The TNFα Signaling Pathway in Glomerular Injury

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Table of Contents

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This thesis is in dedication of my late grandfather, Allen Clifford Dolden, who would have read this manuscript cover to cover, like he did the Globe and Mail every morning.

CONTRIBUTION OF AUTHORS

Nadezda Kachurina prepared the patient serum samples for the focal adhesion complex (FAC) assay. Julie Guillemette cut and cryopreserved the tissue sections from the C57BL/6 mice for the TACE expression studies. Sima Babayeva acquired images from the nephrin internalization studies as well as performed the staining and imaging of the mouse kidney cryo sections for the TACE expression studies. All other experimental work was carried out by Katarina Pessina.

ABSTRACT

Focal segmental glomerulosclerosis (FSGS) is among the leading causes of end stage renal failure in adults (requiring dialysis and/or kidney transplantation) and a cause of nephrotic syndrome in children. The disease is considered primary when it is of idiopathic origin and of those, 30-50% exhibit recurrence (rec-FSGS) of the disease in the allograft following renal transplantation. The immediacy of FSGS recurrence (sometimes within a few hours of transplantation) suggests the presence of a circulating toxic factor that exerts its effects on glomerular epithelial cells (or podocytes). Previously, we established that sera from certain patients with FSGS disrupt focal adhesion complexes in cultured podocytes and that blockade of the tumor necrosis factor alpha (TNFα) pathway can dramatically reduce these toxic effects, including inhibiting cytoskeletal re-organization. We hypothesize that the TNFα signaling cascade is activated in podocytes in response to toxic sera from certain rec-FSGS patients.

To study TNFα mediated glomerular injury *in vitro*, we used immortalized human podocytes and human embryonic kidney 293T cells stably expressing rat nephrin for immunoblotting and immunofluorescence microscopy. *In vivo* studies employed calcium-independent phospholipase A2γ (iPLA2γ) -/- mice in the C57BL/6 background injected with Adriamycin (ADR) to induce podocyte damage and proteinuria.

TNFα converting enzyme (TACE) is a sheddase of the ADAM's family responsible for cleavage and subsequent activation of TNFα. We show an increase in TACE expression in the glomeruli of ADR-treated mice, suggesting a potential role for TACE in podocyte injury and proteinuric disease. Nephrin, a key structural component of the filtration slit diaphragm, is internalized upon treatment of cultured cells with TNFα. We further demonstrate that inhibition

of TACE halted nephrin internalization by maintaining its localization at the surface of cells. Furthermore, when podocytes were treated with rec-FSGS serum, inhibition of TACE prevented the loss of focal adhesion complexes.

In conclusion, inhibition of TACE abrogates the toxic effects of rec-FSGS serum on the podocyte cytoskeleton and induction of nephrin internalization by blocking TNFα signaling. Unveiling the mechanisms of recurrent idiopathic FSGS will allow for development of personalized diagnostic and therapeutic strategies for patients whose disease is mediated by the TNFα signaling pathway, and who may be candidates for anti-TNFα therapy.

RÉSUMÉ

La glomérulosclérose segmentaire et focale (FSGS) est l'une des principales causes d'insuffisance rénale en phase terminale chez les adultes (nécessitant une dialyse et/ou une transplantation rénale) et une cause de syndrome néphrotique chez les enfants. La maladie est considérée comme primaire quand c'est d'origine idiopathique et parmi ceux-ci, 30-50% présentent une récurrence (rec-FSGS) de la maladie dans l'allogreffe après transplantation rénale. La récurrence immédiate de FSGS suggère la présence d'un facteur toxique circulant dans le sang qui exerce ses effets sur les cellules épithéliales glomérulaires (ou podocytes). Nous avons auparavant établi que les sérums de certains patients atteints de FSGS perturbent les complexes d'adhésion focale dans les podocytes en culture cellulaire et qu'un blocage de la voie de signalisation du TNFα peut considérablement réduire ces effets toxiques, y compris l'inhibition de la réorganisation cytosquelettique. Nous émettons l'hypothèse que la cascade de signalisation du TNFα est activée dans les podocytes en réponse à des sérums toxiques provenant de certains patients rec-FSGS.

Pour étudier la lésion glomérulaire induite par le TNFα in vitro, nous avons utilisé des podocytes humains immortalisés et des cellules 293T rénales embryonnaires humaines exprimant de façon stable la néphrine de rat pour immunotransfert et la microscopie par immunofluorescence. Pour les études in vivo ŧ nous avons utilisé des souris calcium-independent phospholipase A2γ iPLA2γ -/- C57BL /6 injectées avec de l'Adriamycine (ADR) pour induire des lésions des podocytes et une protéinurie.

L'enzyme de conversion du TNFα (TACE) est une sheddase de la famille ADAM responsable du clivage et de l'activation subséquente du TNFα. Nous montrons une augmentation de l'expression de TACE dans les glomérules de souris traitées par ADR suggérant

un rôle pour TACE dans la lésion des podocytes et la néphropathie protéinurique. La néphrine, un composant structurel clé du diaphragme à fente de filtration, est endocytée lors du traitement de cellules cultivées avec du TNFα. Nous démontrons en outre que l'inhibition de TACE inhibe l'internalisation de la néphrine en maintenant sa localisation à la surface des cellules. En outre, lorsque les podocytes ont été traités avec du sérum rec-FSGS, l'inhibition de TACE a empêché la perte de complexes d'adhésion focaux.

En conclusion, l'inhibition de TACE abroge les effets toxiques du sérum rec-FSGS sur le cytosquelette des podocytes et l'induction de l'internalisation de la néphrine en bloquant la signalisation du TNFα. Le dévoilement des mécanismes de la FSGS idiopathique récurrente permettra de développer des stratégies diagnostiques et thérapeutiques personnalisées pour les patients dont la maladie est médiée par le TNFα et qui pourraient être candidats à un traitement anti-TNFα.

1.1 INTRODUCTION TO THE GLOMERULUS

1.1.1 The Glomerulus and the Podocyte

Chronic kidney disease (CKD) is defined by a measurable decrease in kidney function for at least 3 months (1). CKD is characterized by being progressive in nature, has widespread etiological associations and as such, its pathogenesis remains under extensive clinical and experimental investigation (1). An aging population presents with a growing incidence of CKD and, as consequence, a large financial burden on health care worldwide (2). The majority of kidney diseases that lead to CKD and require treatment of end-stage renal disease (ESRD) are due to injury of the glomerulus (2). An in-depth understanding of the ultrastructure of the glomerulus and its response to the injury are the key to developing therapeutic targets.

The kidneys participate in a number of functions related to maintaining homeostasis and osmoregulation. They control blood pressure, vitamin synthesis, bone mineralization, erythrocyte development and most importantly, participate in plasma filtration (3). The functional units of the kidney are the nephrons. There are roughly 1 million nephrons within each kidney (2), and they filter up to 20% of the cardiac output (3). The filtering unit of the nephron is the glomerulus (4), a specialized capillary bed fed by an afferent arteriole and collecting into an efferent arteriole (5). This capillary network is contained within the Bowman's capsule which encloses the urinary space (6). The glomerular vasculature is supported by mesangial cells and the mesangial matrix which together form the glomerular tuft (5).

The glomerular capillary wall forms the glomerular filtration barrier and is composed of the podocyte epithelial cell layer, the acellular basement membrane and a fenestrated endothelial

cell layer (7) characterized by individual fenestrate 70-100 nm wide (6). The endothelial cells face the luminal side of the capillary bed (6). The glomerular basement membrane (GBM) is composed of extracellular matrix proteins, including type IV collagen, laminin, entactin/nidogen and proteoglycans (6). Sitting atop the GBM and facing the urinary side of the capillaries are the glomerular epithelial cells, termed podocytes (6). Podocytes are terminally differentiated post-mitotic cells, which maintain a quiescent phenotype (2,8). They arise from mesenchymal progenitor cells in the developing kidney (9). After kidney development is completed, proliferation of podocytes under normal physiological conditions is rarely detected. It is believed that the highly differentiated phenotype of podocytes and their low proliferation rate render them incapable of replacing themselves to compensate for any significant loss (10). As a consequence, injury or loss of podocytes is often irreversible and, thus, is a critical contributor to glomerular disease (2,11).

The cell bodies of podocytes emit lengthy primary processes (12) that form smaller foot processes. The foot processes anchor podocytes to the GBM via multiprotein adhesion complexes that rely on integrins and dystroglycans (8). The structural integrity of foot processes is maintained via an extensive cytoskeleton network consisting of microfilaments (7-9 nm in diameter), microtubules (24 nm) and intermediate filaments (10 nm). The microfilaments are the predominant cytoskeletal component and contain primarily actin filaments which function to maintain podocyte cell shape (8,13). Inside Bowmen's capsule, podocytes are suspended in urinary space and only delicately attached to the GBM. Hence, they are prone to detachment (10).

The foot processes of podocytes are interdigitated along the glomerular basement membrane by specialized adherens junctions organized in a zipper-like fashion and are called slit

diaphragms (SDs) (11) (Figure 1). The unique architecture of podocytes allows them to carry out their specialized functions. One of the main functions of podocytes includes contributing to the permeability properties of the glomerular filtration barrier. Hydrostatic pressure in the glomerular capillary enables ultrafiltration of plasma across the glomerular capillary wall (14). While water and solutes are freely filtered through this barrier, proteins are filtered based on molecular size and charge (8). Furthermore, podocytes provide structural support for the glomerular capillaries (11). By enveloping the capillaries, podocytes contribute to the intraglomerular pressure of the vessels by preventing vessels' distension (12) and maintaining the capillary loop shape (8). Podocytes also synthesize the proteins that make up the SD complex and extracellular matrix components of the GBM.

1.1.2 Slit Diaphragms (SDs)

Podocyte foot processes are interdigitated along the (GBM) by SDs (10). The proteins comprising SD protrude from the sides of the foot processes and bridge the distance approximately 40 nm wide (15). The integrity of the SD complex is maintained by several SD molecules such as cluster of differentiation 2 associated protein (CD2AP), nephrin, podocin, and SD-associated protein filaments such as actin (16). SDs act as a protein sieve and as a barrier to the flow of filtrate. The ~40 nm distance between the adjacent foot processes prevents large proteins from exiting the capillary bed and entering the urine (14). In the case of podocyte injury, these SDs become altered or lost (a condition known as foot process effacement), allowing for proteinuria to occur (15). During foot process effacement, the normal structure of SDs becomes

altered thereby establishing a direct relationship between SD integrity and healthy podocyte function (2).

1.1.3 Nephrin

A key structural component of the SD that bridges interdigitating podocytes was discovered by the Tryggvason's group in 1998 (17). Nephrin is a 180 kDa transmembrane glycoprotein of the Ig superfamily (15) with a short cytoplasmic tail, a transmembrane domain, a fibronectin type 3 domain and eight Ig-like-domains (17). The protein serves as a backbone of the SD and is described as an adhesion protein (15). It anchors to the podocyte's cytoskeletal actin fibers indirectly via link adaptor proteins such as CD2AP (18). Nephrin from one foot process binds the nephrin being expressed from an adjacent foot process across a filtration slit to form the selective filtration barrier (19). Other nephrin functions that have been described include signaling functions that are mediated by intracellular tyrosine residues. Upon binding of Src family protein tyrosine kinase Fyn to the cytoplasmic tail of the nephrin transmembrane domain, the tyrosine residues become phosphorylated (15). When Fyn is knocked out in mice, there is abnormal development of foot processes (20). The process of phosphorylation allows for activation of downstream signaling pathways which act to regulate and maintain cell polarity, cell survival and cytoskeletal organization (21).The Src homology 2 (SH2) domain of Nck adaptor protein binds to three phosphorylated tyrosine residues (Tyr^{1176}, Tyr^{1193}, and Tyr^{1217}) on nephrin (22,23). This interaction is necessary for regulating the actin cytoskeleton dynamics in podocytes. (24). While the SH2 domain binds the phosphorylated motifs, the SH3 domain of Nck mediates interactions with downstream mediators of the cytoskeleton. Therefore, the SH3

domain on Nck provides the link between transmembrane nephrin and the dynamics of the actin cytoskeleton (25).

Nephrin is encoded by the NPHS1 gene. Mutations in NPHS1 can lead to congenital nephrotic syndrome of the Finnish type (15). These findings demonstrate a role for the nephrin gene in hereditary nephrotic syndrome. Its absence leads to a lack of SDs in the glomerulus which weakens the filtration barrier allowing proteins to leak into the urinary space (15). Genetic mutations in the nephrin gene which cause downregulation of its expressions lead to proteinuria, thereby eliciting podocyte injury (26). Injection of monoclonal antibodies against nephrin in rodent models results in proteinuria (27,28). When podocytes are treated with sera from patients who have exhibited recurrence of the disease in the allograft following renal transplantation, there is a dispersion of nephrin from the plasma membrane (29,30). Overall, findings in patients with various glomerular pathologies and in animal models of glomerular disease demonstrate a decrease in expression levels of nephrin (31). Since nephrin influences the dynamics of the cytoskeleton of podocytes via downstream activation pathways, mutations in nephrin result in changes/absence of normal cytoskeleton regulation (32).

Loss of nephrin has been shown to occur either by endocytosis (e.g. in diabetic nephropathy) (31) or by shedding (19). The shedding of nephrin from the plasma membrane of podocytes may occur following cleavage of its extracellular domain by a circulating protease (19). In the endocytotic pathway, nephrin may either be recycled back to the plasma membrane or degraded. These processes are influenced by the regulation of nephrin vesicle trafficking within the cell (33) and as such, the endocytic pathway may play a large part in loss of the protein from the cell surface.

Evidence for the cleavage and subsequent shedding of nephrin arises from a study of preclamptic patients whose sera induced endothelin-1 (ET-1) release from endothelial cells into the cellular supernatant (19). Exposure of podocytes to the supernatant from endothelial cells led to shedding of nephrin extracellular domain into cultured medium (19). It was later confirmed that pre-eclampsia patients demonstrate significant levels of nephrin in their urine, referred to as nephrinuria, while healthy pregnant women do not (34). The rapid loss of nephrin from podocyte cell surface induces cytoskeletal redistribution in cultured podocytes.(19). These findings suggest that pre-eclampsia-induced loss of nephrin from the SD may cause damage to the glomerular filtration barrier that leads to heavy proteinuria seen in the patients (34).

Elucidating the mechanism by which nephrin is lost from the plasma membrane of podocytes may allow for the development of an inhibitor for this process, thereby protecting podocytes from such injury. This inhibition would then present an opportunity for therapeutic targeting of glomerular disease.

1.2 FOCAL SEGMENTAL GLOMERUSCLEROSIS

1.2.1 Histological Description

Glomerulosclerosis is the main cause of chronic end-stage renal disease (8,35). Focal Segmental Glomerular Sclerosis (FSGS) is a common renal glomerular pathology, which frequently progresses to end-stage renal disease, requiring dialysis and or kidney transplantation (36,37). It is among the leading causes of nephrotic syndrome and renal failure in adults, and one of the causes of nephrotic syndrome in children. Nephrotic syndrome is a clinical disorder that displays a urinary loss of protein at least 3 g/day(38), and often progresses to glomerulosclerosis and renal failure. Understanding the molecular mechanisms underlying the onset and progression of FSGS would provide a means to therapeutically target the pathogenesis of the disease.

Previously considered to be a single disease, FSGS is now viewed as a rather non-specific histological pattern that arises due to a wide range of causes (11,39). The pathology of FSGS is most commonly described by progressive lesions in which podocytes begin to detach from the glomerular basement membrane (40). The histological lesions occur due to focal involvement in some but not all glomeruli and display a segmental pattern since only a part of the glomerulus may be damaged (41). The FSGS lesions are characterized by segmental sclerosis, obliteration of the glomerular capillaries and hyalinosis and presence of fibrotic adhesions in the Bowman's capsule (13). These histological changes occur as the cell's response to injury elicited by e.g. an exposure to toxins or to stress (2). (42). Despite the varying causes of the disease, all forms demonstrate injury to and loss of podocytes (11). Injury to podocytes leads to retraction of their foot processes and is associated with changes in the actin cytoskeleton

(43). The underlying molecular mechanisms of podocyte injury are currently under investigation. The severity of foot process effacement varies between injury models and cannot predict the degree of proteinuria observed (44).

1.2.2 Clinical Description and Genetic Causes of FSGS

Another method of classifying FSGS is on the basis of etiology. FSGS may be characterized as either primary (of idiopathic origin) or secondary (caused by known factors)(10). In cases of secondary FSGS, the lesions are brought on by genetic mutations in podocyte genes or by drug-induced nephrotoxicity (45). A subset of pediatric and adult patients acquire FSGS by genetic inheritance or due to sporadic de novo mutations (46). Peak incidence of pediatric nephrotic syndrome, though not necessarily due to FSGS, is ~2 years of age (14).

Patients with nephrotic syndrome are classified as either being responsive to steroid treatment or not responsive, in which case they are categorized as having steroid-resistant nephrotic syndrome (SRNS) (14). SRNS patients develop lesions consistent with a FSGS histological pattern. SSRN patients usually do not progress to FSGS and are more commonly diagnosed as having minimal change disease (MCD) since no change in the glomerulus can be detected under light microscope examination.

Secondary FSGS may also develop as result of severe immunological conditions, immune-mediated glomerulonephritis, arterionephrosclerosis or any other injury to the glomerulus that results in loss of nephrons. As nephrons are lost, the remaining nephrons attempt to compensate for their loss by hyperfunctioning ("hyperfiltration"). The excess energy requirements needed for hyperfunctioning exceeds the amount of metabolic substrate available,

thereby causing inflammation, cellular dedifferentiation, conditions of hypoxia and eventually acidosis and production of reactive oxygen species in the renal tubules (47). However, this is only one of many damage mechanisms to the glomerulus that can occur.

There are both autosomal recessive and dominant forms of hereditary FSGS. Recessive FSGS is generally caused by loss of function mutations while dominantly-inherited FSGS is associated with gain of function mutations. While the recessive form is usually more aggressive and manifests in early childhood, the dominant form is a slower progressing disease and demonstrates later onset (39).

The most common mutations responsible for hereditary FSGS are those in NPHS1 and NPHS2 genes, encoding nephrin and podocin, respectively (48). Both nephrin and podocin are integral components of the SD; thus, in the absence of either protein, the permselective filtration barrier is weakened (49).

Various germ line mutations in the Wilm's Tumor 1 (WT1) gene have been associated with hereditary FSGS (50). The WT1 gene encodes a zinc finger DNA-binding protein that regulates specification of podocyte identity (51). In murine models, heterozygous WT1 mutations that specifically affect binding to DNA and act as dominant-negative toward normal WT1 protein cause proteinuria which progresses to glomerular sclerosis (52). Another gene that has been identified in FSGS is the Crumbs Homolog 2 (CRB2) gene (53). CRB2 has been identified as a critical regulator of epithelial cell polarity (54). Loss of CRB2B in zebrafish compromises podocyte foot process arborization and SD formation (55). Mutations in CRB2 were found in several FSGS patients (50).

Genetic variations associated with APOL1, which encodes apolipoprotein L-1, have been associated with increased risk of FSGS in the African-American population (56-58). In addition,

other genes, when mutated, have been implicated in the onset and/or progression of FSGS: Inverted formin-2 (INF2) encoding formin2) (59), α-actinin-4 (ACTN4) (60), CD2-associated protein (CD2AP) (61), and the MYO1E gene, encoding class I myosin Myo1e (62).

1.2.3 Recurrence of FSGS

Among the patients with FSGS who progress to ESRD and then receive a kidney allograft, 30-50% exhibit recurrence (rec-FSGS) of the disease in the allograft (11). Recurrence may present anytime between a few hours post-transplantation and up to several weeks later (63). Rec-FSGS is often diagnosed by the onset of proteinuria. Proteinuria may occur prior to the appearance of any glomerular lesions which present more gradually. The renal biopsies reveal similar histological findings to the type of FSGS lesions prior to transplantation (13).

Risk factors that are often associated with recurrence of the disease include younger patients (aged 6-15 years), non-black race, heavy proteinuria prior to transplantation and those patients who previously rapidly progressed to ESRD (<3 years) (11). Despite the risk factors above, a clear and precise experimental or clinical method of screening for those patients that will recur following transplantation remains to be determined.

The immediate recurrence of FSGS in the allograft following surgery and diminished proteinuria in response to plasmapheresis provide further evidence for the presence of (an) unknown circulating podocyte toxic factor(s) that elicit(s) its effects by injuring podocytes in primary FSGS (64,65). Additional evidence for this circulating factor includes the occurrence of proteinuria in infants of mothers with FSGS (66). When a recurrent FSGS patient underwent plasmapheresis, urine protein excretion decreased and the patient exhibited remission of the

nephrotic syndrome (67). These lines of evidence support the hypothesis that a circulating factor present in the plasma of patients is responsible for eliciting injury to podocytes.

This unknown factor is believed to be of extra renal origin and likely is an immune system mediator (10). In 1974, Robert Shalhoub (68) was the first to suggest a role for immune cells in damaging the glomerulus. He postulated that an abnormality of T-lymphocyte function resulted in the secretion of some unknown mediator that elicited injury to the GBM. Some years later, in 1986, a patient with relapsing nephrotic syndrome and leukemia due to malignant natural-killer-like-T-cells was studied. This patient's proteinuria subsided following chemotherapeutic treatment of the malignancy (69) thereby strengthening Shalhoub's hypothesis. Since this time, several immunogenic factors including cardiotrophin-like cytokine-1 (70) and soluble urokinase plasminogen activating factor (suPAR) (36,41) have been proposed as the potential "podocyte-toxic" factors. suPAR and its membrane-bound form, uPAR, are involved in intracellular signaling processes related to cell adhesion and migration (71). When mice deficient for uPAR were subcutaneously injected with an expression vector encoding suPAR, the mice exhibited increased αvβ3 integrin signaling in podocytes that was associated with foot process effacement and proteinuria (72). Other studies have suggested a role for nuclear factor kappa-light-chain enhancer of activated B cells (NF-κB) in podocyte injury (73). The NF-κB pathway acts downstream of TNFα activation and is involved in cell migration and other processes (discussed below) (74). As the nature of the circulating factor remains unknown, there are currently no successful treatment regimens for rec-FSGS (40).

Savin (75) further established the role of a circulating factor in glomerular injury of patients by highlighting the recurrence of FSGS following renal transplantation (64), remission of the disease following plasmapheresis (65,67) and detection of proteinuria in children of FSGS

mothers (66,76). These studies provided preliminary data that implicate a role for the TNFα signaling cascade in the progression of rec-FSGS.

In FSGS patients, integrin expression is decreased in podocytes, resulting in detachment of the cells from the GBM (77). This has provided a rationale for our lab to investigate the possibility that a loss of focal adhesion complexes (FACs) is also responsible for podocyte detachment. Our previous work showed a rapid loss of FACs in vitro when human podocytes were treated with rec-FSGS serum (78).

1.3 FSGS AND TNFα IN PODOCYTE INJURY

Inappropriate activation of the TNFα pathway has been associated with the progression of several glomerular diseases (40,79). Injection of TNFα induces glomerular damage and an increase in serum creatinine in rabbits (80). Early work in 1993 by Myers' group demonstrated that there was a greater concentration of TNFα secreted from mononuclear cells of glucocorticoid-resistant FSGS patients as compared to minimal change nephrotic syndrome patients (79). This work led to the notion that antagonists to TNFα may be used to inhibit activation of the TNFα signaling pathway thereby reducing or suppressing renal injury.

In 2009, Leroy (81) was the first to document a case of successful anti-TNFα treatment of a proteinuric patient. The patient's proteinuria was suppressed by treatment with Etanercept (a soluble decoy TNFα receptor) and Infliximab (a chimeric monoclonal antibody against TNFα); both agents act by sequestering TNFα and preventing its binding to the TNFα receptors expressed on the cell surface (81). Some years later, the FONT (Novel Therapies for Resistant FSGS) study group demonstrated a remission of proteinuria in four out of ten patients with primary FSGS following a Phase 1 trial of adalimumab, a humanized TNFα neutralizing antibody (82).

1.4 TNFα SIGNALING

1.4.1 TNFα pathway

The human TNFα gene is found on chromosome 6; TNFα is synthesized as a 212-amino acid type II transmembrane protein. The protein has a soluble 17 kDa extracellular fragment that can be proteolytically cleaved from the surface of the cell by a transmembrane metalloproteinase (45).

TNFα is a major pro-inflammatory cytokine that activates various intracellular signaling cascades by binding to its type I transmembrane receptors TNFRI (p55) and TNFRII (p75) (83). TNFα is most commonly synthesized in activated macrophages, T lymphocytes and natural killer cells (84). Both receptors are activated by the soluble form of TNFα binding the receptor to form a TNFα homotrimer while TNFRI can also be activated by the membrane tethered TNFα. However, the binding affinity is five times greater for TNFRII as compared to TNFRI (85). Downstream pathways include activation of genes involved in the immune response, cell proliferation, antiviral response, growth inhibition and cell death (83). The activation of a specific cellular signaling pathway depends largely on the cell type and a particular cellular context. Once TNFα binds to either of its receptors, it initiates a number of downstream intracellular pathways such as the Jun N-terminal kinase (JNK) pathway, the NF-κB pathway and a pro-apoptotic signaling pathway (83,86) (Figure 2).

After binding of TNFα ligand to its receptor, the receptor becomes associated with the TNFα receptor-associated death domain (TRADD) (87). TRADD recruits Fas to the receptor, the receptor undergoes internalization and subsequent activation of caspase 8 leading to downstream

pro-apoptotic transcriptional changes (87). The NF-κB signaling cascade can also be activated by TNFα binding to either of its receptors. The NF-κB pathway is initiated when NEMO (NFκB essential modulator) phosphorylates IκB, the inhibitory subunit, leading to its subsequent dissociation from NF-κB and degradation. The liberated NF-κB enters nucleus where it activates multiple downstream targets (88). The third common intracellular pathway that is activated upon TNFα signaling is the JNK pathway. JNK-mediated phosphorylation results in enhanced activity of activator protein-1 (AP-1) and specificity protein-1 (SP-1) (89). The downstream intracellular activity that is induced by TNFα is largely dependent on the cellular context and homeostasis (86).

1.4.2 TNFα-converting enzyme

ADAMs (a disintegrins and metalloproteinase) represent a family of type I membrane glycoproteins which display a wide variety of biological roles. They are commonly known to be involved in the release of the ectodomains of membrane-tethered signaling proteins including cytokines, growth factors and their receptors, and adhesion proteins. The mechanisms underlying the cleavage and subsequent shedding of the extracellular domains are currently under investigation (90).

An ADAMS domain consists of an N-terminal prodomain followed by a metalloproteinase domain, a disintegrin domain, followed by a cysteine-rich domain which contains an EGF-like repeat (91), a transmembrane domain and a cytoplasmic domain. The prodomain acts as the protease's inhibitor by maintaining a cysteine switch mechanism. The metalloproteinase becomes activated by a process of nitrosation, alkylation or oxidation which

causes the dissociation of the cysteine linkage with the zinc atom in the active catalytic site. This process releases the prodomain of its inhibition. This activation is mediated by furin (91).

TNFα is produced as a type II transmembrane protein, anchored to the cell surface in its inactive form (92). ADAM 17, commonly referred to as TNFα-converting enzyme (TACE), cleaves TNFα from the cell surface, thereby releasing it in its soluble form into the environment surrounding the cell (91,93). TNFα is just one of over 70 substrates of TACE that have been identified, including VCAM1(vascular cell adhesion molecule-1), ICAM1 (intracellular adhesion molecule-1), TNFRI and TNFRII (94). Due to its sheddase activity, TACE has been implicated in the pathogenesis of several inflammatory diseases including rheumatoid arthritis, inflammatory bowel disease and pulmonary inflammation (74). Deficiency of TACE is embryonic lethal in mice (95) while conditional knockout mice (ADAM17 $^{ex/ex}$) cannot shed membrane anchored ligands into their soluble forms (96).

HYPOTHESIS

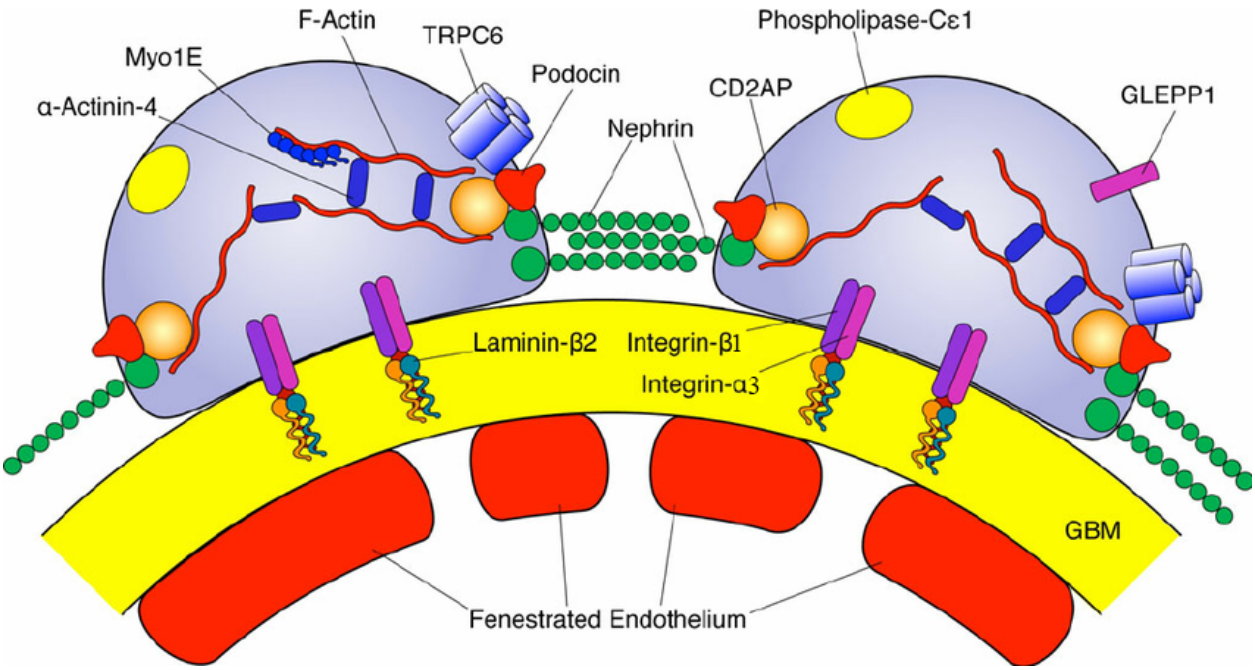
We hypothesize that the TNFα signaling cascade is activated in podocytes in response to toxic sera from rec-FSGS patients. This activation is mediated by prior activation of TACE and leads to subsequent disruption of the cytoskeleton and loss of nephrin from the cell surface.

RATIONALE

To date, recurrent FSGS is incurable and these patients are often not considered to be candidates for repeated renal transplantation, since the disease recurs frequently. Unveiling the mechanisms of idiopathic FSGS (including the recurrent form) will allow for the potential development of personalized therapeutic strategies. We have shown that activation of the TNFα signaling pathway may be associated with the toxic effects on podocytes in glomerular disease, since blockade of the TNFα pathway dramatically reduces these toxic effects restoring nephrin localization and expression at the plasma membrane and cytoskeletal organization in cultured human podocytes exposed to FSGS sera.

Figure 1: Podocyte Foot Process

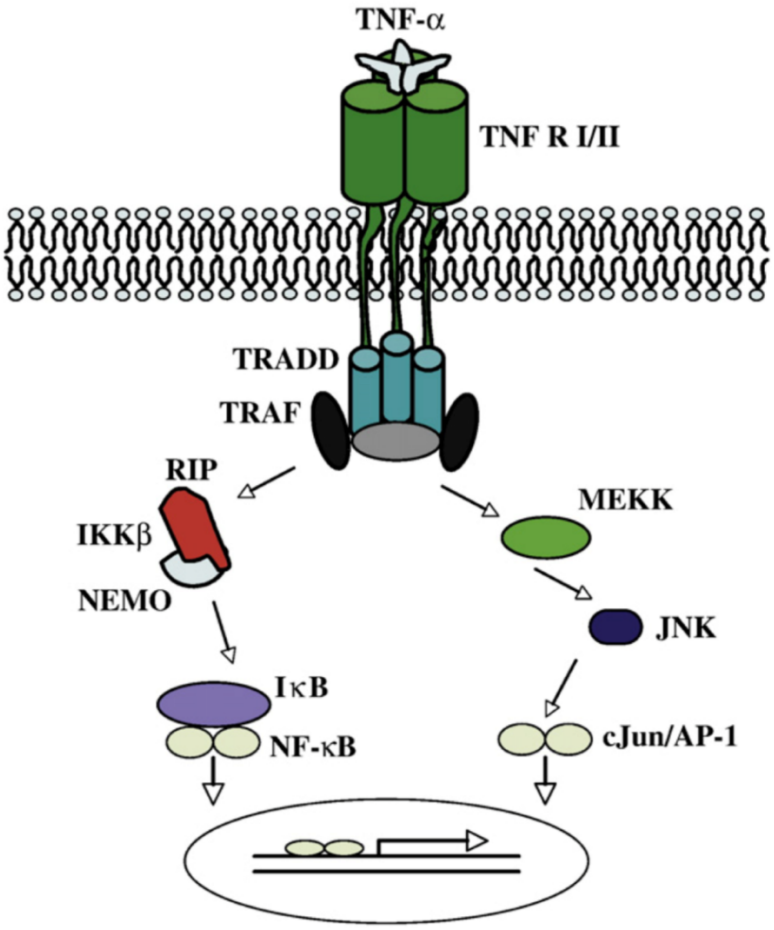
Schematic representation of the podocyte foot process structure and specific protein complexes (SD and adhesions). Cytosolic adaptor protein CD2AP interacts directly with the actin cytoskeleton of podocytes via its multiple binding sites. It is implicated in regulating the dynamics of the actin filaments and trafficking across the plasma membrane (97). Podocytes interact with the GBM via heterodimeric transmembrane integrin adhesion receptors that attach the extracellular matrix to the actin cytoskeleton (98).



Modified from *Simic I, Tabatabaeifar M, Schaefer F. Animals mdoels of nephrotic syndrome. Pediatric Nephrol. 2013* (with permission).

Figure 2. TNFα Signaling Cascade

Binding of TNF-α to either of its receptors (TNFRI or TNFRII) can initiate either pro-apoptotic pathways through its TRADD domain activation or lead to the initiation of the NFκB or JNK signaling pathways. These signaling cascades can then elicit changes in gene expression in the cell.



Modified from *Keigan M. Park, William J. Bowers (2010) Tumor necrosis factor-alpha mediated signaling in neuronal homeostasis and dysfunction. Cellular Signaling* (with permission).

CHAPTER 2: TNFα MEDIATED PODOCYTE INJURY

2.1 PREFACE

2.2 METHODS AND MATERIALS

2.3 RESULTS

2.4 FIGURES

2.1 PREFACE

Recent studies have suggested a role for the TNFα signaling cascade in the pathogenesis of recurrent focal segmental glomerulosclerosis (rec-FSGS). In 2009, Leroy et al. reported the case of a pediatric patient with rec-FSGS who exhibited a measurable reduction in proteinuria and increased eGFR (estimated glomerular filtration rate) following treatment with Infliximab followed by Etanercept, both anti-TNFα agents (81). Similarly, 4 out of 10 idiopathic FSGS patients in the FONT (Novel Therapies for Resistant FSGS) study group cohort demonstrated clinical improvements upon receiving TNFα-blocking therapy (82).

In vitro studies from our lab established that sera from certain patients with FSGS disrupt focal adhesion complexes and induce depolymerization of the actin cytoskeleton in cultured human podocytes. Blockade of the TNFα pathway could dramatically reduce these toxic effects, resulting in reassembly of adhesion complexes and restoration of cytoskeletal organization (40). For this reason, we chose to further investigate the role of the TNFα signaling cascade in podocyte injury in the progression of rec-FSGS. In particular, we chose to explore TNFRI/RII and TACE as potential targets for pathway inhibition.

2.2 METHODS AND MATERIALS

2.2.1 Antibodies Table

Target	Antibody	Source	Dilution/ Concentration
Nephrin (cytosolic domain)	Polyclonal nephrin anti-rabbit (TT 4.4)	Dr. T. Takano (99) McGill University	1:200 for IF 1:100 for WB
Nephrin (total)	Polyclonal nephrin anti-guinea pig	Acris Antibodies Uniprot ID# O60500 Gene ID# 4868	1:150 for cryo staining
TACE	ABT94 (rabbit polyclonal)	Sigma Millipore Cat# ABT94 Lot# Q2190499	1:200 for IF 1:1,000 for WB 1:150 for cryo section staining
Anti-Mouse primary antibody	Alexa Fluor 488 donkey anti-Mouse IgG	Invitrogen Cat# A21202	1:500 for cryo section staining
Alpha Tubulin	Monoclonal Anti-alpha-tubulin	Sigma Millipore Product# T5168 Batch# 103M4773V	1:1,000 for WB
Focal Adhesion Complexes	Monoclonal anti-vinculin (mouse anti-human)	Sigma Millipore Product# V9131 Batch# 034M4809V	1:400 for IF
TNFRI	Monoclonal anti-human TNFRI/TNFRSF1A	R&D Systems Cat #MAB225 Lot# 1P09	0.25 μg/mL
TNFRII	Monoclonal anti-human TNFRII/TNFRSF1B	R&D Systems Cat# MAB226 Lot# AYE05	0.1134 μg/mL
Anti-rabbit primary antibody	Alexa Fluor 568 Goat anti-Rabbit IgG	Invitrogen Cat#A11011	1:500- 1:1000 for IF
Anti-rabbit primary antibody	Peroxidase-conjugated AffiniPure Goat anti-Rabbit IgG	Jackson ImmunoResearch Cat# 111-035-003 Lot# 75609	1:1000 for WB
Anti-mouse primary antibody	Peroxidase-conjugated AffiniPure Goat anti-Mouse IgG	Jackson ImmunoResearch Cat# 115-035-003 Lot# 76936	1:5,000 for WB

2.2.2 Patient Plasma Sera

The National Institute of Diabetes and Digestive and Kidney Disease (NIDDK) provided central repository samples from the patients with steroid-resistant nephrotic syndrome as a part of the auxiliary study of the FSGS clinical trial (FSGS-CT). The auxiliary study was peer-reviewed and approved by the FSGS-CT steering committee. A written consent from all patients or their parent/legal guardian was obtained. Patients were from age 2 to 40 years and included both males and females. The patients were deemed steroid resistant if there was no observable decrease in proteinuria following 4 weeks of steroid treatment. FSGS was confirmed by kidney biopsy. The disease in the majority of patients was of idiopathic origin. All were diagnosed with proteinuria (>1 g/g creatinine) at the time of recruitment. No other major health issues were reported and the patients did not exhibit signs of ESRD. Plasma was collected from patients either during their visit to the clinic or to the hospital. Samples were immediately processed and aliquoted into smaller volumes to avoid multiple freeze/thaw cycles that could otherwise compromise their stability. The samples were then frozen and stored at -80°C.

2.2.3 Tissue Culture and Transfections

Human Podocytes AB 8/13

Conditionally immortalized temperature sensitive human podocytes (hPODs) AB 8/13 were kindly provided by Dr. Moin Saleem of the University of Bristol, UK (100). The hPODs were propagated under permissive conditions at 33°C in T75 tissue culture flasks in Roswell Park Memorial Institute (RPMI) conditioned medium supplemented with 10% Fetal Bovine

Serum (FBS, Wisent), 1% Penicillin/Streptomycin (PS, Wisent) and 100 µg/ml Normocin (Invivogen). Podocytes were trypsinized for 5 minutes with EDTA/Trypsin (Wisent) at 33°C. All assays were carried out at 70-80% cell confluency. Podocytes were plated in 12-well plates containing 80 µg/ml collagen coated 15 mm coverslips (Sigma C3867, 0.01% solution in phosphate-buffered saline, PBS). Podocytes were grown on the coverslips for 3 days at 33°C until they reached 70% confluency. At this point, they were shifted to non-permissive conditions at 37°C and allowed to differentiate for 14 days. During this time, the medium was changed twice weekly.

293/N – Study of Nephrin internalization

293T cells stably expressing rat nephrin (293/N) (a kind gift of Dr. Tomoko Takano, McGill University) were cultured at 37°C in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% Fetal Bovine Serum (FBS, Wisent) and 1% Penicillin/Streptomycin (Wisent) and 300 µg/ml hygromycin (selection marker). 293/N cells were trypsinized for 2 minutes with EDTA/Trypsin (Wisent) at 37°C. Cells were propagated until 80-90% confluence for all assays. Fifteen mm coverslips were coated in 0.02 mg/mL poly-D-lysine for 1 h at 37°C in a 12-well plate. 80,000 293/N cells per well were plated in DMEM/10% FBS/ 1% PS and grown for 48 h. Cells were then starved in DMEM lacking FBS and antibiotics for 30 minutes at 37°C and treated with 10 ng/mL recombinant TNFα (R&D Systems, Minneapolis, MN; MAB610) in DMEM/2% FBS/1% PS for 4 h at 37°C. In certain wells, cells were pre-treated with 500 nM TAPI-0 (Calbiochem, Sigma Millipore, ON) for 30 minutes in DMEM/2% FBS/1% PS at 37°C prior to treatment.

2.2.4. Immunostaining and Imaging

AB8/13 – Phalloidin/Vinculin/DAPI

Podocytes were washed in PBS and then fixed in 4% paraformaldehyde (PFA) for 15 minutes at room temperature. Following fixation, cells were permeabilized in 0.5% Triton-X-100/PBS for 5 minutes. Cells were then blocked in 10% normal goat serum (NGS, Jackson ImmunoResearch Laboratories)/0.1% Triton-X/PBS. Focal adhesion complexes (FACs) were visualized by staining with anti-vinculin antibody. Polymerized actin was visualized with AlexaFluor-568-conjugated Phalloidin (Molecular Probes, 1:40). The nuclei were stained with DAPI (4'-diamidino-2-phenylindole, dilution 1:900, Invitrogen) and the slides were mounted in Prolong Gold Antifade (Molecular Probes). The cells were imaged using the AxioObserved 100 inverted fluorescent microscope (Zeiss) with Axio4.8 software.

293/N – Study of Nephrin internalization

Cells were fixed in 4% PFA for 15 minutes at room temperature and then permeabilized in 0.5% Triton-X for 5 minutes. Cells were blocked in 2% BSA/5% NGS/PBS for 40 minutes. Coverslips were then immunostained with polyclonal anti-nephrin (TT) antibody against the cytoplasmic domain of nephrin for 1 h and then incubated with secondary goat anti-rabbit AlexaFluor 568 antibody along with DAPI (1:900) for 1 h, in the dark. Cells were mounted on coverslips using Prolong Gold Antifade and imaged by AxioObserved 100 inverted fluorescent microscope (Zeiss) using Axio4.8 software. Using ImageJ, a specialized image processing software, the plasma membranes of the cells were traced using the free hand tool and their size was measured in μm^2. Immunofluorescence intensity was quantified as IDVs (integrated density

values) in all cases. IDVs were normalized per measured area. 5 trials were carried out and 30 cells per condition were analyzed in each trial. All cells were compared in blind fashion to the untreated (control) cells incubated in DMEM/2% FBS/1% PS. The treatment conditions for recombinant TNFα on 293/N were determined by a dose-dependent assay and a time-dependent assay.

AB8/13 Stable Nephrin Clones – Nephrin/DAPI

To visualize presence of exogenous nephrin expression in hPODs, the individual clones selected in Zeocin were grown in collagen-coated 12-well plates for 4-5 days. When cells reached 70% confluence, they were fixed in 4% PFA for 15 minutes at room temperature. Cells were stained according the protocol described above.

Podocyte Toxicity Assay

Following a 14 day incubation period, differentiated podocytes were treated with FSGS patient sera. The serum samples were added in RPMI medium at a final concentration of 10%. The cells were then incubated with the serum samples for 20 h. In order to block TNFα signaling, the podocytes were pre-incubated with blocking antibodies against TNFRI and TNFRII for 30 minutes prior to the addition of patients' sera (78). The cells were fixed and stained as described above. Within each experiment, the treated cells were compared to untreated control cells grown in the same 12-well plate. Images of the cells immunostained with anti-vinculin antibody, AlexaFlour-568-conjugated Phalloidin (Molecular Probes, 1:40) and DAPI (1:1000) were obtained on AxioObserved 100 inverted fluorescent microscope (Zeiss) using Axio4.8 software. Cells were quantitatively analyzed by ImageJ. The images were converted to

grayscale to allow for binary measures of immunofluorescence intensity. The perimeter of each cell was traced using the freehand selection tool. The total area of the cell was then measured in μm^2. The grayscale was reversed to obtain a white background and the FACs were set to appear as red. The histogram plots the number of pixels on the Y axis and the intensity of IF from 0 (black) to 255 (maximum red) on the X axis. The threshold was positioned at the end of the histogram using the sliding bar. The quantity of 1-8 μm^2 particles within the perimeters of each cell was counted and normalized as a number of particles per 1000 μm^2. At least 40 cells per condition per patient were analyzed this way (78). The number of FACs in control podocytes was compared to the number of FACs in podocytes treated with patient sera in the presence or absence of TNFRI/II blockade.

2.2.5 Immunoblotting

Lysate preparation

Medium was removed and cells were washed three times in PBS and collected following scraping cells off the plate. The samples were centrifuged at 5,000 rpm for 5 minutes at 4°C. The supernatant was removed and cells were lysed by 10 second sonication in ice cold lysis buffer [50mM HEPES (pH 7.5), 150 nM NaCl, 10% Glycerol, 0.5% Triton-X-100, 1.5 mM MgCl, 1 mM EGTA, 25 mM NaF, 2 mM Na_3VO_4, 10 mM protein phosphate, 1 mM PMSF and 1x protease inhibitor cocktail]. Lysates were then centrifuged (15,000 RPM, 10 minutes at 4°C). The protein concentration in the supernatant was quantified using the Bradford Assay. Measurements were taken in duplicate and the average of both trials was calculated.

Immunoblotting

40-50 μg of cell lysate was loaded per well on a mini-gel electrophoresis apparatus (BioRad). Lysates were run on 7.5% SDS-PAGE for 20 min at 100V and then for 1 h at 120V. Proteins were then transferred from the gel to a nitrocellulose blotting membrane (BioTrace, Pall) at 100V for 1 h, on ice. The membrane was blocked in 5% skim milk (in Tris-buffered saline-Tween) at 22^{o}C for 1 h. The membrane was then incubated with primary antibody overnight at 4°C (see table 2.4.1 for antibody dilutions and concentrations). The next day, the membrane was washed four times for 10 minutes each in TBS-T and then incubated with secondary antibody conjugated with horseradish peroxidase (HRP) in dilutions ranging from 1:1,000-1:5,000 for 1 h at room temperatures in the dark. Following the same washing protocol, proteins were then visualized using enhanced chemiluminescence (ECL, ThermoFisher Scientific).

Adriamycin (ADR) - Mouse Model of FSGS

Male and female calcium-independent phospholipase A2γ (iPLA2γ) -/- mice on the C57BL/6 background (101) were housed at constant temperature with a 12 h light-dark cycle and free access to standard pellet chow and water. The mice were then randomly divided into two equally sized groups, the control group and the ADR-treated animals. At 3-5 months of age, the mice were injected via tail vein with 12 mg/kg of ADR. The control mice were injected with saline solution. Following injection, urine samples were collected every week. Four weeks post injections, the mice were sacrificed and their kidneys were dissected. The kidneys were cryopreserved in OCT compound (Optimal Cutting Temperature, ThermoFisher) at -80°C. The kidneys were then sliced into 4 μm sections and positioned on glass slides.

Immunostaining for TACE/Nephrin/DAPI on adult and embryonic mouse kidney tissues

Slides were brought to 22^{o}C for 20 minutes in a humidified chamber. Adult kidney cryosections were fixed with 4% PFA/PBS for 15 minutes at 22^{o}C. Paraffin-embedded sections generated using E17.5 embryos were used as positive controls. The embryo sections were fixed in 4% PFA/PBS for 5 minutes at room temperature. All kidney sections underwent unmasking of the epitope, the cytoplasmic domain of TACE protein, in 5% NDS/5% NGS/1% BSA/0.1% Triton X/0.05% SDS/PBS for 30 minutes at 22^{o}C followed by washing with 0.05% Tween-20/PBS twice and a subsequent blocking period in 1% BSA/0.05% Triton X/PBS for 30 minutes. TACE was detected with anti-Adam17 antibody (ABT94) in 1% BSA/0.05% Triton X/PBS overnight at 4°C. Anti-nephrin antibody was added to 1% BSA/0.05% Tx/PBS for 1 h and 30 minutes at room temperature. Sections were washed three times with 0.05% Tween-20/PBS for 1 minute followed by washes in PBS twice for 10 minutes. In the dark, sections were stained with AlexaFluor 568 in 2% NDS/1% BSA/0.05% TritonX/PBS for 1 h at room temperature. Sections were incubated in the dark with AlexaFluor 488 in 2% NGS/1% BSA/0.05% TritonX/PBS and DAPI (1:900) for 1 h at room temperature. Sections were then mounted on glass slides using Prolong Gold Antifade (Molecular Probes). Images were acquired by confocal microscopy using Zeiss LSM880 Laser Scanning Confocal microscope and ZEN2010 imaging software.

2.2.6 Statistical Analysis

The Student's t-test (equal variance) was used to calculate statistical significance between two groups. $P<0.05$ was considered statistically significant. One-way ANOVA followed by Bonferroni's adjustment to the Student's t-test (unequal variance), or the Chi Square test were

used to calculate statistical differences among groups. All data are presented as mean and standard error of the mean.

2.3 RESULTS

2.3.1 FAC Assay

The FAC image quantitative assay provides a means to quantify the toxic activity of FSGS serum, as well as the protective effect of TNFα pathway blockade on cells exposed to FSGS serum. FACs are large multi-protein complexes that anchor the cell's cytoskeleton to the extracellular matrix via transmembrane integrin adhesion receptors (102). Dynamic interactions exist between FAC components and the cell's actin filaments (103,104). As such, integrity of FACs is a good indicator of cell and extracellular matrix adhesion (105) and FACs are used to visualize overall cell morphology.

Figure 1 demonstrates the results of the FAC assay for 10 FSGS patients. Of these, 4 (black bars) recurred following renal transplantation (rFSGS), 2 had idiopathic FSGS (idFSGS, did not have transplant at the time of testing) and 4 did not recur post-transplant (nr-FSGS). These results led us to establish the 60% toxicity threshold as demonstrated by the red line. If a patient serum sample induces a loss of more than 40% of FAC rendering fewer than 60% of baseline levels of FAC intact, these patient samples are deemed toxic. Importantly, idFSGS patients whose sera induced this drastic loss of FACs have since recurred in the allograft, unlike the nrFSGS patients whose sera did not elicit FAC's loss below the toxicity threshold. Therefore, this assay is capable of identifying those patients who are at risk of recurrence following renal transplantation.

A larger cohort (96 samples of the NIDDK patients) was analyzed by our laboratory to determine the percentage of "toxic" samples in the extended cohort and to compare the number

of FACs in podocytes following treatment with sera with or without the TNFRI/RII blockade pre-treatment. The latter was done to ascertain percentage of samples in which toxicity could be reversed by TNFα blockade. The FAC numbers in the cells exposed to the FSGS sera with or without TNFα blockade were compared to untreated control cells. Cell morphology was examined by visualization of AlexaFluor-568-conjugated phalloidin (Molecular Probes, 1:40) to label actin filaments. The number of FACs stained by anti-vinculin antibody was assessed quantitatively using a previously established protocol for an unbiased method of screening samples (106).

Untreated hPODs (control) exhibited characteristic features: long stress fibers that extended throughout the cell soma and intact FACs that appeared as dense complexes. In control cells, FACs were evenly distributed throughout the cell along the actin fibers. In contrast, treatment with primary FSGS patient serum led to a disorganization of the actin cytoskeleton. A notable morphological change was evident in treated cells: the cells became elongated in shape; depolymerization of the actin cytoskeleton led to a disassembly of FACs that appeared either clustered together or of diminished density (lesser IF intensity) or lost completely.

The results of 10 of the randomly chosen NIDDK samples from the larger cohort (n=96) are demonstrated in Figure 2. Most patients were pre-transplant and the majority were diagnosed with primary FSGS upon biopsy. Further details of clinical features are described in section 2.2.2. Podocyte pre-treatment with blocking TNFRI and TNFRII antibodies restored to some degree the morphological defects caused by FSGS sera from some but not all patients. (Figure 2A). Of the 10 patients I analyzed, the serum samples from three patients elicited FAC loss below threshold 60%. The blockade of TNFRI and TNFRII significantly reversed the effect of FSGS patient plasma *in vitro* in 7 patients as demonstrated by the significant rescue of FACs in

pre-treated cells (Figure 2B). For patients 1-3, there was no significant rescue of FACs when cells were pre-treated with monoclonal antibodies against TNFRI/RII.

2.3.2. Nephrin Loss from 293/N and Podocytes in FSGS

293/N cells stably express exogenous rat nephrin both at the cell surface and inside the cell. Total nephrin expression can be ascertained by immunoblotting using rabbit anti-nephrin cytoplasmic domain antibody. On SDS-PAGE, nephrin migrates at ~180 kDa; however, in the 293/N cells there is also a nonspecific band at ~170 kDa; this nonspecific band is evident in 293T cells, which do not express exogenous nephrin (Figure 3). When cells were treated with 10 ng of recombinant TNFα for varying periods of time (4, 8 and 16 h), we detected no appreciable differences in the total levels of nephrin by immunoblotting in treated vs untreated cells (Figure 3).

The optimal treatment conditions for determining the effect of recombinant TNFα on nephrin expression and localization in the 293/N cells were established by dose and time dependent assays. First, the TNFα dose-dependent assay displayed a significant internalization of nephrin following treatment with 1 ng/mL, 10 ng/mL and 50 ng/mL of TNFα for 4h (Figure 4A). The time-dependent assay revealed significant internalization of nephrin beginning at 1 h post TNFα treatment, which returned to a non-significant value after 8 h (Figure 4B). This led us to establish the optimal conditions for the assay as 10 ng/mL of TNFα for 4 h because these conditions yielded reliable and consistent results.

Immunofluorescence studies were then carried out using the same anti-nephrin antibody. We show a mean intensity of 36.2% of total nephrin (range ~31-41%) at the plasma membrane of 293/N cells. Upon TNFα treatment, there were no appreciable differences in the total levels of nephrin expression in the TNFα treated vs untreated cells. This indicates that nephrin was not degraded intracellularly. This led us to study nephrin internalization as a possible mechanism for nephrin loss from the cell surface. As such, upon TNFα treatment, we observed a significant internalization of nephrin seen as the decrease in nephrin expression at the plasma membrane and the parallel increase in nephrin expression intracellularly.

293T cells display undetectable levels of endogenous nephrin (Figure 4E), proving specificity of the anti-nephrin antibody in the immunofluorescence experiments. The mean value of nephrin at the plasma membrane in 293/N cells following treatment was 63.6% (±10.2%) of nephrin expression pre-treatment (Figures 4C, 4D and 4F). These results show a mean loss of 36.4% of nephrin expression at the surface of the cell when podocytes were treated with TNFα. Upon TNFα treatment, significant internalization of nephrin was observed, implying that TNFα signaling may result in nephrin endocytosis. TNFα-induced internationalization of nephrin in 293/N cells was blocked by the inhibition of TACE using TAPI-0.

The cells were categorized according to the predominant localization of nephrin expression (surface nephrin versus mixed-localized nephrin versus internal nephrin). Prior to treatment with TNFα, the majority of cells were categorized as having surface expression of nephrin. However, upon treatment, there was a shift towards the mixed nephrin and internalized nephrin categories (Chi-squared test = 7.39836E-75) (Figure 4G).

TAPI-0 is a specific inhibitor of ADAM17/TACE (107). In the presence of TAPI-0, TACE cannot be cleaved to its mature active form and, as consequence, cannot cleave and

subsequently activate TNFα to elicit downstream effects on TNFRI/RII. Additionally, TACE may be able to proteolitically cleave other surface molecules, which may be involved in TNFα signaling (e.g. TNF receptors). We hypothesized that pre-treatment with TAPI-0 would inhibit activation of the TNFα signaling cascade and therefore, would inhibit TNFα- induced nephrin internalization. Upon pre-treatment with TAPI-0, there was a partial yet significant restoration of nephrin expression at the plasma membrane. Mean value of nephrin expression at the plasma membrane was restored to 93.5% (±2.0%) in TAPI-0 pre-treated cells comparing to the control cells that were not incubated with TNFα (Figure 4F). In addition, there was a shift back towards the cells with the surface nephrin expression in the presence of TAPI (Chi-squared test = 3.28E-18) (Figure 4G). Interestingly, 293/N cells treated with 10ng TNFα for 4h display emerging filiopodia (Figure 4H), suggesting a potential increase in cell motility.

2.3.3. Adriamycin-Induced Nephropathy, a Mouse Model of FSGS

Under normal physiological conditions, iPLA2γ -/- mice on the C57/BL6 background demonstrate a substantial loss of podocytes, widening of foot processes and widespread mitochondrial dysfunction (108). However, these ultrastructural changes were not sufficient to induce albuminuria. Studies of podocytes with mitochondrial dysfunction suggest that these cells have enhanced complement mediated cytotoxicity, compared to cells with normal mitochondria (108) hereby implying that a "second hit" to the kidney in the context of mitochondrial impairment might be enough to cause proteinuria in otherwise resistant mice.

Doxorubicin hydrochloride (Adriamycin) is a chemotherapeutic drug that is known to commonly induce renal impairment and proteinuria in rodents, and FSGS 4-6 weeks following

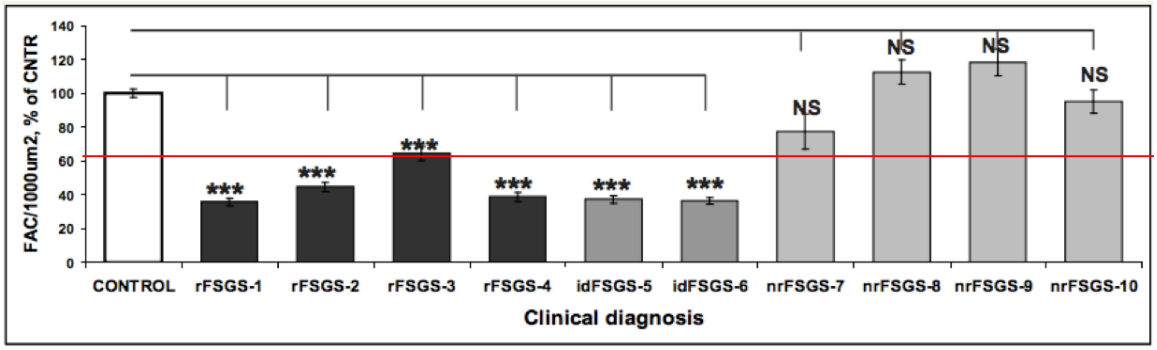
injection (109). The drug induces direct toxic damage to the podocytes and thereby disrupts proper functioning of the glomerular filtration barrier (109,110). Therefore, the injection of Adriamycin was used as a "second hit" to induce albuminuria in the iPLA2γ -/- mice. As expected, the ADR-treated iPLA2γ -/- mice displayed proteinuria.

In order to ascertain whether injury to the glomerular filtration barrier was accompanied by an upregulation of TACE, we quantified the expression of TACE in the glomeruli of mice both treated and untreated with Adriamycin. In kidney sections, glomeruli were detected by the presence of nephrin staining. Both renal tubular and glomerular expression of TACE was seen to increase in mice treated with Adriamycin, as compared to untreated mice (Figure 5A). Therefore, to test whether TACE expression was increased in glomeruli, the relative IF staining intensity in each glomerulus was normalized to the average IF intensity measured in n=3 tubules for each image. Once normalized, we found an approximately 1.5-fold increase in expression of TACE in the glomeruli of proteinuric mice injected with ADR as compared to untreated non-proteinuric controls (Figure 4B). In ADR-treated mice, the staining for TACE appears to colocalize with staining for nephrin, implying that TACE was increased in podocytes, although an increase in TACE in mesangial cells is also a possibility (Figure 5A).

2.4 FIGURES

Figure 1: 60% Toxicity Threshold

Patient serum samples that induce a loss of more than 40% of FACs (fewer than 60% of baseline levels of FACs in the untreated control cells) are deemed toxic (60% toxicity threshold).



White bar indicates control (untreated, healthy patient) sera, black bars indicate recurrent FSGS (rFSGS) patient sera, dark grey bars indicate sera from idiopathic FSGS patients with toxic sera who had not been transplanted at the time of testing (idFSGS)s, and light grey bars are indicative of non-recurrent patient sera (nrFSGS).

Adapted from (78)

Figure 2A: Toxic effects of 10% FSGS serum and rescue by TNFα signaling blockade (anti TNFα RI and RII) in cultured human podocytes.

Actin filaments were visualized by phalloidin staining (red), while focal adhesion complexes were visualized by staining with anti-vinculin antibody (green); and chromatin was stained with DAPI (blue) to visualized nuclei.

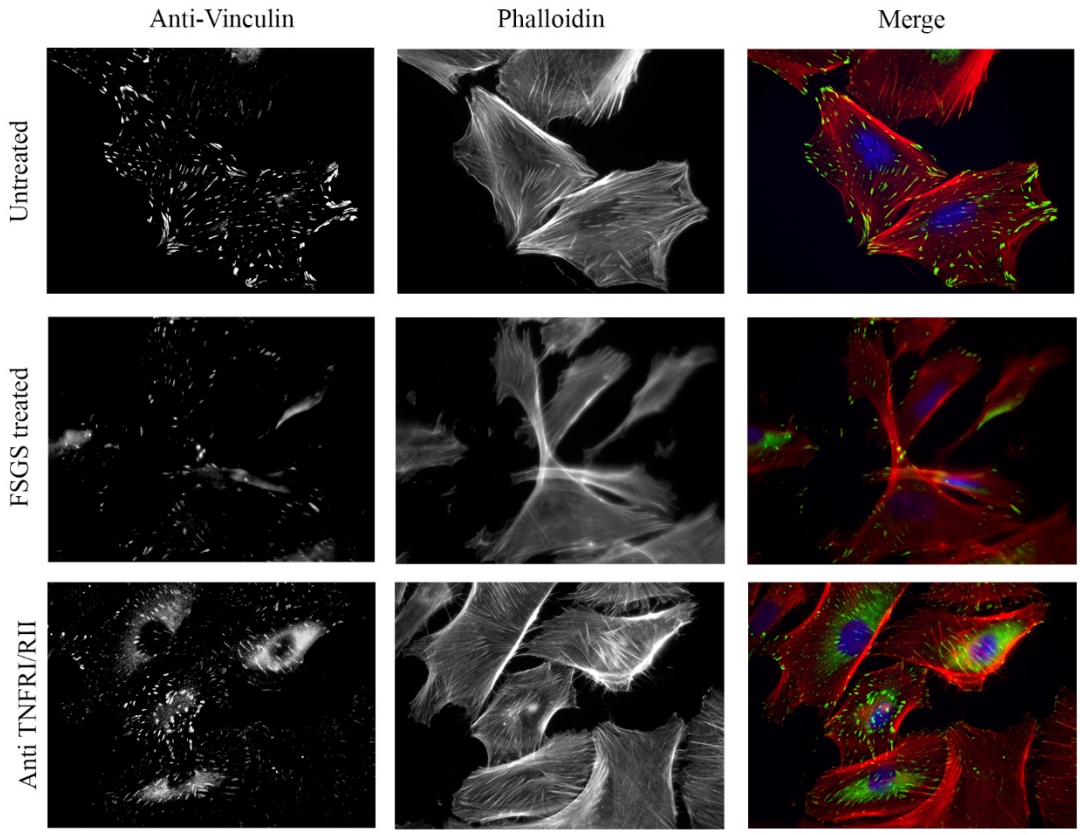


Figure 2B: Quantification of toxic effects and rate of recovery of human podocytes treated with FSGS sera (from 10 idiopathic patients) and anti-TNFRI/RII + FSGS sera

Mean values (±SE) are represented as a percentage of intact focal adhesion complexes per cell (for 30 cells) as compared to untreated control cells set at 100%. (n=3)

*P<0.05

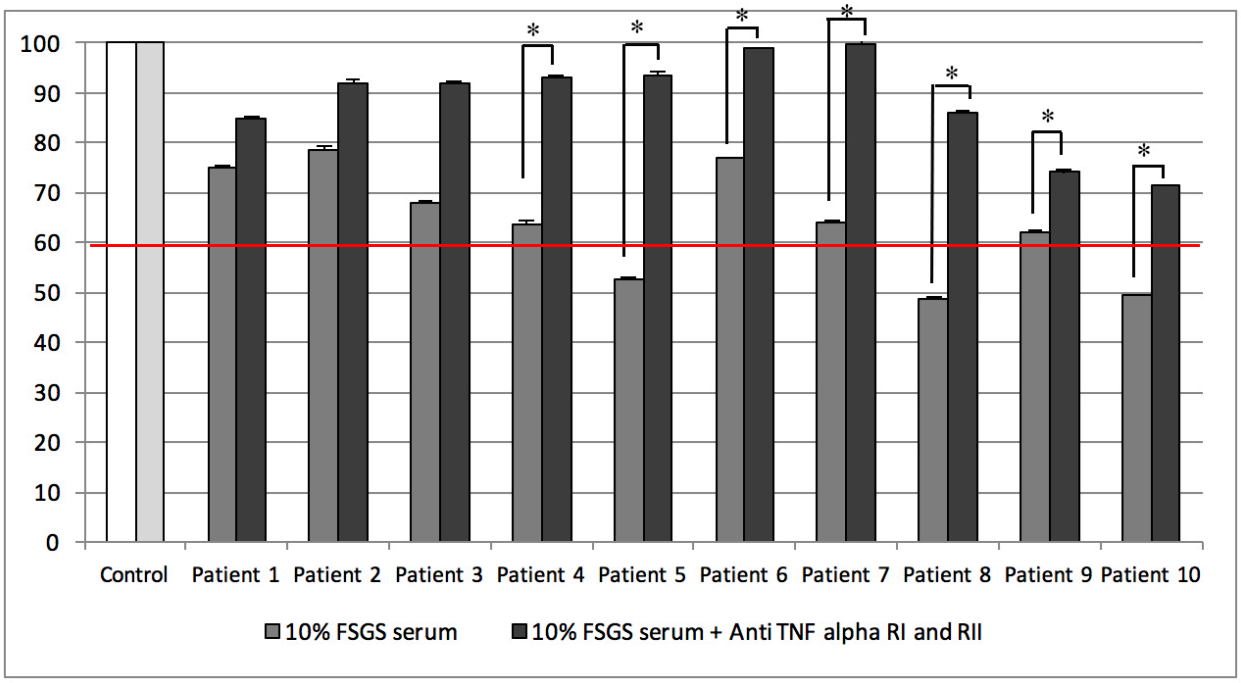
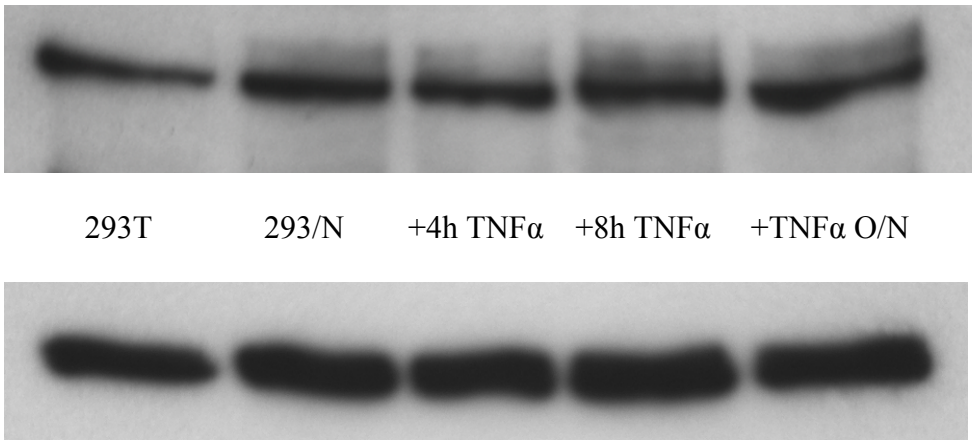
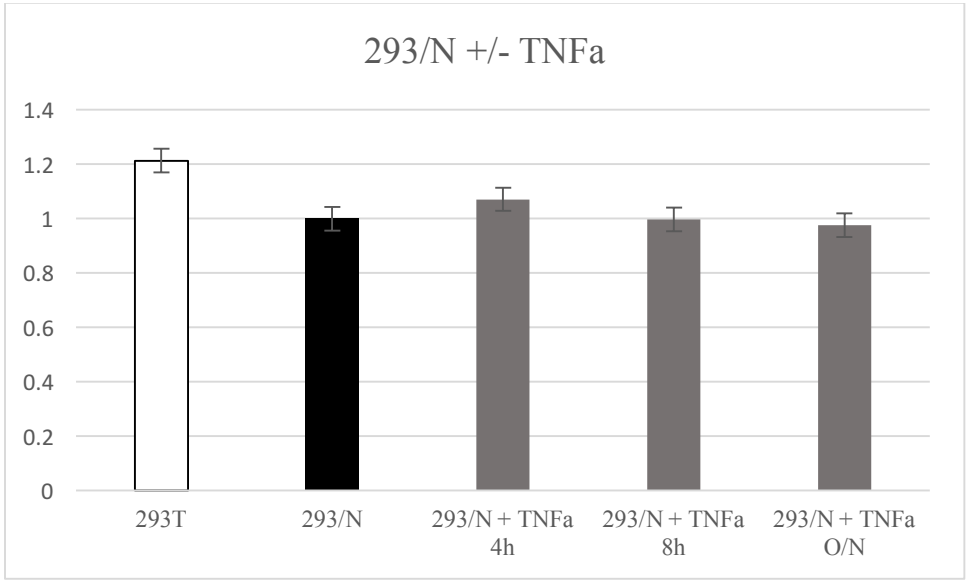


Figure 3: Treatment of 293/N cells with 10 ng of recombinant TNFα for 4 h, 8 h and 16 h demonstrates no appreciable differences in the total level of nephrin (immunoblotting).





Intensity of the nephrin signal was determined by densitometry of the 180kDa band intensity and was normalized to α-tubulin. Results are the average of 3 replicate trials. Nephrin is at 180 kDa and the band at 170 kDa is nonspecific.

Figure 4A: Dose-dependent assay of nephrin internalization following recombinant TNFα treatment of 293/N cells.

Mean values are presented as a percentage (%) of total nephrin at the surface (IF intensity) of the cell prior to treatment. *P<0.05, n=3.

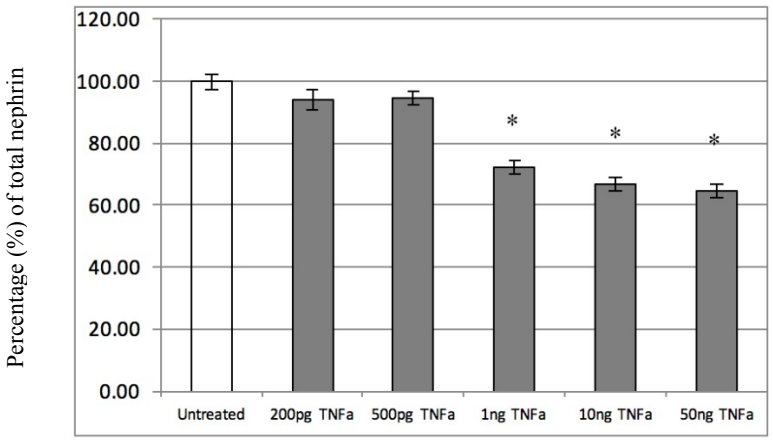


Figure 4B: Time-dependent assay of nephrin internalization following recombinant TNFα treatment on 293/N cells.

Mean values are represented as a percentage (%) of total nephrin at the surface of the cell (IF intensity) prior to treatment. *P<0.05, n=3.

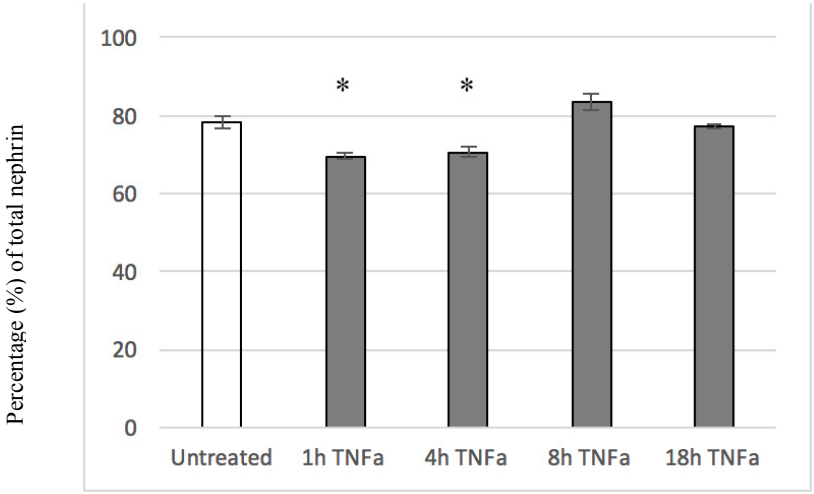
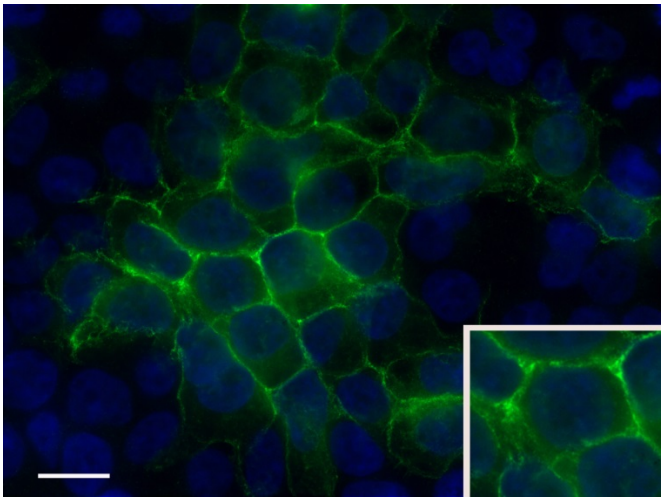


Figure 4C and 4D:



4C: Untreated 293/N cells display the most of their nephrin expression at the plasma membrane. After permeabilization of cells, nephrin was visualized by an antibody against the cytoplasmic domain of nephrin (green) while nuclei were visualized by DAPI (blue). Scale bar is 10 μm.

4D: Upon treatment with 10 ng/mL of recombinant TNFα for 4 h, 293/N cells display internalization of nephrin as visualized by an increase in intracellular staining.

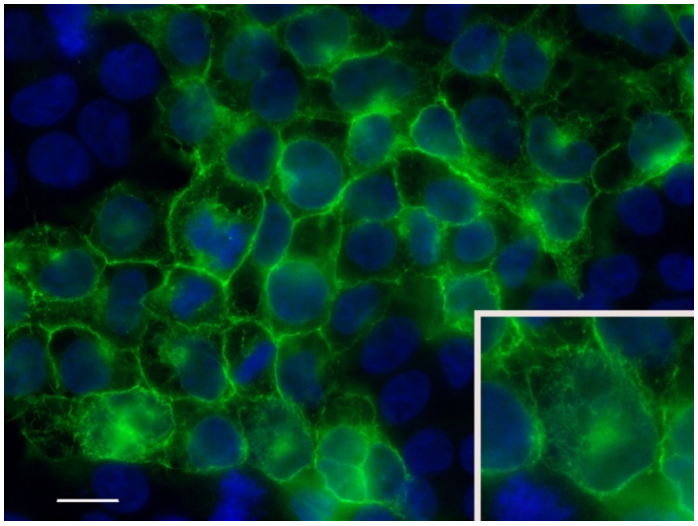


Figure 4E: 293T cells untreated.

293T cells express undetectable levels of endogenous nephrin, visualized by the same anti-nephrin antibody that was used to detect nephrin in 293/N cells (4D-E); nuclei were visualized by DAPI (blue).

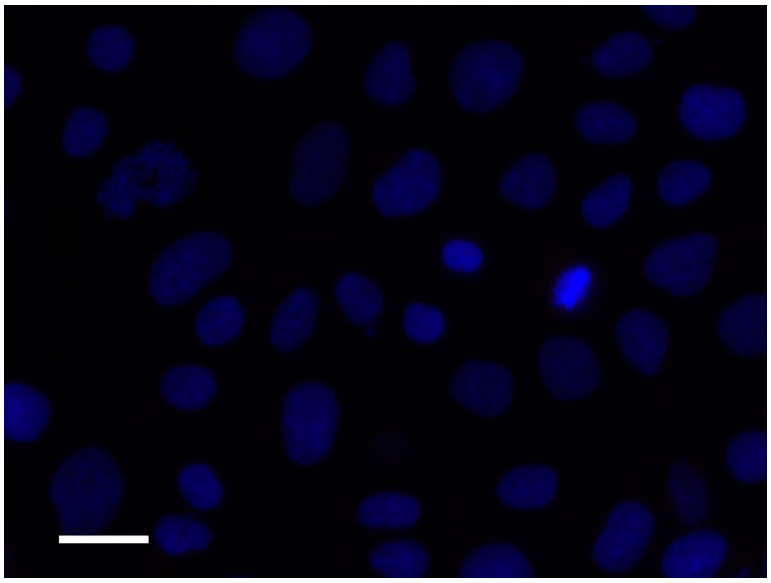


Figure 4F: 293/N cells treated with 10 ng/mL of recombinant TNFα display significant internalization of nephrin. Y axis = Plasma membrane nephrin.

Mean values are presented as a proportion of nephrin expression (IF intensity) compared to untreated, control cells (nephrin level is set at 100%).

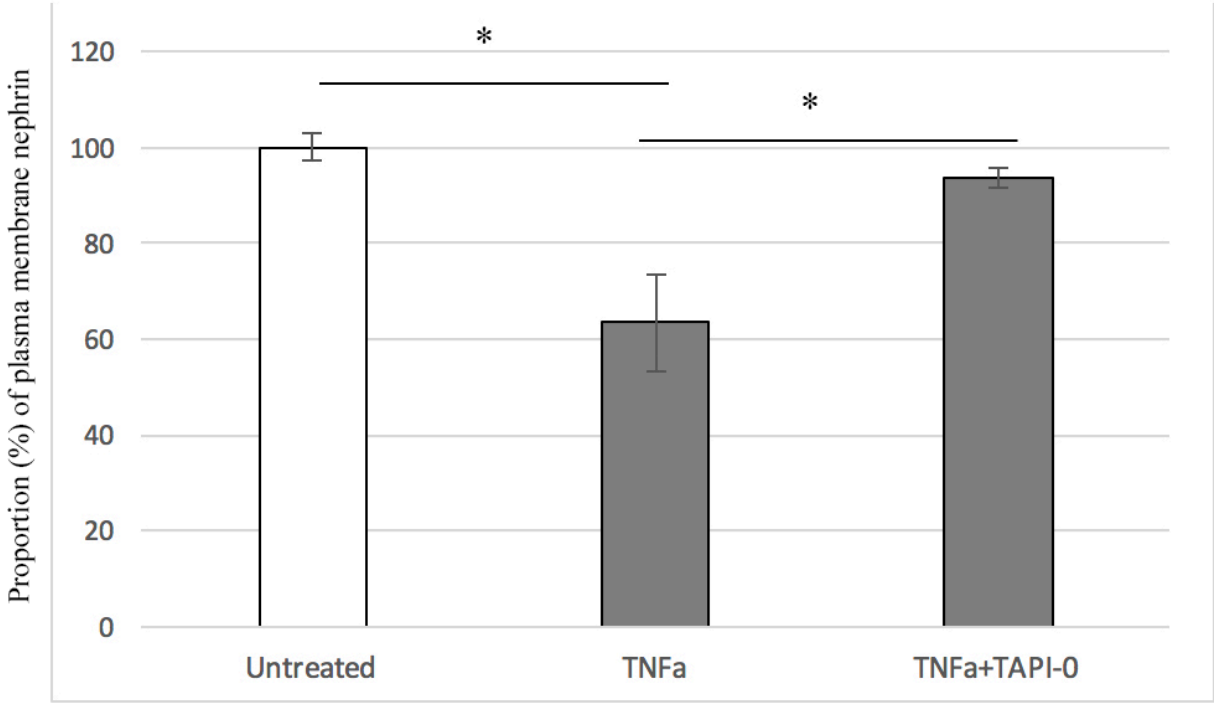
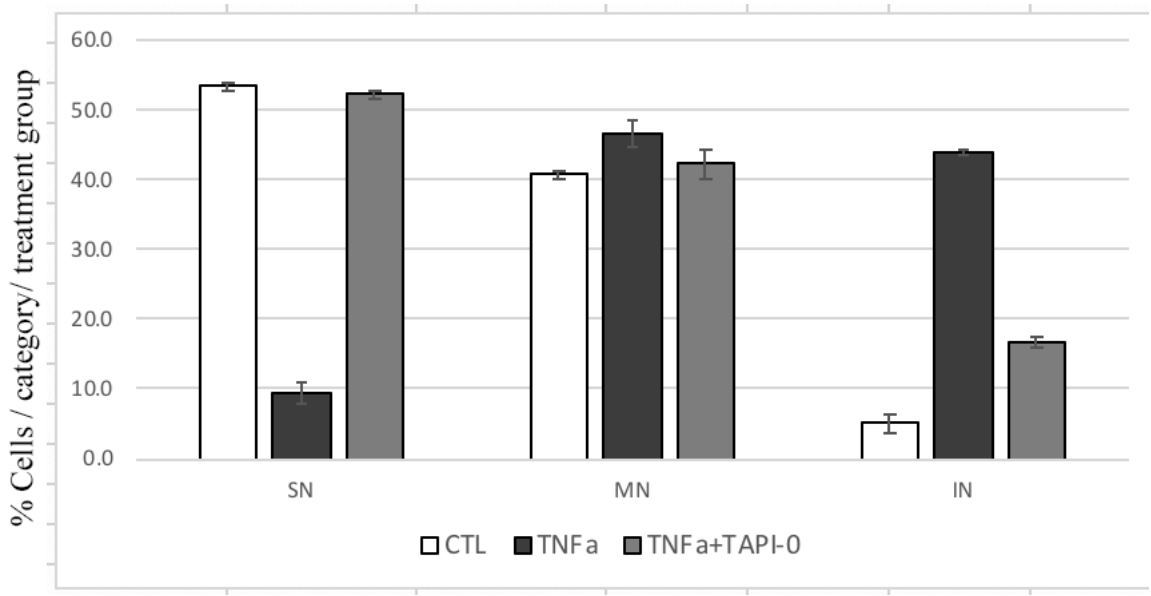


Figure 4G: Untreated control cells most commonly display surface expression of nephrin. Surface nephrin expression is expressed as a percentage of total 100% nephrin expression (y-axis). Upon TNFα treatment, there is more nephrin localized intracellularly. Loss of surface nephrin is abrogated upon pre-treatment with TAPI-0. (n=3)

SN: Most of nephrin is expressed at the surface of the cell

MN: Nephrin expression is fairly equally distributed between the surface of the cell and intracellularly

IN: Most of nephrin is expressed intracellularly

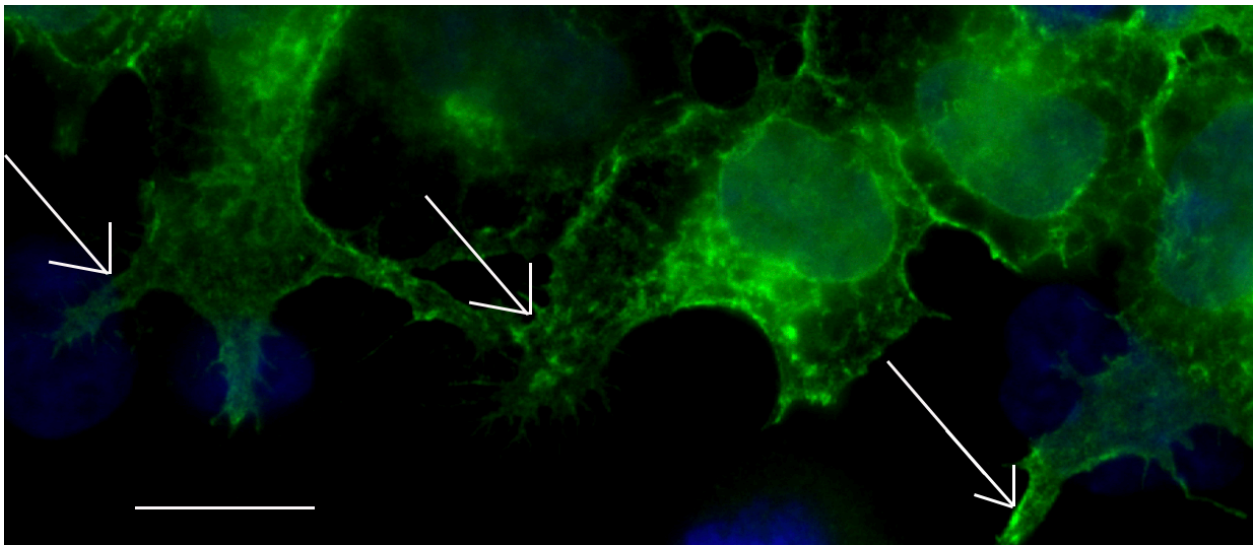


Chi-squared test (CTL vs TNFα) = 7.49E-75

Chi squared test (TNFα vs TNFα + TAPI-0) = 3.28E-18

Figure 4H:

293/N cells treated with 10ng of recombinant TNFα for 4 h were stained with anti-nephrin antibody (green) and DAPI (blue). Scale bar =10μm.



293/N cells treated with recombinant TNFα display emerging filiopodia (arrow heads).

Figure 5A: ADR-treated proteinuric iPLA2γ-/- mice demonstrate an increase in TACE expression in both the glomeruli and the renal tubules.

12 glomeruli were analyzed per mouse. The staining of the glomeruli was normalized to the average staining of 3 renal tubules from the same image.

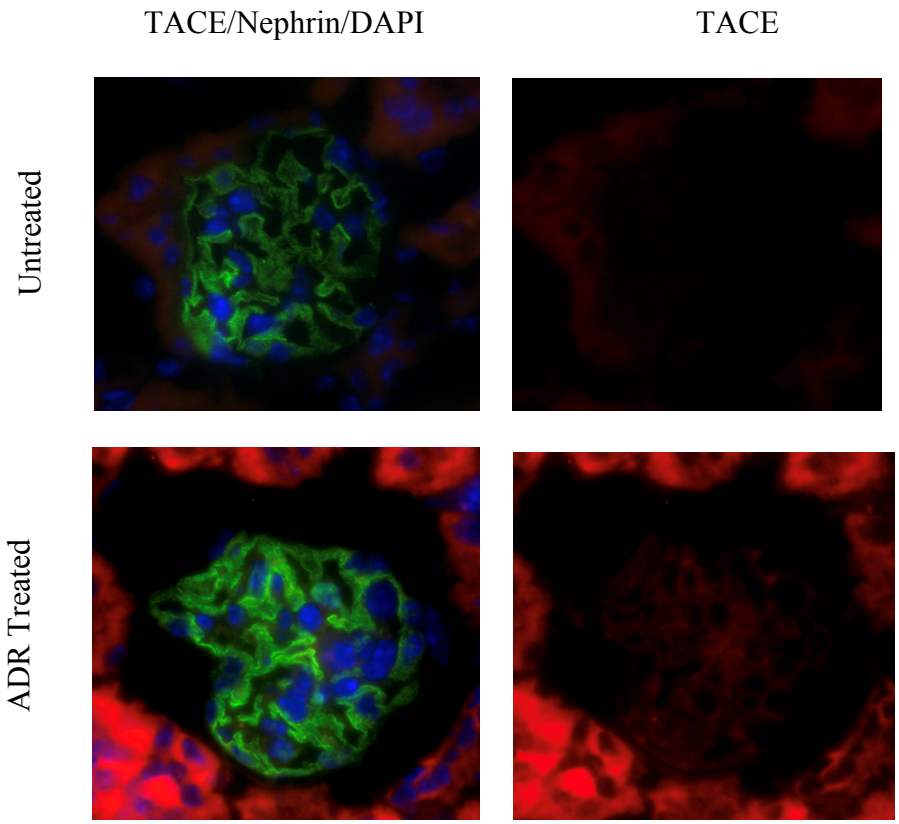


Figure 5B: ADR-treated proteinuric iPLA2γ-/- mice demonstrate an approximately 1.5-fold increase in glomerular TACE expression.

Fold increase in the intensity of TACE/ADAM17 staining in the glomeruli of untreated vs Adriamycin (12 mg/kg) treated iPLA2γ-/- mice, normalized to renal tubular staining (n=4). *P<0.01

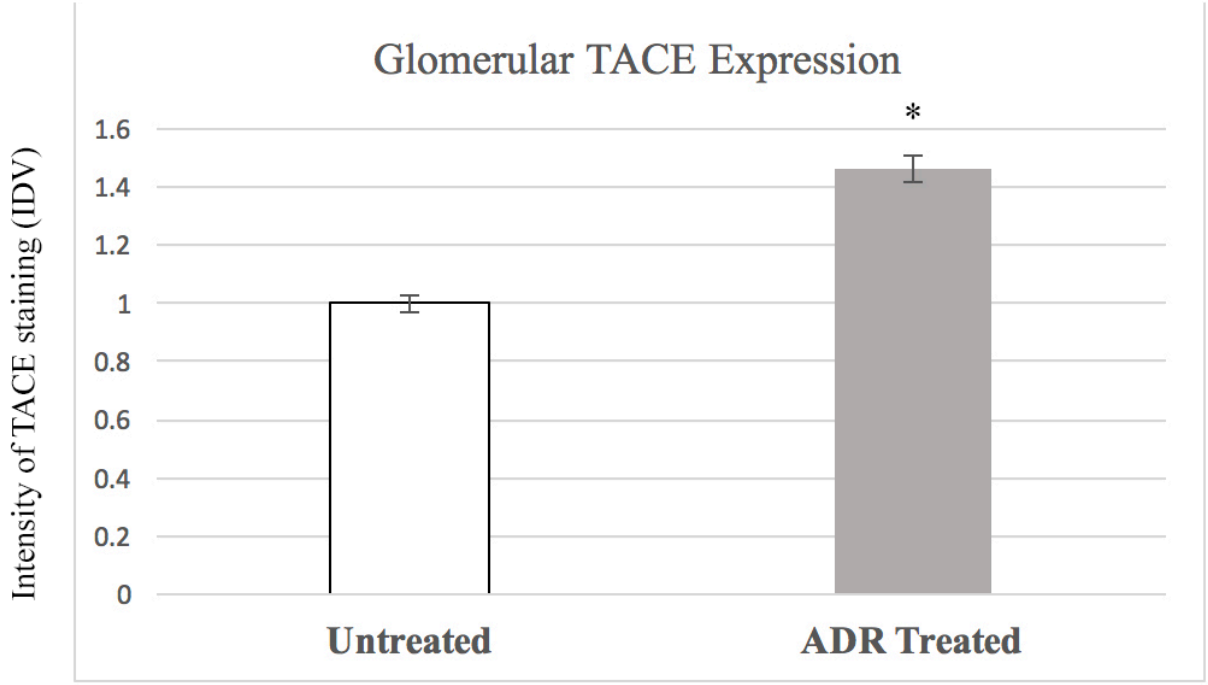
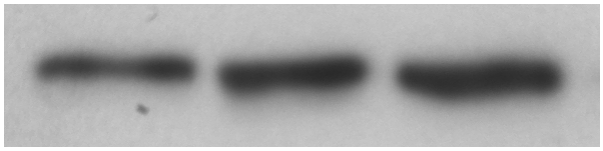


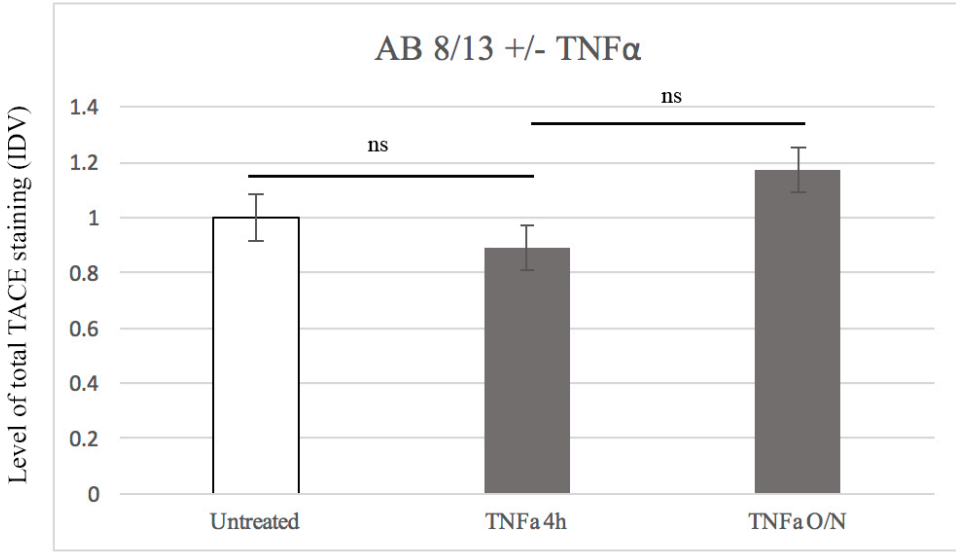
Figure 7: Podocytes treated with 10ng of recombinant TNFα have no significant differences in the total level of TACE expression.

- 120 kDa (immature TACE)
- 100 kDA (mature TACE)

Untreated + TNFα 4h + TNFα ON



- 55 kDa (alpha tubulin)



Relative fold change of total TACE signal was normalized to a housekeeping gene (alpha tubulin). Results are the average of replicated trials (n=3).

CHAPTER 3: DISCUSSION AND FINAL CONCLUSIONS

3.1 Discussion

Our results further validate the role for the TNFα signaling pathway in the progression of glomerular injury, specifically, podocyte injury in FSGS. Our results suggest a role for TACE in this process, as reflected by increased TACE expression in the glomeruli of proteinuric iPLA2γ-/- mice. We further demonstrated that inhibition of TACE reversed the TNFα-induced internalization of the SD protein, nephrin, i.e. nephrin remained on the surface of cells (unpublished). Furthermore, when podocytes were treated with rec-FSGS serum, inhibition of TACE prevented the loss of focal adhesion complexes.

96 FSGS patients were screened using the FAC image quantitative assay. 48/96 or 50% of patient samples elicited toxic effects on hPODs (Figure 1). Of all patients whose sera elicited toxic effects, we blocked the TNFα signaling pathway by pretreatment with anti-TNFα RI and anti-TNFα RII monoclonal antibodies. These patients were then grouped into the following three categories based on the degree of response to the blockade: strong responders, which elicited at least a doubling of FAC (~25% of toxic serum samples), mild responders (~25% of toxic serum samples) and non-responders, which elicited no response (~50% of toxic serum samples). Those patients whose samples showed a strong response to the blockade pre-treatment may benefit from treatment with TNFα inhibitors in the clinic.

The prevention of FAC dissolution observed in hPODs with TNFRI/RII blockade occurs because the blockade of TNFα signaling results in partial restoration of the actin cytoskeleton, overall cell morphology and significant recovery of focal adhesion complexes. Furthermore,

treatment of human podocytes with recombinant TNFα has been shown to cause depolymerization of actin filaments, a reduction in the number of FACs and an overall decrease in cell size (40). As such, TNFα is known to be sufficient to produce the same effects that we observed in podocytes treated with primary FSGS serum. The disruption and loss of FACs observed in hPODs treated with primary FSGS patient serum in vitro is believed to play a role in detachment of podocytes from the GBM in vivo (13).

Nephrin serves as a structural component of the SD complex, thereby, it is critical for the integrity of the filtration slit that forms the selective filtration barrier (66). The absence of nephrin leads to a weakened filtration barrier allowing proteins to leak into the urinary space (63). To determine whether TNFα altered localization and/or expression of nephrin, 293 cells stably expressing rat nephrin were treated with recombinant TNFα. Under baseline conditions, TACE cleaves endogenous TNFα and makes it readily available for activation of its downstream receptors. Under experimental conditions, exogenous TNFα may upregulate endogenous TNFα and TACE may block cleavage of this endogenous TNFα. TAPI-0 inhibits TACE from activating TNFα or TNFRI/RII. Therefore, TNFα cannot bind to its receptors to elicit downstream signaling pathways that involve nephrin loss from the plasma membrane. This work supports a role for TNFα signaling in the loss of nephrin from the surface and a required role for TACE in this process. However, the possibility of nephrin being cleaved from the cell surface and subsequently shed into the conditioned medium was not investigated and therefore cannot be ruled out. TACE cleaves a large number of transmembrane molecules; its cleavage sites analyzed in various proteins proved to be diverse. Therefore, it is difficult to predict in silico which specific molecules might be cleaved by TACE. Although, in theory, it is possible that nephin might be targeted directly by activated TACE (upon TNFa treatment), it is equally likely that

TACE may be acting indirectly on nephrin via activation of the intracellular TNFα pathway and this, in turn, induces nephrin loss from the cell surface. Both scenarios will require further experimental investigation.

It should be noted that 293/N cells treated with recombinant TNFα displayed emerging filopodia (Figure 5G). This is suggestive of the cells displaying a more motile and less adhesion-based phenotype, and is in keeping with the loss of normal adhesion.

In healthy human kidneys, TACE expression is absent in the proximal tubules, moderate in the glomeruli and very strong in the distal tubules (111). However, upregulation of TACE mRNA was noted in the podocytes of diseased kidney tissues (111). To determine whether TACE protein expression was upregulated in proteinuric kidneys, the glomeruli of mice with Adriamycin-induced proteinuria were analyzed. These proteinuric mice display increased expression of TACE in the glomeruli as compared to saline-injected, control mice. Interestingly, TACE expression also increased in the renal tubules of the proteinuric mice, suggesting more widespread changes to a number of renal substructures. For example, tubular epithelial cells may have been injured by albuminuria, resulting in enhanced TACE expression.

In conclusion, inhibition of TACE abrogates the toxic effects of exogenous TNFα on the induction of nephrin internalization. By extension, inhibition of TACE by TAPI may abrogate the toxic effects of rec-FSGS serum such as the endocytosis of nephrin and production of endogenous TNFα. Unveiling the mechanisms of recurrent idiopathic FSGS will continue to allow for the development of personalized diagnostic and therapeutic strategies for patients whose disease is mediated by the TNFα signaling pathway and who may be candidates for anti-TNFα therapy.

3.2 Future Directions

Mechanism of Nephrin Loss from Podocytes Treated with FSGS Sera

Future studies for this project may include determining whether nephrin is internalized in the presence of FSGS sera and whether blockade of the TNFα pathway or TACE inhibition will inhibit nephrin internalization. For these experiments, hPODs stably expressing human nephrin are being produced. Cell surface nephrin may be detected with H-300 anti-nephrin antibody (Sigma Millipore), which recognizes the extracellular domain of nephrin. Podocyte nephrin clones should also be treated with FSGS serum to detect the effect of the serum on nephrin shedding, by quantifying levels of nephrin in the cells and in the conditioned medium by immunoblotting with H-300 antibody.

TACE Proteolytic Cleavage and Activation in an Animal Model of FSGS

In addition, future studies may also refine TACE expression levels in proteinuric mice. The level of TACE expression may be measured by quantitative IF and compared in the glomeruli of proteinuric versus non-proteinuric mice. C57BL/6 iPLA2γ-/- mice when injected with 12mg/kg of Adriamycin become proteinuric. However, wildtype mice on the same background do not exhibit proteinuria. The expression level of TACE in the glomeruli of these mice should be compared to iPLA2γ +/+ ADR-injected mice which do not exhibit proteinuria. It would also be of interest to test whether TAPI fed to the ADR-treated mice ameliorates proteinuria. These experiments will directly test our hypothesis that a local activation (secretion) of TNFα in podocytes in response to ADR injections further exacerbates podocyte injury.

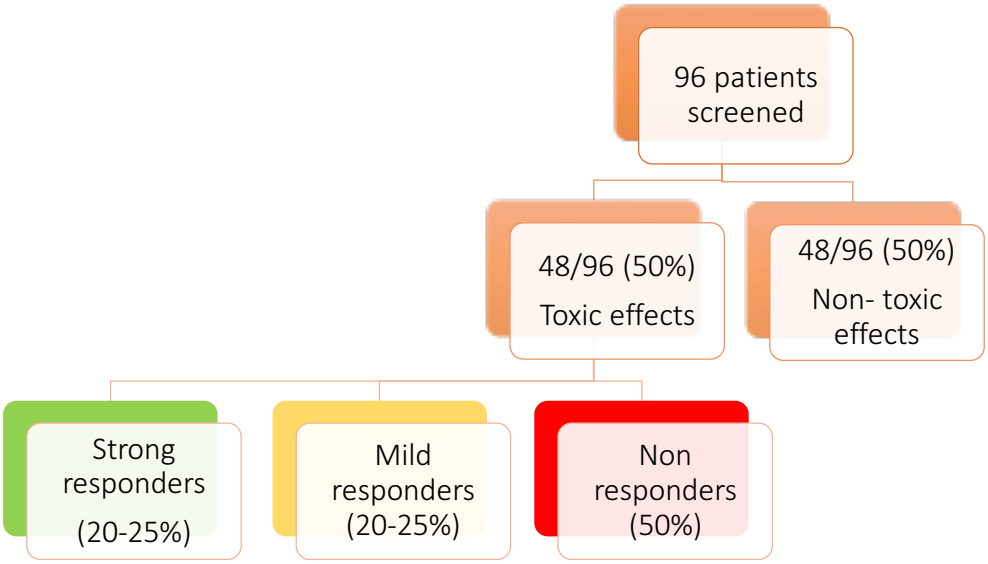
Podocytes should be incubated with FSGS sera (from samples shown to be either responsive or irresponsive to the TNFα blockade). Expression levels of TACE should then be compared between FSGS serum-treated hPODs and untreated hPOD control cells. Translocation of TACE to the cell membrane (and its subsequent activation) depends on phosphorylation at threonine residue T735. Anti-TACE phospho-T735 antibody (Abcam ab60996) may be employed in immunoblot analysis to ascertain whether rec-FSGS plasma increases TACE phosphorylation in hPODs. If FSGS serum works by increasing the active levels of TACE, we would expect to see an increase in the mature form of TACE in FSGS-treated podocytes as compared to controls.

In addition, an ELISA and enzymatic assay should be used to detect total and activated levels of TACE in podocytes exposed to FSGS sera, respectively. ELISA offers a high-sensitivity method of screening for both the total and active concentration levels of TACE in our samples. We would expect these tests to be more sensitive to changes in concentration levels of TACE as compared to IF and immunoblotting.

3.3 Figures for Chapter 3

Figure 1: NIH FSGS Cohort: FAC Assay Results

Ninety-six patients were screened using the FAC image quantitative assay. Forty-eight of the ninety-six (50%) of patient samples elicited toxic effects on hPODs. The TNFα signaling pathway was blocked in those samples that elicited toxic effects. These patients were then grouped into the following three categories based on the degree of their response to the blockade: strong responders, which elicited at least a doubling of FAC (~25% of toxic serum samples), mild responders (~25% of toxic serum samples) and non-responders, which elicited no response (~50% of toxic serum samples).



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