High levels of circulating TNF and its receptors, TNFR1 and TNFR2, predict the progression of diabetic kidney disease (DKD), but their contribution to organ damage in DKD remains largely unknown. Here, we investigated the function of local and systemic TNF in podocyte injury. We cultured human podocytes with sera collected from DKD patients, who displayed elevated TNF levels, and focal segmental glomerulosclerosis (FSGS) patients, whose TNF levels resembled those of healthy patients. Exogenous TNF administration or local TNF expression was equally sufficient to cause free cholesterol–dependent apoptosis in podocytes by acting through a dual mechanism that required a reduction in ATP-binding cassette transporter A1–mediated (ABCA1-mediated) cholesterol efflux and reduced cholesterol esterification by sterol-O-acyltransferase 1 (SOAT1). TNF-induced albuminuria was aggravated in mice with podocyte-specific ABCA1 deficiency and was partially prevented by cholesterol depletion with cyclodextrin. TNF-stimulated free cholesterol–dependent apoptosis in podocytes was mediated by nuclear factor of activated T cells 1 (NFATc1). ABCA1 overexpression or cholesterol depletion was sufficient to reduce albuminuria in mice with podocyte-specific NFATc1 activation. Our data implicate an NFATc1/ABCA1-dependent mechanism in which local TNF is sufficient to cause free cholesterol–dependent podocyte injury irrespective of TNF, TNFR1, or TNFR2 serum levels.
Local TNF causes NFATc1-dependent cholesterol-mediated podocyte injury

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Introduction

Chronic low-grade inflammation and elevated TNF levels have been associated with several diseases, such as rheumatoid arthritis (1), multiple sclerosis (2), and atherosclerosis (3). However, the complex nature of systemic and local TNF has made it difficult to dissect how TNF contributes to disease development and/or progression. This has been further compounded by the fact that commonly used TNF inhibitors may bind to both systemic and local TNF (4). It has been proposed that systemic serum TNF may be spilled over from locally produced TNF within an affected tissue (5). Circulating TNF, TNF receptor 1 (TNFR1), and TNF receptor 2 (TNFR2) are associated with the development (6–8) and the progression of diabetic kidney disease (9–12). However, a causal role for these biomarkers has not been established. Clinical studies have suggested that TNF inhibition may be efficacious in a subset of patients (13, 14) affected by focal segmental glomerulosclerosis (FSGS), a rare kidney disease in which the role of circulating TNF, TNFR1, and TNFR2 in predicting clinical outcome has yet to be established. Nevertheless, these clinical studies were plagued by variable outcomes, suggesting the importance of appropriate patient stratification when studying targeted therapies in highly heterogeneous diseases. Furthermore, as concerns about the high risk for infection and malignancies associated with anti-TNF therapy remain high, the identification of downstream mechanisms and other drug targets will provide new opportunities for the development of safer therapeutic strategies.

Altered cholesterol efflux has been reported to contribute to several diseases, including atherosclerosis (15), nonalcoholic fatty liver disease, and Tangier disease (16). We recently reported that glomeruli isolated from patients with type 2 diabetes and DKD are characterized by reduced expression of ATP-binding cassette transporter A1 (ABCA1) (17), a transmembrane protein involved in cholesterol efflux. These observations suggest that...
local cholesterol accumulation due to decreased cholesterol efflux may contribute to the pathogenesis of DKD. Concurrent with this observation, lipid deposition specifically in podocytes has been observed in kidney biopsies from patients with DKD, and altered glomerular expression of genes involved in lipid metabolism such as ABCA1 correlated with inflammatory markers and estimated glomerular filtration rate (eGFR) (18). Several immunosuppressive regimes are commonly used to treat proteinuric glomerular disorders, such as cyclosporin A (CsA) (19), a pharmacological inhibitor of calcineurin that is an upstream regulator of nuclear factor of activated T cells (NFAT) signaling (20, 21). Interestingly, CsA may be a direct modulator of podocyte function irrespective of its systemic immunological effects (22). Since the ABCA1 promoter region contains an NFAT response element (23), it is possible that NFAT activation is mechanistically linked to ABCA1 expression and cholesterol accumulation in kidney disease.

Based on these clinical and experimental observations, we hypothesized that TNF causes cholesterol-mediated podocyte injury in FSGS and DKD in an NFAT-dependent manner. We show that podocyte apoptosis caused by the exposure of human podocytes to sera from patients with DKD or FSGS is associated with increased podocyte TNF production that is independent of circulating TNF. We further demonstrate with in vitro and in vivo studies that podocyte expression of TNF causes NFAT-mediated ABCA1 repression and reduced sterol-O-acyltransferase 1 (SOAT1) activity leading to free cholesterol-mediated podocyte apoptosis. Thus, our data identify a novel pathway linking key clinical and experimental findings that support targeting of TNF, NFAT, ABCA1, SOAT1, or free cholesterol in patients with FSGS or DKD, irrespectively of circulating levels of TNF, TNFR1, or TNFR2.

Results

Increased serum TNFR1 and TNFR2 levels are detected in DKD and FSGS, whereas increased TNF levels are detected only in DKD. Circulating levels of TNF, TNFR1, and TNFR2 predict chronic kidney disease (CKD) progression in patients with type 1 diabetes (T1D) (9, 11) and type 2 diabetes (10, 12). We studied a small cohort of patients with T1D and kidney disease (DKD+) enrolled in the FinnDiane Study (17) and demonstrated that these patients had elevated TNF, TNFR1, and TNFR2 serum levels when compared with patients with T1D without kidney disease (DKD−) who were matched for age, fasting glycemia, duration of diabetes, total cholesterol, HDL cholesterol, LDL cholesterol, and triglycerides and compared with age- and sex-matched healthy controls (Figure 1, A–C). To determine whether this phenomenon was specific to DKD, we determined serum levels of TNF, TNFR1, and TNFR2 from healthy patient controls (n = 14), patients with nephrotic syndrome (NS, n = 14), and patients with biopsy-proven primary FSGS (n = 6). We found that serum TNF levels were unaltered in patients with NS and FSGS (Figure 1D). Unaltered TNF levels were confirmed in a larger and well-characterized cohort of patients affected by FSGS enrolled in the Nephrotic Syndrome Study Network (NEPTUNE) study (Supplemental Figure 1; supplemental material available online with this article; doi:10.1172/JCI85939DS1). However, serum TNFR1 and TNFR2 were modestly increased in patients with NS and further augmented in patients with FSGS (Figure 1, E and F).
Podocyte expression of TNF and TNFR2 is induced by DKD and FSGS sera as well as by soluble or podocyte-derived TNF. We previously described that human podocytes exposed to DKD+ sera are characterized by a distinct microarray signature compared with podocytes exposed to DKD– and healthy control sera. One of the clusters of genes significantly regulated in podocytes after exposure to DKD+ sera contained genes involved in cytokine–cytokine receptor interaction (17). This observation led us to investigate whether exposure to DKD+ sera may cause changes in the podocyte expression (local expression) of TNF and/or its receptors. Using quantitative real-time PCR analysis, we showed that podocytes exposed to DKD+ sera had significantly increased mRNA expression levels of TNF and TNFR2 compared with controls (Figure 2, A and C), while TNFR1 mRNA expression levels remained unchanged (Figure 2B). Increased TNF expression levels were confirmed by Western blot analysis of DKD+ sera–treated podocytes (Supplemental Figure 2). TNF was increased when podocytes were exposed to sera from patients with diabetes irrespectively of the presence or absence of DKD (Figure 2C).

This mRNA profile was recapitulated in FSGS patient sera–treated podocytes, demonstrating increased TNF and TNFR2 (Figure 2, D and F) with no changes in TNFR1 expression (Figure 2E). These results indicate that increases in the expression of TNF and TNFR2 in sera-treated podocytes occur upon exposure to both DKD+ and FSGS sera irrespectively of serum TNF levels.

We then aimed to determine whether treatment of human podocytes with exogenous TNF would also increase local TNF expression. Similar to what was observed in DKD+ patient sera–treated podocytes, TNF-treated podocytes showed increased local TNF (Figure 2G and Supplemental Figure 3) and TNFR2 expression levels (Figure 2I) without affecting TNFR1 (Figure 2H). To determine the role of local TNF expression in the expression of TNFR1 and TNFR2, lentiviral infection to overexpress TNF (TNFOE) was used (Supplemental Figure 4). Interestingly, increased local TNF expression led to increased expression of TNFR2 (Figure 2L) but not TNFR1 (Figure 2K), similarly to what we observed...
when podocytes were treated with DKD sera, FSGS sera, or recombinant human TNF (Figure 2, A-I).

**Glomerular TNF but not TNFR1 or TNFR2 inversely correlates with eGFR.** To support our in vitro findings, we obtained data of the microarray analysis of glomerular biopsies from patients enrolled in the NEPTUNE study. Serum TNF levels did not correlate with glomerular TNF expression (Figure 3A). Consistent with our findings in DKD and FSGS patient sera–treated podocytes, glomerular TNF expression in the NEPTUNE cohort did not correlate with TNFRI expression (Figure 3B) but positively correlated with glomerular TNFR2 expression (Figure 3C). To determine whether this TNF expression profile was associated with clinical parameters, we performed correlation analysis between eGFR of patients at enrollment and TNF, TNFR1, and TNFR2 in the FSGS and minimal-change disease NEPTUNE cohort. Neither TNFRI expression nor TNFR2 expression correlated with eGFR. However, local TNF expression significantly correlated with reduced eGFR (Figure 3D). These data indicate a possible role for glomerular TNF but not TNFR1 or TNFR2 in the progression of CKD in a subset of patients with FSGS.

**Local TNF causes DKD and FSGS sera–induced podocyte apoptosis.** Consistent with the clinical data supporting a role of TNF in podocyte injury, in vitro experiments demonstrated that TNF but not TNFR1 or TNFR2 causes increased cleaved caspase 3 activity. Podocytes were treated with DKD sera, FSGS sera, or recombinant human TNF (Figure 2, A-I). Consistent with our in vitro findings, we obtained data of the microarray analysis of glomerular biopsies from patients enrolled in the NEPTUNE study. Serum TNF levels did not correlate with glomerular TNF expression (Figure 3A). Consistent with our findings in DKD and FSGS patient sera–treated podocytes, glomerular TNF expression in the NEPTUNE cohort did not correlate with TNFRI expression (Figure 3B) but positively correlated with glomerular TNFR2 expression (Figure 3C). To determine whether this TNF expression profile was associated with clinical parameters, we performed correlation analysis between eGFR of patients at enrollment and TNF, TNFR1, and TNFR2 in the FSGS and minimal-change disease NEPTUNE cohort. Neither TNFRI expression nor TNFR2 expression correlated with eGFR. However, local TNF expression significantly correlated with reduced eGFR (Figure 3D). These data indicate a possible role for glomerular TNF but not TNFR1 or TNFR2 in the progression of CKD in a subset of patients with FSGS.

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Increased local TNF expression in glomeruli from FSGS patients correlates with reduced ABCA1 expression. Analysis by GWAS and microarray analysis of glomeruli of patients with CKD demonstrated that the activation of key inflammatory pathways (24) is shared between clinical and experimental DKD (25). We recently demonstrated that glomerular biopsies from patients with DKD are characterized by reduced ABCA1 expression (17). To determine whether altered ABCA1 expression also occurs in glomeruli from patients with FSGS and is associated with local TNF expression, we analyzed glomerular expression data available from the European Renal cDNA Bank (ERCB) from kidney biopsies of NS patients (26), including FSGS (\(n = 24\)) and minimal-change disease (\(n = 14\)). Increased glomerular TNF expression correlated with reduced ABCA1 expression (Supplemental Figure 9). To further investigate the causative role of ABCA1 in serum-mediated apoptosis, we generated ABCA1-overexpressing (ABCA1OE) podocytes (Figure 4A). Supporting our clinical findings, ABCA1 overexpression prevented DKD and FSGS sera-induced podocyte apoptosis (Figure 4, B and C) and cytotoxicity (Supplemental Figure 5, D and E).

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**Figure 4.** ABCA1 overexpression is sufficient to protect from sera-induced podocyte injury, and TNF treatment alters human podocyte cholesterol homeostasis. (A) Representative Western blot analysis showing that ABCA1-overexpressing (ABCA1OE) podocytes have increased ABCA1 protein compared with empty vector (EV) controls. GAPDH is the loading control. (B) Cleaved caspase 3 analysis in DKD patient sera-treated EV and ABCA1OE. One-way ANOVA; *P < 0.05 compared with all columns. (C) Cleaved caspase 3 analysis in FSGS patient sera-treated EV and ABCA1OE. One-way ANOVA; *P < 0.05 compared with all columns. (D) TNF treatment of cultured human podocytes results in significant reduction of ABCA1-mediated cholesterol efflux compared with untreated controls (C) (\(n = 4\)). One-way ANOVA; ***P < 0.001. (E) TNF treatment of cultured human podocytes leads to increased total cholesterol content compared with untreated controls (\(n = 3\)). Two-tailed Student’s t test; **P < 0.01. (F) TNF treatment of cultured human podocytes leads to reduced esterified cholesterol content compared with untreated controls (\(n = 4\)). Two-tailed Student’s t test; *P < 0.05. (G) TNF treatment of cultured human podocytes leads to reduced SOAT activity compared with untreated controls (\(n = 4\)). Two-tailed Student’s t test; *P < 0.05. (H) Pretreatment with CD prevents TNF-induced caspase 3 activity in cultured human podocytes compared with TNF-treated human podocytes (\(n = 5\)). One-way ANOVA; **P < 0.01, ***P < 0.001.
apoptosis in podocytes isolated from diabetic mice (29), and that ABCA1-dependent cholesterol efflux impairment in DKD is associated with podocyte apoptosis (17), we tested the hypothesis that TNF causes podocyte apoptosis by reducing ABCA1-mediated cholesterol efflux to ApoA1. As expected, TNF treatment and TNFOE reduced ABCA1 mRNA expression (Supplemental Figure 10). To determine the effect of TNF on ABCA1-mediated cholesterol efflux via ApoA1, podocytes were exposed to [3H]-cholesterol in the presence or absence of TNF, and ApoA1-mediated cholesterol efflux was measured. TNF treatment of podocytes significantly reduced ApoA1-mediated cholesterol efflux (Figure 4D). Quantitative enzymatic analysis of cholesterol content revealed significant increases in total cholesterol content in TNF-treated cells compared with untreated controls (Figure 4E). This occurred in association with a significant reduction in the esterified cholesterol pool (Figure 4F). To further define the mechanism by which TNF reduces the esterified cholesterol pool, podocytes were exposed to [14C]-oleic acid, and SOAT1 activity was measured by assessment of [14C] incorporation into the esterified cholesterol pool. SOAT1 activity was significa-
cantly reduced in TNF-treated podocytes compared with controls (Figure 4G). As free cholesterol accumulation may be cytotoxic (30–32), we assessed caspase 3 activity as a measure of apoptosis. As shown in Figure 4H, TNF treatment of human podocytes significantly induced apoptosis, which was prevented by depletion of free cholesterol with methyl-β-cyclodextrin (CD). Using the same [3H]-cholesterol labeling assay as described above, we demonstrate that ABCA1OE podocytes were similarly protected from TNF-mediated impairment of cholesterol efflux when compared with empty vector control transfected podocytes (Figure 5A). Additionally, ABCA1OE podocytes were protected from TNF-induced (Figure 5B) and TNF0E-induced (Supplemental Figure 11) apoptosis.

Esterified cholesterol accumulation due to reduced ABCA1-mediated cholesterol efflux is not sufficient to cause podocyte apoptosis. To assess whether decreased ABCA1 expression is sufficient to cause injury, we generated podocytes with knockdown of ABCA1 (siABCA1) (Supplemental Figure 12). Using the [3H]-cholesterol labeling assay described above, we showed that siABCA1 podocytes are characterized by significantly reduced ApoA1-mediated cholesterol efflux when compared with empty vector control transfected podocytes (Figure 5A). Additionally, ABCA1OE podocytes were protected from TNF-induced (Figure 5B) and TNF0E-induced (Supplemental Figure 11) apoptosis.

Figure 6. Injection of murine recombinant TNF causes cholesterol-mediated albuminuria. (A) Glomerular TNF expression is significantly increased in BALB/cJ mice 6 hours after injection with murine recombinant TNF (TNF) compared with control mice (C) (n = 4 per group). Two-tailed Student’s t test; *P < 0.05. (B) Bar graph analysis of total cholesterol content in kidney cortices of TNF-injected mice and in C mice (n = 6 per group). Two-tailed Student’s t test; *P < 0.05. (C) Transmission electron microscopy analysis of n = 3 mice per group treated with either vehicle (left panels) or TNF (right panels). TNF treatment resulted in segmental foot process effacement (arrowheads) compared with vehicle controls. Focally, podocytes and parietal epithelial cells undergoing apoptosis are detectable, as characterized by condensed chromatin and electron-dense cytoplasm (asterisks) with mitochondrial swelling. Scale bars: 0.5 μm in top panels, 0.2–0.25 μm in bottom panels. (D) The urinary albumin-to-creatine ratio was significantly higher in TNF-injected mice compared with C mice 6 hours after injection. Pretreatment with HPBCD 24 hours before TNF administration significantly reduces albuminuria compared with TNF-treated mice (n = 6 per group). One-way ANOVA; *P < 0.05, **P < 0.01. (E) The urinary albumin-to-creatine ratio is significantly higher in podocyte-specific ABCA1-deficient mice treated with TNF (KO + TNF) compared with WT mice treated with TNF (WT + TNF) 6 hours after TNF administration (n = 4). Two-tailed Student’s t test; *P < 0.05.

of the HCS LipidTOX staining and its cell segmentation in Figure 5E shows that siABCA1 had more lipid droplets compared with siCO. Quantitative analysis of images with Acapella Analysis Software revealed a significant increase in the number of neutral lipid droplets per cell in siABCA1 podocytes compared with siCO (Figure 5F). However, the accumulation of esterified cholesterol was not associated with increased podocyte apoptosis (Figure 5H). Overall, these data suggest that decreased ABCA1 expression and esterified cholesterol accumulation per se are not sufficient to cause podocyte apoptosis.

Free cholesterol-mediated apoptosis requires reduced ABCA1-mediated cholesterol efflux in combination with reduced cholesterol esterification activity via SOAT1. Since TNF reduced ABCA1-mediated cholesterol efflux and SOAT1 activity, we investigated whether shifting the balance from esterified to free cholesterol accumulation in siABCA1 podocytes would cause apoptosis. Podocytes were treated with a pharmacological inhibitor of SOAT activity (SI). Quantitative analysis of HCS LipidTOX staining revealed fewer lipid droplets per cell in SI-treated siABCA1 podocytes compared with untreated siABCA1 controls (Figure 5G), suggesting reduced accumulation of esterified cholesterol in the form of lipid droplets. SI treatment did not increase apoptosis in siCO podocytes, whereas it significantly increased apoptosis in siABCA1e podocytes (Figure 5H). These results support a causal role of free rather than esterified cholesterol accu-
calcineurin 1 (RCAN1) expression in podocytes exposed to sera from individual patients with FSGS (Figure 7). TNF causes NFAT activation and NFAT-mediated ABCA1 repression leading to podocyte apoptosis. (A) Quantitative RT-PCR analysis of regulator of calcineurin 1 (RCAN1) expression in podocytes exposed to sera from individual patients with FSGS (n = 6) and healthy patient (C) controls (n = 3). One-way ANOVA; *P < 0.05 vs. C. (B) TNF causes luciferase activity in mouse podocytes stably transfected with a cDNA coding for the luciferase gene under the control of an NFAT response element compared with untreated controls (n = 3). Two-tailed Student’s t test; *P < 0.05. (C) Quantitative RT-PCR analysis of RCAN1 expression in normal human podocytes exposed to TNF (n = 3) shows increased RCAN1 expression in TNF-treated podocytes (TNF) compared with untreated podocytes (C). Two-tailed Student’s t test; *P < 0.05. (D) Quantitative RT-PCR analysis of ABCA1 expression demonstrating that pretreatment of human podocytes for 1 hour with CsA and continued during TNF treatment (TNF + CsA) prevents TNF-mediated ABCA1 repression compared with TNF-treated cells (n = 4). One-way ANOVA; **P < 0.01. (E) Bar graph analysis showing that pretreatment with CsA and continued during TNF treatment prevents TNF-induced cleaved caspase 3 activity compared with TNF-treated cells (n = 4). One-way ANOVA; *P < 0.05, **P < 0.01. (F) Injection of murine recombinant TNF increases glomerular NFAT-mediated luciferase activity in an NFAT-luciferase reporter mouse model compared with vehicle-treated controls (C) 6 hours after injection (n = 4 per group). Two-tailed Student’s t test; ***P < 0.001. (G) The urinary albumin-to-creatinine ratio was significantly higher in TNF-injected BALB/c mice compared with C mice 6 hours after treatment. Pretreatment with CsA 24 hours before TNF administration significantly reduces albuminuria compared with TNF-treated mice (n = 5 per group). One-way ANOVA; **P < 0.01, ***P < 0.001.

mulation in podocyte apoptosis and are further strengthened by the observation that free cholesterol depletion with CD prevented SI-induced apoptosis in siABCA1 cells (Figure 5).

TNF-induced albuminuria in mice is associated with increased kidney cholesterol content and is aggravated in mice with a podocyte-specific Abca1 deletion. To determine whether TNF causes podocyte injury in vivo, 6-week-old BALB/c mice were injected i.v. with 0.250 mg/kg of recombinant murine TNF. Glomeruli isolated from these mice showed a significant increase in Tnf expression levels (Figure 6A) compared with those from vehicle-treated control mice. Quantitative enzymatic analysis of total cholesterol content in kidney cortex revealed a moderate but significant increase in total cholesterol content in TNF-treated controls (n = 4). Ultrastructural analysis revealed that TNF treatment caused segmental podocyte foot process effacement. Morphological changes including condensation of nuclear chromatin, electron-dense cytoplasm with loss of organelles, and mitochondrial swelling suggesting focal acute injury of a subset of podocytes were observed (Figure 6C). These changes were accompanied by a significant increase in the albumin-to-creatinine ratio 6 hours after TNF injection in comparison with vehicle-injected controls (Figure 6D), but did not result in podocyte loss (Supplemental Figure 13). To investigate whether cholesterol depletion strategies would exert antialbuminuric effects, mice were injected s.c. with 2-hydroxypropyl-β-cyclodextrin (HPBCD) (4,000 mg/kg) as described (17) 18 hours before TNF injection. As expected, cholesterol depletion with Hydroxypropyl-β-cyclodextrin reduced TNF-mediated albuminuria (Figure 6D). In order to further support a link between TNF-induced albuminuria and podocyte ABCA1 expression, we injected recombinant murine TNF into podocyte-specific Abca1 knockout mice, which did not have albuminuria at baseline (Supplemental Figure 14). Mice were sacrificed 6 hours after TNF administration. At time of sacrifice, podocyte-specific Abca1 knockout mice had a significantly increased urinary albumin-to-creatinine ratio compared with controls (Figure 6E). These results indicate that TNF-induced albuminuria is influenced by cholesterol content in podocytes.

TNF-induced NFAT activation in mice results in cholesterol-mediated apoptosis. It was recently described that the proximal promoter region of the Abca1 gene has 2 functional NFAT response elements within a 100-bp region upstream of the transcriptional start site that is conserved between mice and humans (23). The functional consequence of this interaction is not well understood. NFAT activation is an important mediator of glomerular damage (33). Since regulator of calcineurin 1 (RCAN1)
expression, a downstream target of NFAT (34), was increased in podocytes exposed to sera from patients with FSGS (Figure 7A), we tested the hypothesis that downregulation of ABCA1 expression by TNF occurs through an NFAT-mediated mechanism. To assess the effect of TNF treatment on NFAT activation, we used a reporter cell line established from mouse podocytes where the luciferase gene is under the control of an NFAT response element (35). Exposure of this cell line to recombinant murine TNF enhanced NFAT promoter activity, leading to increased luciferase activity (Figure 7B) and RCAN1 expression (Figure 7C), similar to what was observed in TNFOE (Supplemental Figure 15). Finally, to establish a link between TNF-mediated ABCA1 repression and TNF-induced NFAT activation, we treated normal human podocytes with TNF in the presence or absence

Figure 8. ABCA1 overexpression or treatment with HPBCD can partially prevent NFAT-mediated albuminuria. (A) Electron microscopy analysis of double-transgenic (DT) mice fed doxycycline chow for 4 days resulted in segmental foot process effacement (arrowhead) compared with single-transgenic controls (ST). Scale bars: 0.5 μm. (B) Total cholesterol content in kidney cortices of DT and ST mice (n = 6 per group) treated with HPBCD or vehicle control. One-way ANOVA; *P < 0.05. **P < 0.01. (C) Urinary albumin-to-creatinine ratio in DT mice treated with HPBCD or vehicle before doxycycline chow feeding (n = 6 per group). Two-tailed Student’s t test; *P < 0.05. (D) Urinary albumin-to-creatinine ratio in triple transgenic (TT), inducible podocyte-specific constitutively active NFAT mice overexpressing ABCA1, compared with DT controls after 4 months of doxycycline chow feeding (n = 3 per group). Two-tailed Student’s t test; *P < 0.05. (E) Analysis of WT1 podocytes per glomerular profile in DT and TT mice fed control or doxycycline diet for 4 months (n = 3). One-way ANOVA; *P < 0.05 compared with all columns. (F) Representative periodic acid–Schiff (PAS) staining (original magnification, ×20) of kidney sections from DT and TT mice fed doxycycline or control diet for 4 months. (G) Mesangial expansion scores on PAS-stained kidney sections from DT and TT mice fed doxycycline or control diet for 4 months (n = 3 per group) as assessed by 2 blinded, independent investigators. One-way ANOVA; *P < 0.05 compared with all columns. (H) Bar graph analysis of serum creatinine levels from DT and TT mice fed doxycycline or control diet for 4 months (n = 3 per group). One-way ANOVA; *P < 0.05 compared with all columns. (I) Bar graph analysis of picrosirius red–stained kidney sections from DT and TT mice fed doxycycline or normal diet (n = 3 per group). One-way ANOVA; *P < 0.05 compared with all columns. (J) Model of proposed mechanism of local TNF–induced podocyte injury in FSGS and DDK.

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of CsA, a known inhibitor of podocyte NFAT activity (22). We observed that TNF-mediated Abca1 repression (Figure 7D) and induction of apoptosis (Figure 7E) were prevented by CsA.

Systemic TNF administration in mice causes NFAT-mediated albuminuria that can be partially prevented by CsA. To determine whether TNF administration causes NFAT activation in vivo, we used a transgenic reporter mouse model expressing luciferase under the control of nine NFAT response elements from the IL-4 promoter (36). Following i.v. TNF injection, glomeruli were isolated and luciferase activity was assessed after 6 hours. TNF-treated mice showed increased glomerular luciferase activity compared with vehicle-treated controls (Figure 7F). Although systemic TNF administration may impose off-target effects, our data confirm an effect of TNF on the glomerulus and support a direct effect on podocytes.

To determine whether pharmacological inhibition of calcineurin and downstream NFAT signaling could prevent TNF-induced albuminuria, CsA was administered orally (50 mg/kg) to 6-week-old BALB/cj mice 18 hours before TNF administration. As in our previous experiments, TNF administration caused significant albuminuria 6 hours after injection, which was partially prevented by CsA (Figure 7G).

Taken together, these results support a role for podocyte NFAT activation in TNF-induced albuminuria.

Podocyte-specific NFAT activation causes albuminuria that can be attenuated by HPBCD. Our in vitro experiments suggested that NFAT activation causes podocyte injury by altering cholesterol homeostasis in an ABCA1-dependent manner. To confirm these observations in vivo, we used a transgenic mouse model with podocyte-specific inducible (podocin-rtTA) overexpression of constitutively active NFATc1 (Nfatc1mouse). The Nfatc1mouse transgene was generated by mutation of the Nfatc1 gene at 21 positions, leading to the substitution of alanine residues for serine (37). Nfatc1mouse mice were previously reported to develop albuminuria 4 days after induction of Nfatc1mouse (DT) expression by doxycycline compared with single-transgenic controls (ST) (35). In fact, electron microscopy analysis revealed podocyte foot process effacement in DT mice compared with ST controls (Figure 8A) but no histological changes by light microscopy analysis of H&E staining (Supplemental Figure 16). To examine the role of altered podocyte cholesterol homeostasis in NFAT-mediated podocyte injury, 6-week-old Nfatc1mouse mice were fed doxycycline chow for 4 days, and cholesterol content in the kidney cortex was analyzed. DT mice showed significantly increased cholesterol content (Figure 8B) in the kidney cortex compared with ST controls. As expected, DT mice developed albuminuria, which was partially prevented by administration of HPBCD (Figure 8C).

These results support a causative role of kidney cholesterol accumulation in the development of NFAT-mediated albuminuria. In order to confirm that NFAT-mediated albuminuria is ABCA1 dependent, we performed a rescue experiment and generated triple-transgenic mice with podocyte-specific Nfatc1mouse activation and glomerular Abca1 overexpression (TT), which were fed doxycycline chow for 4 months. Our data demonstrated that NFAT-mediated albuminuria (Figure 8D) and podocyte loss (Figure 8E) can be prevented in TT mice. Additionally, TT mice were protected from mesangial expansion (Figure 8, F and G), elevated serum creatinine (Figure 8H), and increased picrosirius red staining (Figure 8I). These data strongly support that NFAT activation plays a causal role in ABCA1-dependent cholesterol accumulation leading to podocyte injury and the development of albuminuria.

Discussion

Our study provides several novel findings. First, we demonstrate the dissociation between systemic and local TNF pathway activation in the pathogenesis of podocyte injury. These findings challenge the translatability of systemic TNFR1 and TNFR2 from their strong role as biomarkers to a potential role as therapeutic targets. Second, we have used a unique translational approach combining clinical and experimental research to dissect the causal role of glomerular TNF expression in podocyte injury in DKD and FSGS, which are discordant in the level of circulating TNF. Third, we describe for the first time to our knowledge the opposing roles of free (proapoptotic) and esterified cholesterol (antiapoptotic) as key mediators of TNF-induced podocyte injury.

Although CKD per se is considered a chronic inflammatory disorder (38), the contribution of inflammatory mediators to kidney damage remains largely unknown. Recent studies have identified increased serum TNF, TNFRI, and TNFR2 to predict the progression of DKD (9, 10, 12), but a causal role of these cytokines in the development and/or progression of DKD has not been established. Our data indicate that TNF but not TNFRI or TNFR2 causes podocyte apoptosis (Figure 3G). Furthermore, as podocytes exposed to DKD and FSGS sera show increased local TNF expression, and TNF inhibition prevents patient sera–mediated podocyte apoptosis (Figures 2 And 3) irrespectively of serum TNF levels, our data support the importance of locally expressed TNF as a major driver of podocyte injury. In fact, elevated local TNF expression was shown to reflect disease severity better than systemic TNF levels in several inflammatory diseases (39, 40), and reduced renal TNF expression was shown to correlate with reduced albuminuria independently of serum TNF levels in mice (41). This supports that circulating TNFRI/TNFFR2 may represent a compensatory mechanism to elevated local TNF.

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expression. The potential circulating factors in FSGS that may affect glomerular TNF expression remain to be identified. While soluble urokinase-type plasminogen activator receptor (suPAR) correlates with circulating TNF in lupus erythematosus (44) and causes podocyte injury in FSGS (45), the ability of circulating suPAR to affect local TNF expression remains to be established. Similarly, it would be interesting to determine whether circulating autoantibodies including anti-CD40 (46) or cytokine receptor-like factor-1 (CRLF-1) (47), which are linked to proteinuria in FSGS, may cause TNF-mediated podocyte injury. Additionally, the TLR4 ligand lipopolysaccharide, which causes acute podocyte injury and is elevated in sera from patients with DKD (48, 49), is also necessary for trafficking of TNF to the plasma membrane (50). Finally, the possibility that other local factors contribute to TNF pathway activation in podocytes exposed to patient sera should also be considered. Therefore, it is likely that a combination of local and systemic factors may contribute to modulation of local TNF pathway activation, irrespectively of circulating TNF.

Recent experimental evidence suggests that local TNF causes inflammasome activation (51) and is associated with altered cholesterol homeostasis (52). Our clinical observations demonstrate that increased glomerular TNF expression in patients with FSGS correlates with reduced eGFR in the NEPTUNE cohort (Figure 3D) and with reduced ABCA1 expression in the ERCB cohort (Supplemental Figure 9), suggesting a role of local TNF in the pathogenesis of glomerular diseases. The ability of TNF to inhibit ABCA1 expression and function in podocytes (Figure 4) may be cell-type specific, as in macrophages TNF expression positively correlates with ABCA1 expression and ABCA1-mediated cholesterol efflux (27). While TNF causes NF-κB activation in Caco-2 cells and macrophages, the discrepant effect of TNF on ABCA1 expression (upregulated in macrophages and downregulated in Caco-2) suggests that mechanisms other than NF-κB may modulate ABCA1 expression (53). In fact, p38 has been described as a key mediator linking TNF to increased ABCA1 expression in macrophages (54). Therefore, it seems possible that TNF signals primarily through p38 in macrophages or that macrophages and podocytes express different p38 isoforms leading to converse effects on ABCA1 transcription (55).

Since knockdown of ABCA1 did not lead to podocyte injury in vitro (Figure 5) and in vivo (Supplemental Figure 14), it is likely that a second hit is required to cause podocyte injury. In fact, while reduced ABCA1 expression alone is not sufficient to cause podocyte injury, simultaneous pharmacological inhibition of the conversion of free cholesterol to esterified cholesterol in ABCA1 knockout podocytes was sufficient to cause cell injury (Figure 5). In further support of this second-hit model, our in vivo data demonstrate that while Abca1 knockout mice are not albuminuric at baseline, they are more susceptible to TNF-induced albuminuria (Figure 6). However, the highly complex nature of cholesterol-mediated cell injury in vivo requires further investigation, as other studies yielded conflicting results showing either protective or detrimental effects of esterified cholesterol accumulation in atherosclerotic phenotypes (56). Our data suggest that, in podocytes, irrespectively of the amount of esterified cholesterol that accumulates, cell injury is mainly caused by free cholesterol. While SOAT1 activity is likely to be tightly regulated to maintain cholesterol homeostasis in normal podocytes, the possibility that altered SOAT1 activity contributes to podocyte injury in disease conditions warrants further investigation. It would be interesting to determine whether TNF-induced podocyte injury is linked to the formation of cholesterol crystals, a phenomenon that was recently shown to be prevented by HPBCD in macrophages in the setting of atherosclerosis (57).

Our data also support a role for TNF in activating NFAT signaling leading to altered cholesterol metabolism via reduced ABCA1 expression, thus contributing to kidney injury. Although inhibition of NFAT with CsA may directly rescue podocyte dysfunction in glomerular injury (22), long-term treatment with calcineurin inhibitors is associated with significant toxicity (58). Thus, the identification of new downstream targets of NFAT activation in podocytes is crucial to the development of new therapeutic strategies. Interestingly, 4-day feeding of doxycycline chow in DT (podocyte-specific constitutively active Nfatc1nuc) mice is characterized by altered cholesterol homeostasis, podocyte foot process effacement, and albuminuria that can be partially prevented by cholesterol deple-tion (Figure 8). On the contrary, Nfatc1nuc mice (DT) fed doxycycline chow for 4 months developed podocyte loss, glomerulosclerosis, elevated serum creatinine, and increased collagen staining. Overexpression of ABCA1 in Nfatc1nuc mice (TT) was sufficient to prevent this CKD-like phenotype. As our short-term and long-term mouse models demonstrate efficacy of HPBCD and ABCA1 overexpression, it seems feasible to propose that HPBCD or drugs that increase ABCA1-mediated cholesterol efflux may represent a new class of drugs to treat patients affected by proteinuric glomerular disorders. Our data also suggest that cholesterol-targeted therapies may have a beneficial effect in diseases of nonglomerular origin that respond to CsA treatment, such as rheumatoid arthritis or psoriasis. Further investigations are required to understand whether agents facilitating podocyte cholesterol efflux maintain their benefi-cial effects in the setting of established CKD.

In conclusion, our results uncover a novel mechanism where cholesterol-mediated podocyte injury is the result of increased endogenous TNF expression, thus possibly contributing to the development of FSGS and DKD. To our knowledge, this is the first study showing that local TNF contributes to lipid-mediated podocyte injury in FSGS and DKD by a dual mechanism that requires both reduced ABCA1 expression and reduced SOAT1 activity. The fact that this occurs irrespectively of sera TNF levels challenges the idea of using circulating TNF, TNFR1, and TNFR2 for the implementation of patient-specific targeted therapies. We also demonstrate a novel role for NFAT as a downstream target of TNF in podocytes. NFAT acts as a transcriptional repressor of ABCA1 expression, and overexpression of ABCA1 is sufficient to prevent NFAT-induced podocyte injury and albuminuria, providing the rationale to study agents targeting cholesterol efflux in proteinuric kidney diseases. Additional studies are needed to investigate the role of TNF- or NFAT-mediated dysregulation of cholesterol homeostasis in the context of nonglomerular diseases.

**Methods**

**Study approval.** All study protocols involving mice were approved by the University of Miami’s IACUC (Miami, FL, 2016). The University of Miami (UM) has an Animal Welfare Assurance on file with the Office
of Laboratory Animal Welfare, NIH (A-3224-01, effective November 24, 2015). Additionally, UM is registered with the US Department of Agriculture Animal and Plant Health Inspection Service, effective December 2014, registration 58-R-007. As of October 22, 2013, the Council on Accreditation of the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC International) has continued UM’s full accreditation. All study protocols involving human samples were granted IRB exemption since the samples were collected under an IRB-approved research study for which informed consent was obtained and there was no access to identifiable information (Human Subjects Research Office, Miami, FL, 2012, and Human Research Protection Program, Ann Arbor, MI, 2015). Appropriate safeguards were in place to protect subject confidentiality and privacy.

Analysis of patient sera and kidney biopsies. Three cohorts were used for this study: (a) serum samples from 10 healthy controls, 10 patients with TID, and 10 patients with TID and kidney disease from the Finnish Diabetic Nephropathy (FinnDiane) Study (17); (b) 14 serum samples from patients with steroid-dependent NS (Supplemental Table 1); and (c) 6 serum samples from patients with biopsy-proven FSGS (59). Serum TNF, TNFR1, and TNFR2 were measured according to the manufacturer’s instructions (Quantikine HS ELISA human TNF-α, Quantikine ELISA human TNF-R1/ TNFRSF1B, and Quantikine ELISA human sTNFR1/TNFRSF1A; R&D Systems) or Luminex Assay (Eve Technologies, Canada) using previously described methods (60). For glomerular expression, kidney biopsy specimens from patients with minimal-change disease (n = 24), and patients with FSGS (n = 14) were obtained from the ERCB, and specimens from minimal-change disease patients (n = 42) and FSGS patients (n = 48) were obtained from the Nephrotic Syndrome Study Network (NEPTUNE). Biopsy samples represent those that were available for expression analysis and that passed quality control measures at the time of study. ERCB and NEPTUNE gene expression data were normalized and quantified on a probe set level using custom CDF annotations on Affymetrix U133 and Affymetrix ST 2.1 platforms, respectively, as previously described (26, 61).

Podocyte culture. Human podocytes were cultured as previously described (62). Briefly, podocytes were grown under permissive conditions at 33°C temperature in media containing 0.01 mg/ml recombinant human insulin, 0.0055 mg/ml human transferrin (substantially iron-free), and 0.005 µg/ml sodium selenite, then thermoshifted to 37°C and differentiated for 14 days. Stable podocyte cell lines expressing siABCA1 or nontargeting siRNA (Applied Biological Materials) were developed by lentiviral infection. Clones were obtained by limited dilution and expanded under puromycin selection. ABCA1 knockdown in different clones was confirmed by Western blotting and analysis of ABCA1-mediated cholesterol efflux. The clone with the most efficient knockdown for ABCA1 was chosen for further experiments. A stable cell line expressing GFP-labeled ABCA1 (63) (vector gift from Michael Fitzgerald) or GFP alone was developed by transfection with Fugene (Promega) followed by cell sorting. A stable cell line expressing siTNF (Applied Biological Materials) was developed by lentiviral infections followed by puromycin selection.

Differentiated podocytes were serum restricted for 24 hours before treatments. For pharmacological inhibition experiments, podocytes were treated for 1 hour with cyclosporin A (CsA, 0.1 µg/ml; Sigma-Aldrich), methyl-β-cyclodextrin (CD, 5 mmol/l; Sigma-Aldrich), TNFR2 (R2B, 3 µg/ml; R&D Systems), or TNFR1 antibody (RIB, 10 µg/ml; Santa Cruz Biotechnology) where indicated, followed by treatment with recombinant human TNF (100 ng/ml; R&D Systems), recombinant human TNFRI or TNFR (125 ng/ml; R&D Systems), Sandoz 58-032 (SI, 4 µM; Sigma-Aldrich), empty vector or TNF-over-expressing lentivirus (Origene), or 4% patient sera for 24 hours. Some experiments were completed with pooled patient sera as indicated. Podocytes were analyzed as described below.

Apoptosis analysis. Apoptosis was assessed using the Caspase-3 Fluorometric HTS Assay Kit (Biotium) or ApoTox-Glo Triplex Assay (Promega) according to the manufacturer’s instructions. Briefly, differentiated podocytes were treated as indicated, and caspase 3 activity was determined after 2 hours at excitation 470 nm, emission 520 nm for the former, or excitation 400 nm, emission 505 nm for viability and luminescence for 1 second for caspase 3 activity for the latter. Additionally, cytotoxicity was measured using the ApoTox-Glo Triplex Assay according to the manufacturer’s instructions, with excitation 485 nm and emission 520 nm. Values were expressed as fold change in comparison with controls.

Cholesterol efflux and SOAT activity assays. To measure cholesterol efflux from differentiated human podocytes, a previously described method was performed with some modifications (64). Podocytes were labeled with 1 µCi/ml [3H]-cholesterol in medium with 2% FBS for 16 hours; cells were washed with PBS and then incubated in RPMI containing 10 µg/ml ApoA1 (Millipore) for 6 hours. An aliquot of medium was recovered for measurement of radioactivity by scintillation counting. Podocytes were washed twice with PBS, followed by cellular lipid extraction with hexane/isopropanol 3:2 (vol/vol). Lipids were separated by thin-layer chromatography in heptane/diethylether/methanol/ acetic acid 80:30:3:1.5 (vol/vol/vol/vol) to quantify free and esterified cholesterol radioactivity by scintillation counting. Cellular proteins were dissolved in 0.1% SDS in 0.1 M NaOH and quantified by bicinechonic acid assay (BCA assay) method. Cholesterol efflux to medium containing serum was expressed as percentage of total cell cholesterol by scintillation counting as previously described (65).

Esterification of cellular cholesterol by SOAT was measured as previously described with modifications (64). Differentiated podocytes were incubated with 2 µCi/ml [4-14C]-oleate for 1 hour at 37°C. Cellular lipids were extracted and separated by thin-layer chromatography (TLC) as described above, followed by scintillation counting.

Determination of cholesterol mass. To determine total cholesterol mass, differentiated podocytes were grown in 100-mm plates. Cellular lipids were extracted as described above. Total cholesterol content was measured using the Amplex Red Cholesterol Assay Kit (Invitrogen) following manufacturer instructions and normalized to cell protein content using the BCA method.

To determine esterified cholesterol content, differentiated podocytes were grown in 100-mm plates. Cellular lipids were extracted and lipids were separated by TLC as described above. Esterified cholesterol was re-extracted from the TLC plates with chloroform and evaporated under a stream of N2 gas. Esterified cholesterol was determined by enzymatic conversion to free cholesterol using the Amplex Red Cholesterol Assay Kit (Invitrogen).

High-content lipid droplet quantification. Following treatment, differentiated podocytes were fixed with 4% paraformaldehyde/sucrose. Cells were stained with HCS LipidTOX Red (Invitrogen) and HCS CellMask Blue (Invitrogen) according to the manufacturer’s instructions. Cells were imaged by the Opera High Content Screening.
System using the ×20 confocal lens. Acapella High Content Imaging and Analysis software (PerkinElmer) was used for analysis. Nuclei and cellular segmentation was detected using standard detection modules in the UV channel. Within the detected cytoplasm region, neutral lipid droplets were detected in the red fluorescence channel using intensity thresholds. From the cell segmentation and lipid droplet images, the number of lipid droplets per cell was determined with analysis of more than 300 cells per well.

**Quantitative real-time PCR.** RNA was extracted from differentiat-ed podocytes using the RNeasy Mini Kit (Qiagen). Reverse transcription was performed using gScript cDNA SuperMix (Quanta) according to the manufacturer’s protocols. Quantitative real-time PCR (RT-PCR) was performed using the StepOnePlus system (Applied Biosystems) with PerfeCTA SYBR Green FastMix (Quanta) using the ΔΔct method.

**Western blot analysis.** Cell lysis was performed with 3-[3-chol- amidopropyl]dimethylammonio]-1-propanesulfonic acid (CHAPS) buffer. Western blot analysis was performed as previously described (59) using the following primary antibodies: anti-GAPDH (ab1001, 1:10,000; Calbiochem), anti-ABCAl (ab18180, 1:1,000; Abcam), or anti-TNF (6945, 1:1,000; Cell Signaling); or the following secondary antibodies: anti-mouse IgG HRP (W042B, 1:10,000; Promega) or anti-rabbit IgG HRP (W041B, 1:10,000; Promega).

**Luciferase assay.** NFAT reporter podocyte cell lines were previously generated (35). Briefly, mouse podocytes that stably express the pGL4.30 reporter plasmid (Promega) with luc2P firefly luciferase gene under the control of the NFAT response element were generated by hygromycin selection. Podocytes were differentiated for 14 days and treated as described above. Cells were lysed using the Bright-Glo Luciferase Assay System (Promega), and luminescence was measured on a SpectraMax L microplate reader (Molecular Devices) and normalized to protein content by NanoOrange Protein Quantification Kit (Invitrogen) using the manufacturer’s instructions with slight modifications.

**Animal studies.** Thirty-six female mice (BALB/cj, stock number 000651) at the age of 5–6 weeks were purchased from Jackson Laboratory. Mice were treated with pharmacological agents as indicated and 24 hours before TNF treatment. Pharmacological agents were used at the following concentrations: hydroxypropyl-beta-cyclodextrin (HPBCD), 4,000 mg/kg, s.c. (Sigma-Aldrich); CsA, 50 mg/kg, oral-ly administered by hygromycin selection. Podocytes were differentiated for 14 days and treated as described above. Cells were lysed using the Bright-Glo Luciferase Assay System (Promega), and luminescence was measured on a SpectraMax L microplate reader (Molecular Devices) and normalized to protein content by NanoOrange Protein Quantification Kit (Invitrogen) using the manufacturer’s instructions with slight modifications.

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Author contributions

CEP conceived the project, performed the in vitro and in vivo experiments, analyzed the data, and wrote the manuscript. FL, GMD, JM-D, AM, MG, TH, OK, and TH performed some of the in vitro and in vivo experiments. ML, MIL, C. Forsblom, HR, and P-HG collected patient samples and clinical and laboratory data and performed some of the experiments involved in the analysis of patient samples. AJM designed and performed experiments related to cholesterol determination and cholesterol efflux. C. Faul and AS provided models and expertise for in vivo experiments. GWB conceived 1 of the techniques used. S. Merscher and AF conceived the project, designed and supervised the study, analyzed the data, and edited the manuscript. S. Martini analyzed glomerular expression data available from the European Renal cDNA Bank (ERC B). AF is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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