, the RN-induced direct effects on podocytes would point this drug more towards a non-preferred indication in podocytopathies. However, more research is necessary to delineate the multifunctional effects of dose- and time-dependent effects of RN on podocytes, especially when given in combination with calcineurin inhibitors.

Summary

There is an overwhelming amount of data emphasizing the pivotal role of podocytes on proteinuria in many different forms of glomerular diseases. The anti-inflammatory and immunosuppressive action of glucocorticoids, calcineurin inhibitors and mTOR inhibitors may only play a minor role in modulation of podocyte biology and promotion of glomerular repair mechanisms. Instead, these drugs have direct effects on podocytes through regulation of some cytokines and several signalling pathways relevant for cytoskeletal stability, cell maturation and survival. Furthermore, the expression and distribution of key components of the slit diaphragm and the cytoskeleton are regulated (Figure 1). However, the data on direct effects of immunosuppressive agents on proteinuria induced by podocyte dysfunction remain controversial, and more research is necessary to differentiate the multifactorial effects especially regarding time and dose of treatment and the effects according to the type of glomerular pathology.

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References


**Abstract**

Recent trials, and meta-analyses, have cast further doubt on the clinically desirable and safe range for increasing haemoglobin in chronic kidney disease using erythropoiesis-stimulating agents. In this article, I review the current dilemmas we face, suggest key clinical and biological research priorities, and conclude that we need to be brave enough to admit our present shortcomings, and then perhaps adopt a more patient-focused, individualized approach to anaemia management.

**Keywords:** anaemia; epoetin; mortality