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TNFR2 interposes the proliferative and NF- κ B-mediated inflammatory response by podocytes to TNF- α

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Abstract

The development of proliferative podocytopathies has been linked to ligation of TNFR2 expressed on the renal parenchyma; however, the TNFR2 positive cells within the kidney responsible for podocyte injury are unknown. We detected *de novo* expression of TNFR2 on podocytes prior to hyperplastic injury in crescentic glomerulonephritis of mice with nephrotoxic nephritis, and in collapsing glomerulopathy of Tg26^{HIV/nl} mice, *kd/kd* mice, and humans. We further found that serum levels of soluble TNF- α and TNFR2 correlated significantly with renal injury in Tg26^{HIV/nl} mice. Thus, we asked whether ligand binding of TNFR2 on podocytes *ex vivo* precipitates the characteristic proliferative and pro-inflammatory diseased podocyte phenotypes. Soluble TNF- α activated NF- κ B and dose-dependently induced podocyte proliferation, marked by expression of the podocyte G₁ cyclin and NF- κ B target gene, cyclin D1. Microarray gene and chemokine protein expression profiling showed a marked pro-inflammatory NF- κ B signature, and activated podocytes secreting CCL2 and CCL5 induced macrophage migration in transwell assays. Neutralization of TNFR2 on podocytes with blocking antibodies abrogated NF- κ B activation and the induction of cyclin D1 by TNF- α , and identified TNFR2 as the primary receptor that induced I κ B α degradation, the initiating event in NF- κ B activation. These results suggest that TNFR2 expressed on podocytes and its canonical NF- κ B signaling may directly interpose the compound pathogenic responses by podocytes to TNF- α , absent other TNFR2 positive renal cell-types in proliferative podocytopathies.

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DISCLOSURE/DUALITY OF INTEREST

None.

Keywords

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Mounting evidence suggests that podocytes, known susceptible targets of disparate immune mediators causing proteinuria (1–3), may themselves subsequently elaborate effectors such as chemokines, cytokines, and growth factors that further amplify and shape the pattern of glomerular injury (3–7); however, the molecular pathways responsible for these compound diseased podocyte phenotypes and how they contribute to the development of specific podocytopathies are not well understood (8, 9). In most other non-lymphoid cell-types that serve as both targets and effectors of immunologic challenges, the resulting phenotypes are often induced by activated NF- κ B, transcription factors of paramount importance in directing localized inflammatory responses (10, 11). This fact raises the possibility that immunologic challenges which potently activate NF- κ B in podocytes are candidates to both precipitate and aggravate proteinuric diseases (12).

With regard to the proliferative podocytopathies, crescentic glomerulonephritis and collapsing glomerulopathy (CG) (13), the tumor necrosis factor- α (TNF- α)-TNF receptor 2 (TNFR2) axis has emerged as a candidate pathway contributing to the compound proliferative and pro-inflammatory diseased podocyte phenotypes within these lesions (14, 15). The TNF receptor family and its ligands are major membrane proximal signaling axes that robustly activate NF- κ B (11), of which TNF- α is required for the development of crescentic glomerulonephritis in several animal models (16). In addition to its well-known role in priming pro-inflammatory tissue responses (10, 11), TNF- α can also function as a potent mitogen (17, 18), and recent studies in mice with crescentic glomerulonephritis from nephrotoxic nephritis showed that, of its two receptors, TNFR1 and TNFR2, the pathogenic contribution from TNFR2 predominates (19). Chimeric mice bearing TNFR2^{+/+} bone marrow-derived cells and TNFR2^{-/-} renal cells were protected from hyperplastic podocyte injury, albuminuria, and glomerulosclerosis despite intact immunity and glomerular deposition of IgG (19). This protection did not occur in TNFR2^{+/+} mice lacking TNFR1 (19), also supporting observations in kidney explants that apoptotic *versus* proliferative responses of the renal parenchyma to TNF- α segregate with renal expressed TNFR1 *versus* renal expressed TNFR2, respectively (20). Moreover, the characteristic recruitment of inflammatory mononuclear phagocytes that intimately admix with diseased podocytes (21, 22), aggravating glomerular injury and proteinuria (23), was absent in the chimeras. This suggests that TNFR2 signaling by resident glomerular cells also contributes to detrimental chemoattraction of leukocytes.

The premise that this pathogenic role of the TNF- α -TNFR2 axis in crescentic glomerulonephritis may extend to CG is now well-grounded. Multiple early reports, including observations of their morphologic patterns co-existing in nephrotoxic nephritis (24), suggested that crescentic glomerulonephritis and CG develop along closely linked pathomechanisms (reviewed in ref. 8). Recently, careful cell lineage-tracing studies extended and solidified this premise (25, 26). Nearly identical admixtures of hyperplastic

podocytes (visceral and parietal), parietal “transitional” podocyte progenitors, and glomerular epithelial stem cells, along with recruited mononuclear phagocytes, populate and form the extracapillary proliferative lesions in crescentic glomerulonephritis and CG (25, 26). Intriguingly, CD44, a known NF- κ B target gene induced by TNF- α that coordinately increases the affinity of CD44 for its ligands (27), was shown to be a glomerular epithelial cell injury marker in these studies (26).

These seminal observations leave unresolved whether activation of TNFR2 on podocytes *versus* TNFR2 on other renal cell-types contributes to podocyte injury in the proliferative podocytopathies. Nevertheless, podocytes have been documented to pathogenically respond to TNF- α . Podocytes challenged briefly with TNF- α *ex vivo* undergo cytoskeletal reorganization (28), shed and down-regulate nephrin (29, 30), produce reactive oxygen species and inducible nitric oxide synthase (iNOS) (31, 32), up-regulate TNFR2 (33) and Toll-like receptor 2 (TLR2) expression (34), and activate p38MAPK signaling (35), a pathway downstream of TNFR2 (15). Unlike TNFR1 which is basally expressed by most cells at steady-state, including podocytes (36), TNFR2 expression is largely restricted to leukocytes, and in normal animal and human kidneys, podocytes do not express detectable TNFR2 (14, 15). However, recent examination of TNFR2 expression in glomeruli from patients with acutely rejecting renal allografts (37) and IgA nephropathy (33) showed that TNFR2 can be induced on podocytes. This suggests that TNFR2 may serve to directly interpose podocyte injury to TNF- α during inflammatory renal states *in vivo*, even in the absence of other TNFR2 positive renal cell-types. Thus, we ask here whether ligand binding of TNFR2 on podocytes *ex vivo* precipitates the characteristic proliferative and pro-inflammatory diseased podocyte phenotypes.

MATERIALS AND METHODS

Mice

All animal studies were approved by the respective Institutional Animal Care and Use Committees. The C57Bl/6 (B6) nephrotoxic nephritis model of crescentic glomerulonephritis (41), the heterozygous FVB/N Tg26 HIV-1 (Tg26^{HIV/nl}) transgenic mouse model of CG (42), and the B6 *kd/kd* model of CG (43), have been characterized and described in detail. Kidneys from nephrotoxic nephritis mice were collected 3 days after injection of antibody, before the onset of significant hyperplastic glomerular injury. Serum, urine, and kidneys were collected from Tg26^{HIV/nl} mice ranging between 2–4 months of age, and urinalysis and serum chemistries were performed as previously described (61). Mice were maintained under specific pathogen free conditions.

Microscopy

Immunohistochemical detection of TNFR2 was performed on kidney sections from nephrotoxic nephritis mice, Tg26^{HIV/nl} mice, *kd/kd* mice, normal wild-type FVB/N and B6 mice, and on archival human kidney biopsies diagnosed with CG (n=10), primary focal segmental glomerulosclerosis (n=10), and normal kidney on post-transplant protocol biopsy (n=2). All studies on human tissues were Institutional Review Board-approved. 3 μ m-thick paraffin sections underwent low pH antigen retrieval (Dako, Carpinteria, CA, USA)

followed by staining for TNFR2 (1:100; rabbit polyclonal, GeneTex, Irvine CA, USA) using 3,3'-diaminobenzidine staining kits from Vector Laboratories (Burlingame, CA, USA) as per the manufacturer's protocols. Immunofluorescence detection of TNFR2 on differentiated podocytes on glass cover-slips was performed after challenge with TNF- α (2.5 ng/ml) for 24 h to enhance TNFR2 expression. Podocytes were fixed with 60% acetone/3.7% formaldehyde for 20 min at -20°C and stained for TNFR2 (1:100; rabbit polyclonal, GeneTex) using polyclonal FITC-labeled swine anti-rabbit antibody (1:20; Dako, Carpinteria, CA, USA) for secondary detection. Quantitative histopathology on periodic acid-Schiff stained slides of kidneys from Tg26^{HIV/nl} mice was performed as previously described (43).

Cell Culture

The conditionally-immortalized podocyte cell culture system, originally developed by Mundel and colleagues (47) and used previously by us for culturing normal (48) or human immunodeficiency virus (HIV)-expressing Tg26^{HIV/nl} podocytes (52), was employed for studies on podocytes *ex vivo*. The protocol for developing undifferentiated or differentiated podocytes in cell culture has been described in detail (47). The proliferative response of podocytes to soluble recombinant mouse TNF- α (R&D Systems, Minneapolis, MN, USA) or vehicle (phosphate buffered saline, pH 7.4) was determined by CellTiter 96 Non-Radioactive Cell Proliferation assays (Promega, Madison, WI, USA). Triplicate wells of 2000 podocytes per well, either growth-restricted (37°C with no interferon- γ for 24 h) or differentiated (37°C with no interferon- γ for 14 d), were treated for 6 d with 2-fold dilutions of TNF- α (10 ng/ml to 5 pg/ml) or vehicle under continuing growth-restricted conditions prior to CellTiter assays. Additional wells of differentiated podocytes were treated with TNF- α (2.5 ng/ml) or vehicle for 6 d, fixed with 60% acetone/3.7% formaldehyde at -20°C for 20 minutes, and stained with 0.4% trypan blue to visualize cell density and morphology by light microscopy. Changes in protein expression by differentiated podocytes following challenge with or wash-out of TNF- α (2.5 ng/ml) or vehicle were evaluated by Western blot. 50 μg of protein lysates underwent denaturing polyacrylamide gel electrophoresis and immunoblotting for cyclin D1 (clone SP4, LabVision, Fremont, CA, USA), TNFR2 (rabbit polyclonal, GeneTex), and tubulin- β (rabbit polyclonal, LabVision, Fremont, CA, USA), using horse radish peroxidase-conjugated antibodies for secondary detection. Western blot band intensity by chemiluminescence (Supersignal West Femto Maximum Sensitivity Substrate, Thermo Fisher Scientific, Milwaukee, WI, USA) was quantified on a ChemiDoc EQ system (BioRad Laboratories, Hercules, CA, USA) using QuantityOne software (BioRad Laboratories). All experiments were repeated three separate times for significance.

Evaluation of both baseline mRNA levels and parallel changes in mRNA expression with TNF- α treatment by quantitative reverse transcription-polymerase chain reaction (RT-PCR) was performed by the New York University Cancer Institute Genomics Facility with primers designed by Primer 3 (software available at http://www-genome.wi.mit.edu/genome_software/other/primer3.html). Primer sequences for target genes were as follows: Cyclin D1: forward, CACAACGCACTTCTTTCCA reverse, TCCAGAAGGGCTTTCAATCTG; iNOS: forward, AGCTGAACTTGAGCGAGGAG reverse, GGAAAAGACTGCACCGAAGA; and WT1: forward

ACCATCTGAAGACCCACACC, reverse, ACAACTGTGCCACCGACAG. Primer sequences for internal controls were as follows: β -2-microglobulin: forward, CTGACCGGCTGTATGCTAT reverse, TATGTTCCGGCTTCCCATTCT; glyceraldehyde-3-phosphate dehydrogenase: forward, GGCATTGCTCTCAATGACAA reverse, CCCTGTTGCTGTAGCCGTAT; and hypoxanthine phosphoribosyl-transferaseI: forward, TGTGTTGGATATGCCCTTG reverse, GGCTTTGTATTTGGGCTTTTCC. For each triplicated quantitative RT-PCR reaction, 10 ng of cDNA generated by iScript cDNA Synthesis reagents (BioRad Laboratories) from whole podocyte RNA (collected by TRIAGENT, Molecular Research Center, Cincinnati, OH, USA) underwent amplification and PCR product detection on an ABI 7900 SDS cyclor (Applied BioSystems, Foster City, CA, USA) using Power SYBR Green PCR Master Mix reagents (Applied BioSystems). Determination by the 2^{-Ct} method of the fold-change in expression of each target gene relative to 3 geometrically averaged internal reference genes, previously shown to increase the accuracy of quantitative RT-PCR (62), was performed as described previously (63). Data are presented as fold change in expression relative to baseline expression of each gene.

NF- κ B assays

Normal podocytes and HIV-expressing Tg26^{HIV/ml} podocytes (52), cultured under the conditions described above, were examined with a functional assay for NF- κ B activation using a standard reporter assay in 24 well plates (2.5×10^4 cells/well) as previously described (52). This assay involves transient transfection of a plasmid (pNF- κ B SEAP, Clontech, Mountain View, CA, USA) expressing the reporter gene SEAP (secreted alkaline phosphatase) under the control of an NF- κ B-dependent promoter. 24 h after transfection, media was removed and replaced with media containing either vehicle (phosphate buffered saline), TNF- α (20ng/ml), lipopolysaccharide (O55:B5, 1ng/ml, Sigma, St. Louis, MO, USA), or combinations containing rat anti-mouse TNF- α -neutralizing IgG (20 μ g/ml, clone MP6-XT22, eBiosciences, San Diego, CA, USA) or TNF- α -neutralizing soluble TNFR1 Fc-fusion protein (5 μ g/ml, R&D Systems). After 24 h, SEAP levels in conditioned media were determined by chemiluminescence (BD Biosciences, San Jose, CA, USA) as previously described (52). Reporter assay results were confirmed by Western blotting or RT-PCR of native genes regulated by NF- κ B (TNFR2, iNOS, or Cyclin D1).

Neutralization of TNFR1 or TNFR2 on podocytes was achieved with monoclonal hamster anti-mouse IgG blocking TNFR1 (clone 55R-170, BioLegend, San Diego, CA, USA) or blocking TNFR2 (clone TR75-54.7, BioLegend), originally developed by Sheehan *et al* (54). Non-specific monoclonal hamster IgG (clone HTK888, BioLegend) was used as control. We confirmed (data not shown) that these blocking antibodies do not have agonistic activity when bound to the receptors, as shown previously (54) and stated by the manufacturer. Differentiated podocytes were challenged with vehicle (phosphate buffered saline) or TNF- α for 24 h in the absence or presence of 2-log dilutions (1000 ng/ml to 0.01 ng/ml) of each antibody prior to collection of media or RNA for SEAP measurements or quantitative RT-PCR analyses, respectively. All studies were performed at least three times in triplicate for statistical analysis.

Measurement of I κ B α degradation in podocytes, evaluated by Western blotting as previously described (52), was also done in parallel with SEAP measurements during blocking of TNFRs with neutralizing antibodies. As above, podocytes were pretreated with neutralizing antibodies (1000 ng/ml) followed by stimulation with TNF- α (20 ng/ml) for 24 hours. Due to the normally high rate of I κ B α turnover, cells were re-stimulated with TNF- α for 30 minutes before lysis. The Western blotting studies were repeated three times for statistical analysis.

Gene microarray profiling

RNA was extracted from quiescent, differentiated normal podocytes prior to challenge with TNF- α , or after a 48 h treatment with TNF- α (2.5 ng/ml), on three separate occasions. 100 μ g of RNA from each extraction was hybridized on Affymetrix Mouse 430A 2.0 genes chips (New York University Cancer Institute Core Genomics Facility). Normalization, data filtering, and identification of statistically significant differential mRNA abundance, defined as 2-fold or greater on average between quiescent and TNF- α -activated podocytes, were performed as previously described (64).

Migration and cytokine assays

Migration assays were performed on transwells (24 well format, 5.0 μ m uncoated polycarbonate) using the murine macrophage/monocyte cell line RAW264.7 (American Type Culture Collection, Manassas, VA, USA; clone TIB-71). Chemoattractants were 3 day conditioned media from normal or Tg26^{HIVnl} podocytes, stimulated and unstimulated with TNF- α . 550 μ l of conditioned media, 0.2 μ m filtered prior to use, were applied to the lower transwell chamber. RAW264.7 cells were washed three times in phosphate buffered saline, suspended in media containing 0.5% fetal bovine serum, and applied to the upper chamber (10⁵ cells in 100 μ l). Chambers were incubated for 4 hours at 37°C. Cells remaining in upper chambers were gently removed, and migrated cells were fixed and stained with a Hema 3 staining kit (Thermo Fisher Scientific) as per the manufacturer's protocol. Stained cells were manually counted in 6 randomly selected fields, and each experiment was performed three times.

Chemokines from the conditioned media were quantified using a bead-based, antigen-antibody multi-analyte profiling assay (LINCOplex, Millipore, Billerica, MA, USA) analyzed on a Luminex LabMAP (Luminex Corp, Austin TX, USA). Mouse serum TNF- α levels were quantified using a Fluokine multi-analyte profiling assay (R&D Systems) analyzed on a Luminex LabMAP. Mouse serum sTNFR levels were quantified using colorimetric enzyme linked immunosorbant assays (Quantikine, R&D Systems). All samples were tested in duplicate and duplicates were averaged prior to statistical analysis.

Gene silencing by RNAi

TNFR2 expression was reduced in podocytes by RNAi using commercially-available, pre-made RNAis (ON-TARGETplus SMARTpool L-043973, Dharmacon). A non-targeting RNAi was used as a control (ON-TARGETplus D-001810, Dharmacon). The RNAis were transfected into cells as recommended by the manufacturer and extent of knockdown was determined by Western blotting. RNAi transfected cells were used for experiments including

the SEAP reporter assay for functional NF- κ B activation and quantification of cell proliferation as described above. For NF- κ B reporter assays, the reporter plasmid was co-transfected with the RNAi. Each experiment was performed three times in duplicate.

Statistics

Unless otherwise stated, numerical and graphical data are expressed as mean \pm standard deviation. Comparisons were made using the two-tailed *t* test. Correlations between TNF- α and sTNFR2, and between sTNFR2 and renal indices of CG were evaluated by calculating Pearson correlation coefficients (R values) and through simple linear regression. Significance was accepted at the 0.05 level of probability.

RESULTS

TNFR2 expression by podocytes in proliferative podocytopathies

TNFR2 is normally expressed by several leukocytes subsets; however, other cell types including renal epithelial and endothelial cells can be induced to express TNFR2 by TNF- α itself, interleukin-1 family cytokines, and several other pro-inflammatory mediators (14, 15, 33, 38). Because the extracellular domain of membrane TNFR2 is rapidly cleaved and shed (39, 40), we employed an antibody reactive to its intracellular domain to detect TNFR2 *in situ* in the crescentic glomerulonephritis of B6 mice with nephrotoxic nephritis (41), and in the CG of Tg26^{HIV/nl}, one of the best studied models of CG (42), B6 *kd/kd* mice, a non-HIV model of CG (43), and human biopsies. Immunohistochemical staining showed a distinct pattern of *de novo* expression of TNFR2 by podocytes in crescentic glomerulonephritis and CG. Podocytes in normal FVB/N mice (Figure 1A) and normal B6 mice (not shown) did not have detectable TNFR2. In contrast, podocytes in nephrotoxic nephritis kidneys (Figure 1B), in Tg26^{HIV/nl} kidneys (Figure 1C, D), and in *kd/kd* kidneys (Figure 1E, F) clearly expressed TNFR2 prior to the development of hyperplastic injury in glomeruli. In full coronal sections, adjacent glomeruli at varying stages of hyperplastic injury and fibrosis were identifiable and highlighted the differential expression of TNFR2 (Figure 1D, F).

Contrary to recent reports suggesting shared pathomechanisms in CG and crescentic glomerulonephritis (8, 25, 26), CG has been classically described as a variant of primary focal segmental glomerulosclerosis, a lesion characterized by podocyte loss, not hyperplastic injury (44). We therefore examined TNFR2 expression in 10 human biopsies with CG, in 10 human biopsies with primary focal segmental glomerulosclerosis, and in 2 human protocol transplant biopsies showing normal kidney. As described by others (33), TNFR2 was not detected on podocytes in normal human kidneys, nor was it detected on podocytes in primary focal segmental glomerulosclerosis (Figure 1G). In CG, however, TNFR2 was detected on human podocytes in glomeruli without evidence of significant hyperplastic injury and capillary collapse at the time of biopsy (Figure 1H, I). These well-preserved glomeruli were infrequent, being present in only 2 of the 10 archival cases studied, indicative of the state of parenchymal injury at clinical presentation of CG. Taken together, this pattern of *de novo* expression of TNFR2 by podocytes in animals and humans prior to significant hyperplastic glomerular injury suggests that its engagement by TNF- α may contribute to directly precipitating the proliferative podocytopathies.

TNF- α and TNFR2 in CG

The development of crescentic glomerulonephritis is well-studied for its dependency on TNF- α (16), an innate immune and T helper type 1 (Th1) effector cytokine; however, this has not been explored in CG. Nevertheless, reports of CG predominate with patients afflicted by pre-existing Th1-polarizing disorders originating outside the kidney, the most prevalent being HIV infection, suggesting a causal link similar to crescentic glomerulonephritis (8, 44). Thus, we studied this clinical correlate in detail in Tg26^{HIV/nl} mice, which display abnormally elevated serum TNF- α (45) from the co-opting of TNF- α expression control by HIV-1 gene products (46). Serum levels of soluble TNF- α and its' soluble (s) TNFRs (shed from membranes to delimit the activity of TNF- α (39, 40)), and the extent of renal dysfunction and injury was evaluated in diseased Tg26^{HIV/nl} mice and non-transgenic littermates. Compared to non-transgenics, diseased Tg26^{HIV/nl} mice had significantly higher levels of serum TNF- α , sTNFR1 and, most prominently, sTNFR2 (Figure 2A, B), consistent with the up-regulation, cleavage, and shedding of TNFR2 during inflammation mediated by TNF- α (39, 40). Serum levels of TNF- α correlated significantly with sTNFR2 (Figure 2C). Since sTNFR2 is a more robust and dynamic surrogate of TNF- α activity on target tissues than sTNFR1 (39, 40), sTNFR2 was compared to indices of CG in a large cohort of Tg26^{HIV/nl} mice exhibiting a wide range of renal disease (Table 1). This analysis showed that the levels of sTNFR2 correlated significantly with the degree of podocyte proliferation, podocyte injury (by desmin expression), global glomerular capillary collapse and sclerosis, tubulointerstitial microcysts and inflammation, proteinuria, and blood urea nitrogen, but not the milder histopathological parameters (segmental glomerulosclerosis and collapse) or the dyslipidemia of nephrotic syndrome (serum triglycerides and cholesterol levels). The strength of the association data was determined with Pearson's correlation coefficients, and were found to be good (Table 1, R values between 0.5 to 0.8) for proteinuria, blood urea nitrogen, global glomerulosclerosis, podocyte injury, and tubulointerstitial inflammation, whereas the association data was weaker (R values less than 0.5) but significant for the two key features of CG, global glomerular collapse (R=0.464) and podocyte hyperplasia (R=0.347), as well as tubular microcysts (R=0.383). In composite, these results demonstrated a significant and overall good correlation between TNF- α expression and activity and the development of renal disease in Tg26^{HIV/nl} mice. Taken together with the *de novo* expression of TNFR2 on podocytes, we hypothesized that TNF- α -TNFR2 interaction induces the characteristic proliferative and pro-inflammatory diseased podocyte phenotypes *ex vivo*.

Proliferative phenotype of podocytes to TNF- α

TNF- α is a cytokine promoter of proliferation and transformation for many epithelial and other cell-types, particularly during chronic immune activation and inflammation (17, 18). To address this possibility for podocytes *ex vivo*, we employed the podocyte cell culture system developed by Mundel and colleagues (47) and utilized previously by us (48) to study their phenotypic response to prolonged challenges with soluble TNF- α . Differentiated podocytes exhibited robust, dose-dependent proliferation to soluble TNF- α quantified using a standard proliferation assay (MTS assay, Figure 3A). These cultures had abundant mitotic figures and foci formation by day 6 of TNF α treatment (Figure 3B, C). TNF- α similarly

induced proliferation of undifferentiated podocytes (Figure 3A, growth restricted). Notably, however, unlike undifferentiated podocytes growing under permissive conditions (dependent on a temperature sensitive-SV40 T antigen, a mechanism of viral transformation), which down-regulate expression of WT1 (47), differentiated podocytes induced to proliferate *via* soluble TNF- α retained gene expression of WT1, as previously observed by Takano *et al* (32). Quantitative RT-PCR analysis of podocytes proliferating with TNF- α *versus* podocytes proliferating *via* SV40 T showed that WT1 expression was increased ($+1.16\pm 0.03$ fold) *versus* decreased (-22.17 ± 0.50 fold), respectively, when compared to quiescent, differentiated podocytes without TNF- α stimulation. TNF- α stimulation of differentiated podocytes did not reactivate the temperature sensitive-SV40 T antigen (Supplemental Figure 1).

Pathogenic cell-cycle engagement of quiescent (G_0) podocytes in the proliferative podocytopathies is driven by expression of the G_1 cyclin, cyclin D1 (48, 49), a known target gene of TNF- α (17, 18) and of NF- κ B (50, 51). Thus, we selected cyclin D1 as a proliferation marker of TNF- α -TNFR2 signaling by podocytes and studied its gene expression by quantitative RT-PCR before, during, and after (*i.e.*, wash-out) challenge with soluble TNF- α . iNOS, a previously-identified NF- κ B target gene of TNF- α in podocytes (32), was used as a positive control. As with iNOS, cyclin D1 was up-regulated during challenge with TNF- α and returned to near baseline following wash-out of TNF- α , with baseline defined as the expression prior to challenge with TNF- α (Table 2). This was also evident in the pattern of protein expression of cyclin D1 (Figure 3D, E). Together, these results indicate that TNF- α can serve as a mitogenic cytokine for podocytes, even when the podocytes were in a differentiated, quiescent state.

Pro-inflammatory NF- κ B activation in podocytes by TNF- α

While unknown for podocytes, signaling via the TNF- α -TNFR2 axis activates the pro-inflammatory transcription factor NF- κ B in other cell types (11). We have previously shown NF- κ B is persistently activated in HIV-expressing Tg26^{HIV/nl} podocytes (52) resulting in a higher baseline activation level compared to normal podocytes. We therefore studied NF- κ B activation in both normal and Tg26^{HIV/nl} podocytes following challenges with TNF- α *ex vivo*. As expected, stimulation of both normal and Tg26^{HIV/nl} podocytes with soluble TNF- α induced or further increased NF- κ B activation, respectively (Figure 4). Blocking the soluble TNF- α with neutralizing antibody or chimeric TNFR1 abrogated this NF- κ B activation, showing the specificity of the recombinant TNF- α preparation and its lack of contamination by endotoxin, as control lipopolysaccharide (LPS)-induced NF- κ B activation was not countered by blocking TNF- α (Figure 4).

TNF- α is well-characterized for inducing NF- κ B-mediated pro-inflammatory priming of tissues leading to recruitment of leukocytes (11). To capture the broad repertoire of gene responses whereby podocytes may interpose this effect of TNF- α in the proliferative podocytopathies, RNA from podocytes challenged with TNF- α were profiled on Affymetrix Mouse 430A 2.0 microarray genes chips. Compared to quiescent podocytes, 183 annotated genes were induced 2-fold or greater in podocytes activated by TNF- α (Supplemental Table 1). Not surprisingly, the NF- κ B cell-cycle target gene, cyclin D1, and a large host of

downstream mitotic genes (*e.g.*, cyclin E1, Ki-67, PCNA) involved in cell-cycle progression, DNA replication, cytokinesis, and suppression of apoptosis (*e.g.*, survivin) of podocytes were induced by TNF- α . The expression of pro-inflammatory genes was also pronounced and included TNFR2, multiple chemokines, cell adhesion and receptor proteins (including TLR2 and CD44, as recently described (26, 34)) antigen presenting molecules, acute phase reactants, and transcription factors and regulators of the NF- κ B family itself (Table 3), but not TNF- α . Cross-referencing with known target genes of activated NF- κ B showed that the vast majority of these pro-inflammatory genes fall into this category.

Because recruited inflammatory mononuclear phagocytes intimately admix with diseased podocytes in CG (8) and crescentic glomerulonephritis (21, 22), we sought to validate the known functionality of the NF- κ B-induced macrophage chemokines, CCL2 (MCP-1) and CCL5 (RANTES) (53), present in this NF- κ B target gene signature. Conditioned media from normal and Tg26^{HIV/nl} podocytes induced macrophage migration, and with TNF- α stimulation, this chemoattractive effect was significantly increased in both cell types (Figure 5). The conditioned media contained readily detectable CCL2 and CCL5 protein (Table 4) each secreted 2–3 fold greater by Tg26^{HIV/nl} podocytes, consistent with their higher degree of NF- κ B activation (Figure 4). In comparison, other candidate NF- κ B-dependent chemokines were also readily detected but did not show the same differential secretion by Tg26^{HIV/nl} podocytes (Table 4), suggesting that HIV-expressing podocytes may harbor specific functional consequences on leukocyte recruitment. Similar to the mRNA expression profiling results above, TNF- α was not detectable in conditioned media from normal or Tg26^{HIV/nl} podocytes, suggesting NF- κ B activation or soluble TNF- α treatment did not induce new TNF- α production. Together, these results suggest that podocytes can directly serve as effectors of the pro-inflammatory and chemoattractive effects of TNF- α in diseased glomeruli.

Neutralization of TNFR2 on podocytes

Since TNFR2 is expressed by podocytes prior to hyperplastic injury in proliferative podocytopathies, we neutralized TNFR2 on podocytes to determine whether its engagement is required for the activation of NF- κ B and the induction of cyclin D1. Application of the TNFR2 blocking antibody, TR75-54.7, originally developed by Sheehan *et al* (54), 30 minutes prior to challenge with TNF- α , dose-dependently abrogated the functional activation of NF- κ B (Figure 6A) at concentrations shown previously to neutralize TNFR2 (54), whereas an isotype-matched control antibody had no effect. In contrast, the TNFR1 blocking antibody, 55R-170, also developed by Sheehan *et al*, did not abrogate functional activation of NF- κ B at concentrations shown previously to neutralize TNFR1 (54). This inability of TNF- α to activate NF- κ B with neutralization of TNFR2, paralleled the loss of induction by TNF- α of the NF- κ B target genes, cyclin D1 (Figure 6B) and iNOS (Figure 6C), the latter studied as a known positive control.

We showed previously that degradation of I κ B α , the key cytoplasmic repressor of canonical NF- κ B activation, is associated with the NF- κ B-dependent proliferative responses of podocytes in HIV-associated CG (52). Proteasomal degradation of I κ B α is the key initiating event following membrane receptor engagement that induces the NF- κ B activation cascade.

Thus, we studied the degradation of I κ B α in podocytes following challenge with TNF- α using similar receptor neutralization strategies as in Figure 6. NF- κ B activation was significantly reduced with neutralization of TNFR2, not TNFR1, and their combined neutralization did not significantly differ from neutralization of TNFR2 alone (Figure 7A). Parallel examination of total I κ B α protein by Western blotting showed that I κ B α degradation was significant only through the engagement of TNFR2, with minimal degradation mediated by TNFR1 (Figure 7B). Together, these studies mechanistically link selective ligation of TNFR2 with the proximal signaling events and functional gene expression from TNF- α -induced activation of NF- κ B in podocytes.

To confirm the receptor neutralization studies, similar experiments for NF- κ B activation and proliferation were conducted in which TNFR2 expression was reduced by RNAi-mediated gene silencing. Using RNAi, expression of TNFR2 was reduced by 64% compared to mock transfected cells (Figure 8A). Upon stimulation with TNF- α , the RNAi knocked-down cells showed significant reductions in both proliferation (68% reduction, Figure 8B) and NF- κ B activation (69% reduction, Figure 8C). The reductions in both proliferation and NF- κ B activation are consistent to the level of reduction in TNFR2 expression and support their mechanistic link to TNFR2 function in podocytes.

DISCUSSION

The immune induction of compound diseased podocyte phenotypes, in which podocytes are both targets and effectors of inflammatory challenges, is increasingly recognized as an important factor in the glomerular injury of several podocytopathies (3–9). We show here that TNFR2 expressed by podocytes can directly interpose the proliferative (target) and pro-inflammatory and chemoattractant (effector) phenotypes -- all characteristic of diseased podocytes in the proliferative podocytopathies -- induced by TNF- α . This extends the seminal observations by Vielhauer *et al* of the requirement for renal expressed TNFR2 to precipitate disease (19), and suggests that the capacity of the TNF- α -TNFR2 axis to injure podocytes may not necessitate other TNFR2 positive renal cell-types. These results do not, however, exclude possible contributions from other TNFR2 positive cell-types or pro-inflammatory mediators to podocyte injury in the proliferative podocytopathies *in vivo*, a question that will require selective deletion of TNFR2 from podocytes but not from other renal and non-renal cell-types to address.

The exact mechanisms for the *de novo* expression of TNFR2 by podocytes in the proliferative podocytopathies and in other inflammatory glomerular diseases (33, 37) remain to be determined. TNFR2 can be up-regulated by extrinsic factors such as pro-inflammatory cytokines (*e.g.*, by TNF- α as shown here), growth factors, and microbial products (39, 40). In support of this possibility, CG is often diagnosed in patients afflicted by extra-renal Th1-polarizing disorders (44), suggesting that these extrinsic mechanisms may be operative for podocytes. Alternatively, an intrinsic defect of podocytes may lead to the expression of TNFR2, as was shown by its induction *via* hypoxia-inducible factors in the podocyte-restricted von Hippel Lindau null model of crescentic glomerulonephritis (4) or by the proposed mitochondrial-hypoxia-inducible factor pathway in the B6 *kd/kd* model of CG (8). In these latter instances, TNFR2 positive podocytes may become quite susceptible to

otherwise “clinically silent” levels of TNF- α . This was demonstrated when CG in B6 *kd/kd* mice was eliminated when the mice were housed in a germ-free environment, indicating the “benign” specific-pathogen free Th1 environment, which has no effect on normal mice, can itself precipitated CG in B6 *kd/kd* mice (55). In any respect, contributions from synergistic pathogenic co-factors, such as underlying genetic predispositions in patients susceptible to CG (44), likely determines the pattern of glomerular injury following TNFR2 ligation on podocytes.

The Tg26^{HIV/nl} model of CG exemplifies this multifactorial pathogenesis associated with the TNF- α -TNFR2 axis in glomerular injury. The development of renal disease in Tg26^{HIV/nl} mice requires both the FVB/N genetic background (56) and expression of the HIV transgene (57). Within the FVB/N background, however, neutralization of TNF- α in Tg26^{HIV/nl} mice has been shown to silence HIV transgene expression and its associated pathologies (45), demonstrating the sensitivity of the HIV promoter to NF- κ B activated by TNF- α (58). We show here an overall good correlation between TNF- α , TNFR2, and the development of renal disease in Tg26^{HIV/nl} mice; thus, the TNF- α -TNFR2 axis in Tg26^{HIV/nl} mice may serve not only to precipitate disease phenotypes but also to up-regulate expression of HIV gene products known to further exacerbate tissue injury and the activation of NF- κ B itself (59). This would be expected to create pathogenic feed-forward NF- κ B loops in target cells such as podocytes (Figure 9). Indeed, the previously described HIV-expressing podocytes from Tg26^{HIV/nl} mice utilized here demonstrate persistent activation of NF- κ B, and suppressing this activation reduces rates of podocyte proliferation (52). The fact that TNF- α further exacerbated this persistent NF- κ B activation supports the paradigm that HIV can prime the cytokine responsiveness of infected cells (46).

This compounding of podocyte injury via NF- κ B is not unusual due to the multiple pathways that activate NF- κ B and the large number of NF- κ B target genes which then further amplify NF- κ B signaling (10–12). Virus-induced or somatic mutations causing NF- κ B dysregulation, stimulated by feed-forward NF- κ B loops from cytokine signaling, have long been known to couple and precipitate or accelerate cellular transformation (60). Our microarray gene profiling showed that podocytes respond similarly to TNF- α , up-regulating several NF- κ B transcription family members amongst other target genes that together perpetuate further activation of NF- κ B. In summary, these results add to that of others identifying the TNF- α -TNFR2 axis as a compelling pathway for further study of pathogenic NF- κ B signaling in proliferative podocytopathies.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

B6	C57BL/6
CG	collapsing glomerulopathy
HIV	human immunodeficiency virus
iNOS	inducible nitric oxide synthase
LPS	lipopolysaccharide
RT-PCR	reverse transcription-polymerase chain reaction
SEAP	secreted alkaline phosphatase
sTNFR	soluble tumor necrosis factor receptor
Th1	T helper 1
TLR	Toll-like receptor
TNF-α	tumor necrosis factor alpha
TNFR	tumor necrosis factor receptor

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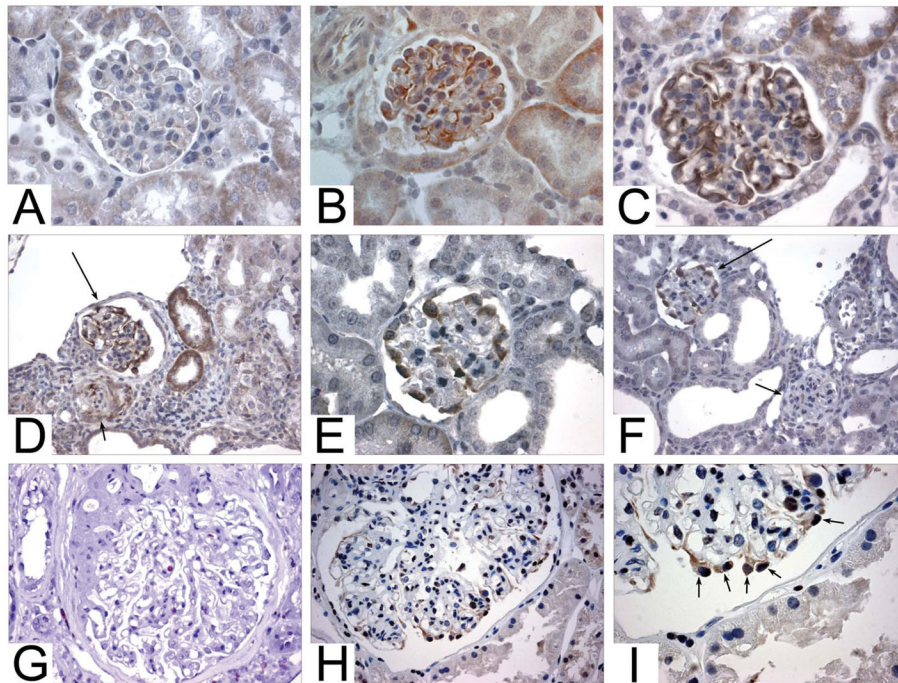


Figure 1. Expression of TNFR2 by podocytes in proliferative podocytopathies

(A) Lack of TNFR2 expression by podocytes in a normal FVB/N kidney. (B) TNFR2 expression by podocytes in the glomerulus from a nephrotoxic nephritis kidney prior to significant hyperplastic injury. (C) TNFR2 expression by podocytes in a diseased Tg26^{HIV/nl} kidney in a glomerulus with little to no hyperplastic injury. (D) Differential TNFR2 expression by podocytes in a diseased Tg26^{HIV/nl} kidney in adjacent glomeruli, one (long arrow) with little to no hyperplasia, the other (short arrow) with marked hyperplasia and fibrosis. (E) TNFR2 expression by podocytes in a diseased *kd/kd* kidney in a glomerulus with little to no hyperplastic injury. (F) Similar to panel D, differential TNFR2 expression by podocytes in a diseased *kd/kd* kidney in adjacent glomeruli, one (long arrow) with little to no hyperplasia, the other (short arrow) with marked hyperplasia. (G) Lack of TNFR2 expression by podocytes in a human biopsy diagnosed with primary focal segmental glomerulosclerosis. (H) TNFR2 expression by podocytes in a human biopsy diagnosed with CG in a glomerulus with little to no hyperplastic injury. (I) Higher power view of podocytes (arrows) in panel H. Magnifications: $\times 400$ in A, B, C, E, I; $\times 200$ in D, F, G, H.

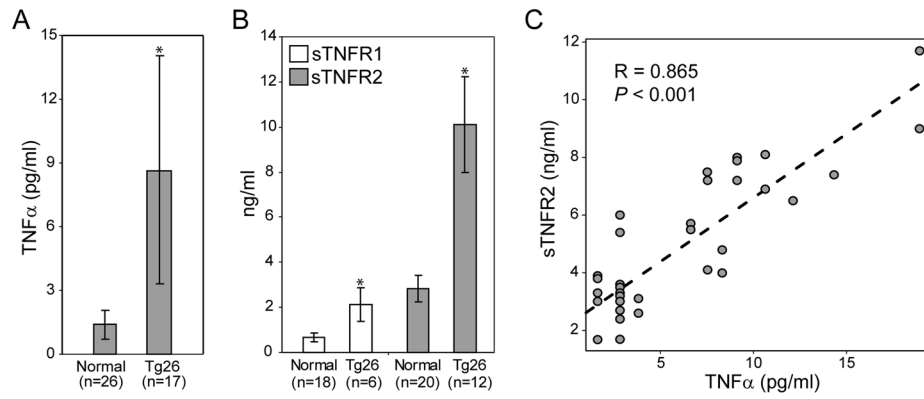


Figure 2. Levels of soluble TNF- α , sTNFR1, and sTNFR2 in serum of Tg26^{HIV/nl} mice
 Concentrations of (A) soluble TNF- α and (B) sTNFR1 and sTNFR2 in serum of diseased Tg26^{HIV/nl} mice and normal non-transgenic littermates. (C) Linear regression analysis and scatter plot demonstrating a significant correlation between serum levels of soluble TNF- α and sTNFR2 levels in normal and Tg26^{HIV/nl} mice with a range of severity of renal disease.

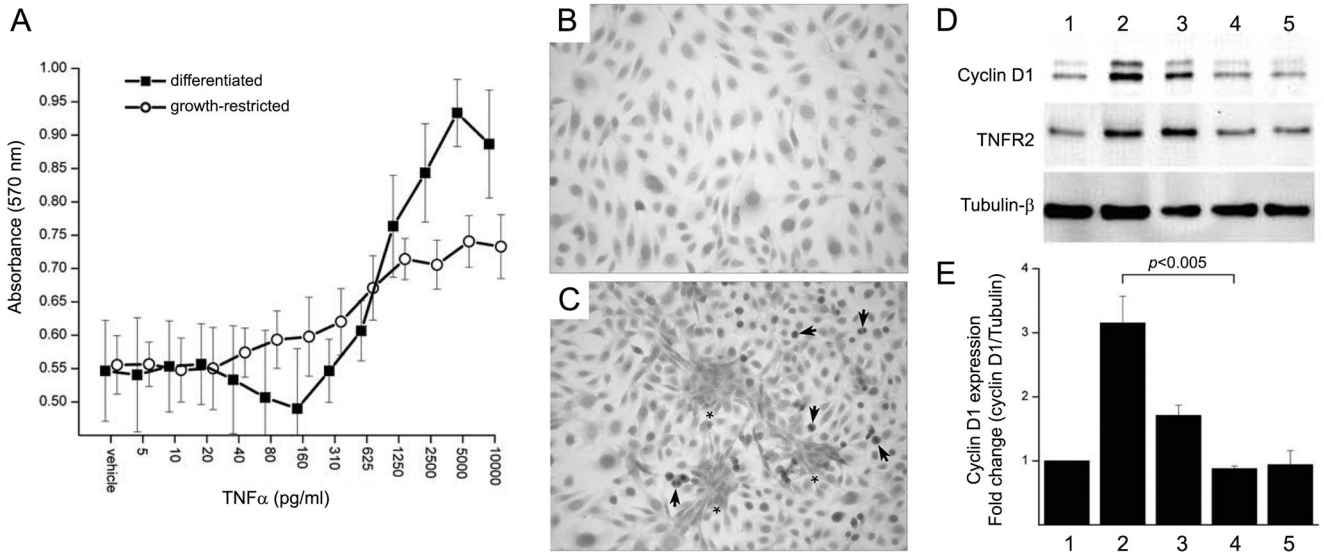


Figure 3. Proliferative response of podocytes to soluble TNF- α

(A) Cell proliferation assay showing dose-dependent proliferation of podocytes, either differentiated for 14 days or growth-restricted for 1 day, to a 6 day challenge with vehicle or 2-fold increasing doses of soluble TNF- α . (B) Representative monolayer of differentiated podocytes challenged with vehicle for 6 days, then fixed and stained with trypan-blue. (C) Representative changes in the density and morphology of differentiated podocytes challenged with TNF- α (2.5 ng/ml) for 6 days, then fixed and stained with trypan blue. Several mitotic figures (arrows) and foci (above asterisks) are present. Magnification: $\times 100$ in B and C. (D) Representative Western blot analyses of the changes in cyclin D1 protein expression by differentiated podocytes lysed after a 6 day challenge with TNF- α (2.5 ng/ml; lane 2) or vehicle (lane 4), or lysed at day 12 after a 6 day challenge with and then a 6 day wash-out of TNF- α (2.5 ng/ml; lane 3) or vehicle (lane 5). Lysates from differentiated podocytes prior to challenge with TNF- α at day 0 (lane 1) are included. The induction of TNFR2 by TNF- α is also shown as a positive control. (E) Fold-change in the protein expression of cyclin D1 relative to tubulin- β under the conditions of panel D.

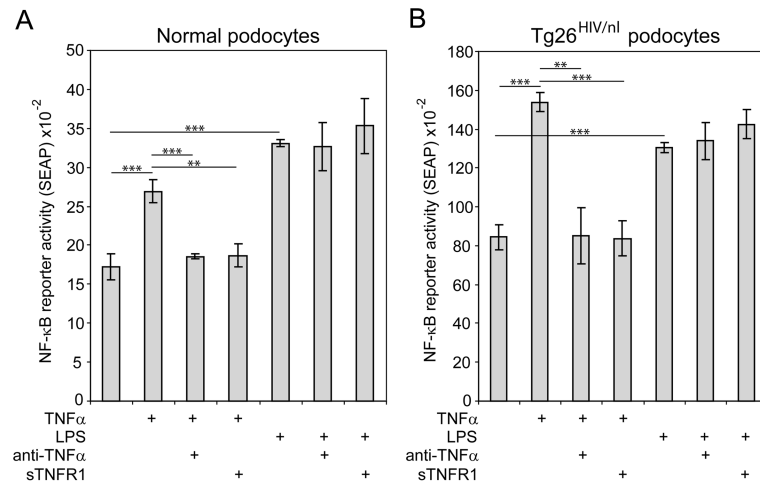


Figure 4. Functional activation of NF- κ B in podocytes by soluble TNF- α
 (A) Normal and (B) Tg26^{HIV/nl} podocytes were transfected with a NF- κ B-dependent SEAP reporter plasmid to functionally assay NF- κ B transcriptional activation. Stimulation with soluble TNF- α or LPS strongly induced NF- κ B activity in both normal and Tg26^{HIV/nl} podocytes. Neutralization of TNF- α with either antibody or soluble chimeric TNFR1 eliminated NF- κ B activation by TNF- α but not by LPS (** $P < 0.005$, *** $P < 0.001$).

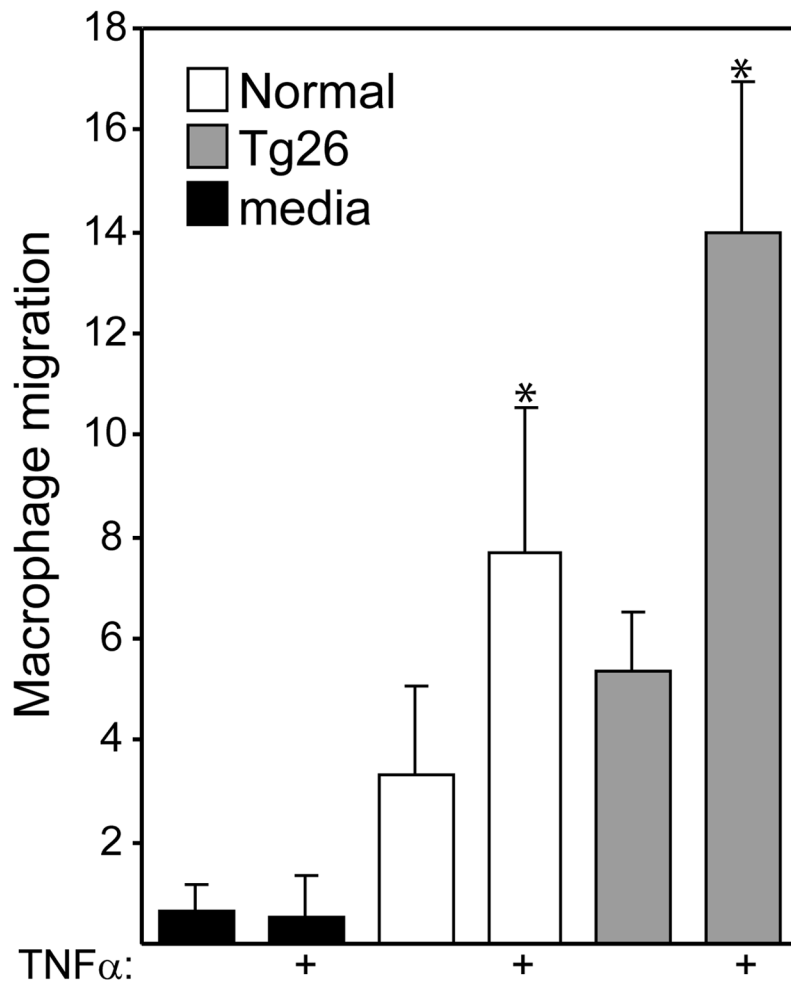


Figure 5. Chemoattraction of macrophages to podocytes-derived chemokines

Migration of monocytic cells (murine cell line RAW264.7) was used as a functional assay for chemokine secretion by podocytes. Unconditioned media (media) and conditioned media from normal and Tg26^{HIV/nl} podocytes, either untreated or treated with TNF- α , were tested for the ability to chemoattract macrophages in standard transwell migration assays, quantified as the number of migrated macrophages per high powered field. The identification and concentration of chemokines in the media was determined using a multi-analyte profiling assay (see Table 4) ** $P < 0.01$ compared to respective conditioned media without TNF- α stimulation.

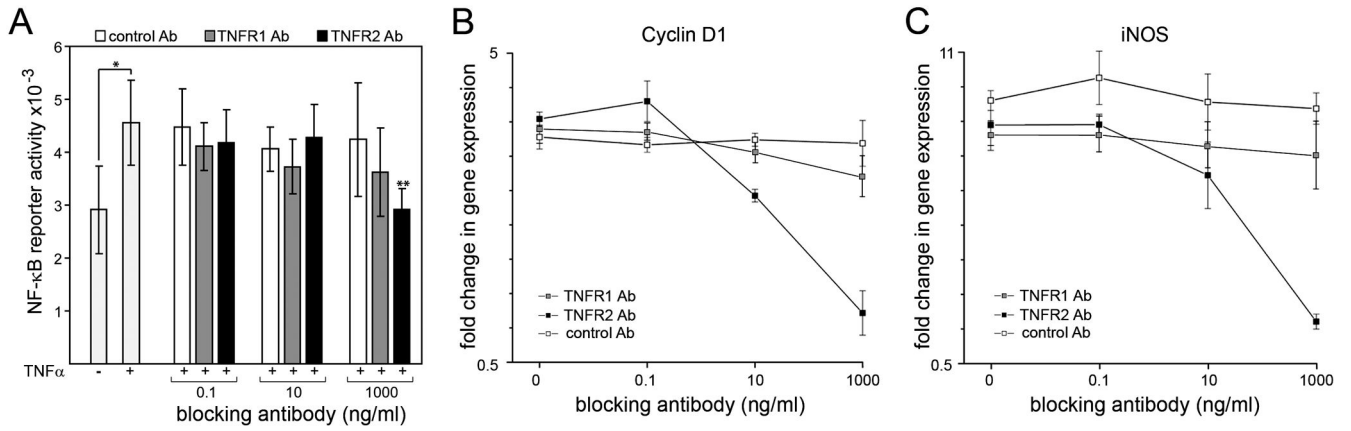


Figure 6. Neutralization of TNFR2 on podocytes and responses to TNF- α

(A) Functional NF- κ B activation was assessed in transient transfection of an NF- κ B-dependent SEAP reporter. After transfection, normal podocytes were treated 30 minutes prior to a 24 hour challenge with TNF- α with blocking antibodies for TNFR2 (clone TR75-54.7), TNFR1 (clone 55R-170), or an isotype-matched control IgG (* P <0.05; ** P <0.02 compared to TNF- α treated). In parallel experiments on normal, differentiated podocytes, the fold-change in gene expression of (B) cyclin D1 and (C) iNOS induced by TNF- α during neutralization of TNFR1 or TNFR2 was compared by quantitative RT-PCR to the level of gene expression in podocytes prior to challenge with TNF- α .

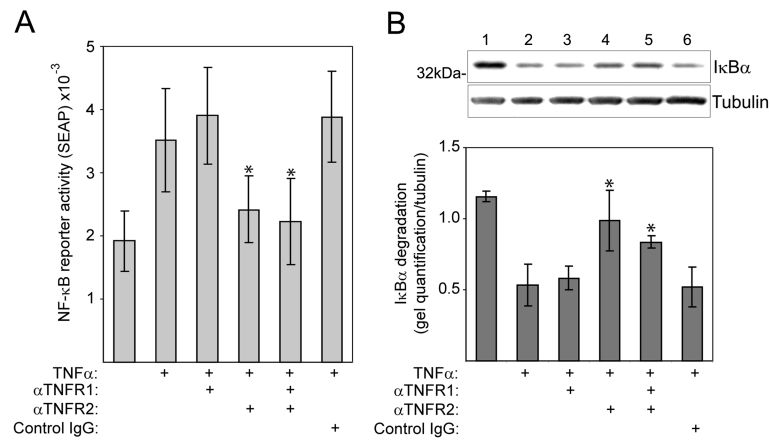


Figure 7. TNF receptor specificity for NF-κB activation

(A) Similar to Figure 6, functional NF-κB activity in podocytes following challenge with TNF-α using blocking antibodies was assessed using the SEAP reporter assay. (B) Western blotting for IκBα degradation in podocytes following TNF-α induction in the same experiments presented in panel A. Shown is a representative Western blot for IκBα that was re-probed for α-tubulin, a control protein, and a graph of the quantification of all gels normalized to α-tubulin expression. Lanes 1–6 correspond to the treatments shown below the bars on the graph (* $P < 0.05$ compared to TNF-α treated).

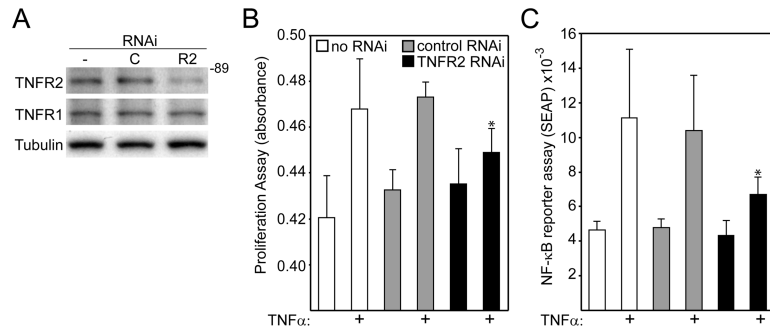


Figure 8. Effect of TNFR2 gene silencing on proliferation and NF-κB activation

Podocytes were mock transfected (no RNAi) or transfected with a non-target control or TNFR2-specific RNAi followed by treatment with TNF- α . (A) Reduction in expression of TNFR2 by RNAi was evaluated by Western blotting; a representative gel is shown and quantification indicated a reduction of 64% by TNFR2 (“R2”) RNAi compared to mock transfected cells (“-”). Control (“C”) RNAi showed an 8% reduction compared to mock transfected cells. Expression of TNFR1 was unchanged; Tubulin was used as a loading control. (B) Effect on cell proliferation with each RNAi was tested in experiments similar to Figure 3. Graphs are a composite of three experiments that resulted in an average reduction of 68% (**P* 0.05 compared to control treated with TNF- α). (C) Effect on NF- κ B activation with each RNAi was tested in experiments similar to Figure 4. Graphs are a composite of three experiments that resulted in an average reduction of 69% (**P* 0.05 compared to control treated with TNF- α).

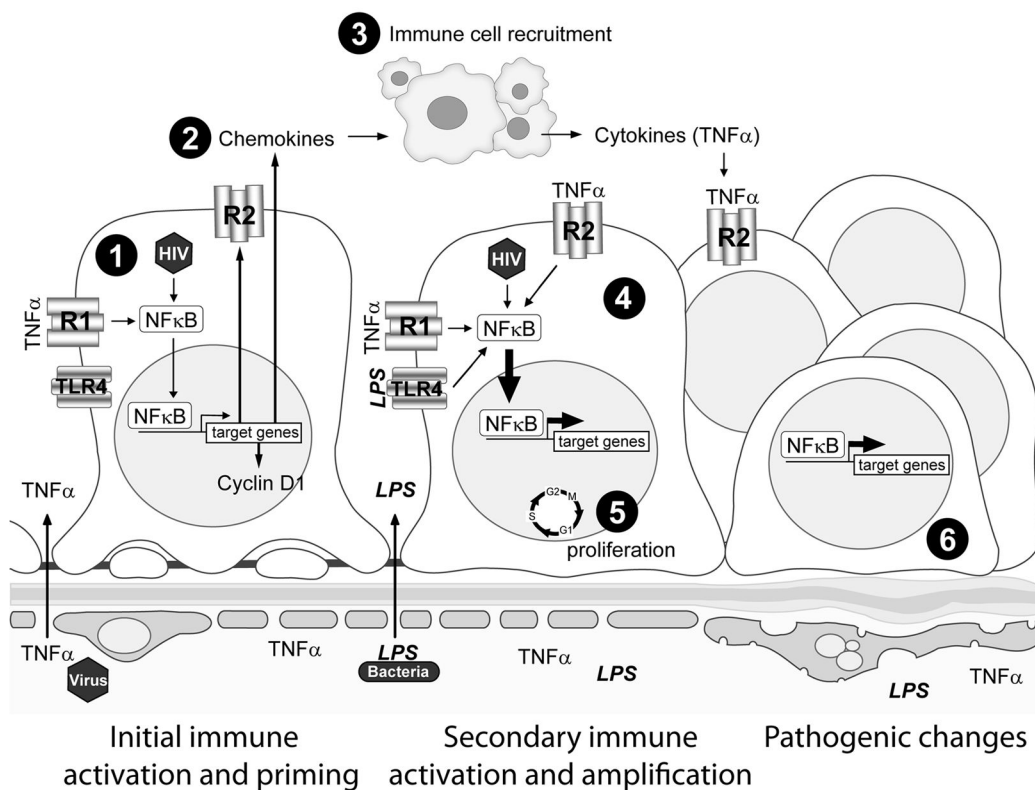


Figure 9. Proposed model of inflammatory amplification of NF-κB activation in podocytes during the pathogenesis of proliferative podocytopathies

Normal podocytes at steady-state have low levels of NF-κB activation, with the majority of NF-κB being sequestered in the cytoplasm through binding its cytoplasmic inhibitor IκB. (1) Pro-inflammatory challenges to quiescent podocytes, such as by TNF-α, initiate NF-κB signaling through degradation of IκB and translocation of NF-κB to the nucleus. In collapsing glomerulopathy, HIV-1 proteins can also initiate this NF-κB signaling (45). (2) As a result, multiple NF-κB target genes, including cyclin D1, TNFR2, and various chemokines, are up-regulated further priming the inflammatory responsiveness of podocytes. (3) Primed podocytes expressing immunomodulating proteins *de novo* are now more susceptible to further activation of NF-κB by multiple factors (e.g., TNF-α, bacterial LPS) either from the circulation, from infiltrating immune cells, or possibly produced from other renal cell types, thereby amplifying NF-κB signaling. (4) This reinforces cell-cycle progression, contributing to (5) the pathologic changes characteristic of proliferative podocytopathies.

Table 1Correlation of sTNFR2 levels with indices of renal disease in Tg26^{HIV/nl} mice (n=38).

<u>Covariates</u>	<u>R</u>	<u>P</u>
Renal function		
urine (protein/creatinine)	0.535	<0.001
blood urea nitrogen	0.693	<0.001
serum cholesterol	-0.097	0.564
serum triglycerides	-0.304	0.064
Pathology		
segmental glomerular collapse	0.133	0.425
global glomerular collapse	0.464	0.003
segmental glomerulosclerosis	0.176	0.292
global glomerulosclerosis	0.589	<0.001
podocyte hyperplasia	0.347	0.033
glomerular desmin expression	0.630	<0.001
tubulointerstitial inflammation	0.542	<0.001
tubular microcysts	0.383	0.018

Table 2Fold-changes in the gene expression of cyclin D1 in response to challenge with TNF- α .

gene	TNF-α challenge day 2	TNF-α challenge day 4	TNF-α challenge day 6	day 6–12 wash-out day 12
cyclin D1	3.21 \pm 0.09	2.90 \pm 0.04	1.89 \pm 0.01	1.14 \pm 0.02
iNOS	18.21 \pm 0.68	14.61 \pm 1.67	13.24 \pm 2.67	1.56 \pm 1.01

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Table 3Representative pro-inflammatory genes induced in podocytes by TNF- α

Genbank ID	Gene	Chemokines
BC019961	Cxcl16	chemokine (C-X-C motif) ligand 16
NM_019494	Cxcl11	chemokine (C-X-C motif) ligand 11
NM_021274	Cxcl10	chemokine (C-X-C motif) ligand 10 ^a
NM_023785	Cxcl7	chemokine (C-X-C motif) ligand 7
NM_009141	Cxcl5	chemokine (C-X-C motif) ligand 5 ^a
NM_008176	Cxcl1	chemokine (C-X-C motif) ligand 1 ^a
AF099052	Ccl20	chemokine (C-C motif) ligand 20 ^a
NM_021443	Ccl8	chemokine (C-C motif) ligand 8 ^a
AF128193	Ccl7	chemokine (C-C motif) ligand 7 ^a
NM_013653	Ccl5	chemokine (C-C motif) ligand 5 ^a
AF065933	Ccl2	chemokine (C-C motif) ligand 2 ^a
AF010586	Cx3cl1	chemokine (C-X3-C motif) ligand 1 ^a
<u>Adhesion proteins and receptors</u>		
BB250384	Vcam1	vascular cell adhesion molecule 1 ^a
X66083	Cd44	CD44 ^a
AF079222	Hmmr	hyaluronan mediated motility receptor
NM_010743	Il1rl1	IL-33 receptor, ST2 ^a
NM_011905	Tlr2	toll-like receptor 2 ^a
M60469	Tnfrsf1b	TNFR2, p75 ^a
<u>Growth factors</u>		
X03019	Csf2	GM-CSF, colony stimulating factor 2 ^a
<u>Antigen presentation proteins</u>		
BC003476	Ii	Ia-associated invariant chain ^a
M29881	H2-Q7	histocompatibility 2, Q region locus 7 ^a
NM_010395	H2-T10	histocompatibility 2, T region locus 10 ^a
M33151	H2-Q10	histocompatibility 2, D region locus 1 ^a
<u>Acute phase reactants</u>		
NM_011315	Saa3	serum amyloid A 3 ^a
K02782	C3	complement component 3 ^a
X67668	Hmgb2	high mobility group box 2
L38281	Irg1	immunoresponse gene 1
<u>NF-κB factors and regulators</u>		
NM_009046	Relb	RelB ^a
AF155372	Nfkb2	NF- κ B2, p52/p100 ^a
NM_019777	Ikbke	IKK ϵ ^a

Genbank ID	Gene	Chemokines
NM_010907	Nfkbia	I κ B α ^a
AK011965	Nfkbie	I κ B ϵ ^a
NM_009397	Tnfaip3	TNFAIP3 ^a
AJ242777	Tnip1	A20 binding inhibitor of NF- κ B, ABIN-1 ^a

^a target genes of activated NF- κ B (www.nf-kb.org)

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Table 4

Chemokine protein concentrations in media conditioned by podocytes

	WT (n=7)	Tg26 (n=6)	
CCL2	2.9 ± 0.8	9.2 ± 1.1*	ng/ml
CCL3	13.6 ± 9.9	23.0 ± 8.0	pg/ml
CCL5	89.4 ± 42.8	189.6 ± 46.4*	pg/ml
CXCL1	2.7 ± 0.8	3.2 ± 0.7	ng/ml
CXCL10	3.8 ± 3.0	3.5 ± 1.0	ng/ml

* $P < 0.001$

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