Fine-tuning of NFκB by Glycogen Synthase Kinase 3β directs
the fate of glomerular podocytes upon injury

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Abstract

NFκB is regulated by a myriad of signaling cascades including glycogen synthase kinase (GSK) 3β and plays a Janus role in podocyte injury. In vitro, lipopolysaccharide or adriamycin elicited podocyte injury and cytoskeletal disruption, associated with NFκB activation and induced expression of NFκB target molecules, including pro-survival Bcl-xL and podocytopathic mediators like MCP-1, cathepsin L and B7-1. Broad range inhibition of NFκB diminished the expression of all NFκB target genes, restored cytoskeleton integrity, but potentiated apoptosis. In contrast, blockade of GSK3β by lithium or TDZD-8, mitigated the expression of podocytopathic mediators, ameliorated podocyte injury, but barely affected Bcl-xL expression or sensitized apoptosis. Mechanistically, GSK3β was sufficient and essential for RelA/p65 phosphorylation specifically at serine 467, which specifies the expression of selective NFκB target molecules, including podocytopathic mediators, but not Bcl-xL. In vivo, lithium or TDZD-8 therapy improved podocyte injury and proteinuria in mice treated with lipopolysaccharide or adriamycin, concomitant with suppression of podocytopathic mediators but retained Bcl-xL in glomerulus. Broad range inhibition of NFκB conferred similar but much weakened antiproteinuric and podoprotective effects accompanied with a blunted glomerular expression of Bcl-xL and marked podocyte apoptosis. Thus, the GSK3β dictated fine-tuning of NFκB may serve as a novel therapeutic target for podocytopathy.

Keywords

glomerulus; proteinuria; podocyte; nuclear factor κB; glycogen synthase kinase 3β; apoptosis

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DISCLOSURE

All the authors declared no competing interests.
stage renal failure and for cardiovascular morbidity and mortality.\(^1\)\(^–\)\(^3\) Converging evidence suggests that podocyte injury is a major culprit for proteinuria and progressive glomerular sclerosis.\(^4\)\(^–\)\(^7\) However, the exact mechanism responsible for podocyte injury still remains obscure. Research data recently have pinpointed the central pathogenic role of nuclear factor \(\kappa B\).\(^8\)\(^,\)\(^9\)\(^,\)\(^10\) In glomerular disease, \(\text{NFkB}\) signaling is indispensable for podocyte expression of a multitude of \(\text{NFkB}\) target molecules involved in podocytopathy. These include proinflammatory cytokines\(^11\),\(^12\), like MCP-1, as well as lysosomal protease cathepsin L and co-stimulatory molecule B7-1, all of which function at the interface between innate/adaptive immunity and podocyte cytoskeletal remodeling.\(^13\)

\(\text{NFkB}\) activation is a complex and highly orchestrated process that undergoes multiple key steps and offers several levels for inhibition.\(^14\) Once activated, \(\text{NFkB}\) transcriptional activity is further finely regulated by inducible post-translational modifications, including acetylation, methylation, and phosphorylation.\(^15\),\(^16\) Thus, \(\text{NFkB}\) subunits with different phosphorylation patterns may recruit different transcriptional cofactors, direct distinct profiles of gene expression, and result in varying and even opposing pathobiological effects.\(^8\),\(^17\) Inhibition of \(\text{NFkB}\) by maneuvers that intercept proximal signaling of \(\text{NFkB}\) pathway at a level above its nuclear activity blocks the general effects of \(\text{NFkB}\) and would be consired as broad range inhibition, because it unselectively suppresses the expression of a broad range of \(\text{NFkB}\) target genes,\(^14\) including those critical for biological homeostasis, and often results in a lot of undesirable side effects.\(^18\)\(^–\)\(^20\) These maneuvers consist of genetic knockout of RelA/p65, glucocorticoids\(^21\), and chemical inhibitors, including scavengers of reactive oxygen species, like pyrrolidine dithiocarbamate (PDTC)\(^22\), proteosome inhibitors, and protease inhibitors, like N-tosyl-L-phenylalanine chloromethyl ketone (TPCK)\(^23\). Indeed, corticosteroids are very effective in inducing remission of proteinuric glomerulopathies, but commonly causes severe side effects due to suppression of \(\text{NFkB}\) dependent cell survival, proliferation and differentiation.\(^24\) To this end, therapeutic targeting of signaling cascades that fine-tune \(\text{NFkB}\) has become an attractive strategy to precisely control the \(\text{NFkB}\) response.

Recent studies from this and other groups\(^25\)\(^–\)\(^27\),\(^28\) suggest that fine-tuning of \(\text{NFkB}\) activation involves GSK3\(\beta\), a multitasking kinase situated at the nexus of a complex cell signaling network. GSK3\(\beta\) specifically dictates human RelA/p65 phosphorylation at serine 468, which is equivalent to serine 467 of mouse RelA/p65 by sequence homology, and thereby directs the transcription of selective \(\text{NFkB}\) target molecules involved in kidney disease.\(^28\) This study examined the role of GSK3\(\beta\) in controlling \(\text{NFkB}\) activation in liposaccharide (LPS) or adriamycin (ADR) induced podocyte injury by using selective GSK3\(\beta\) inhibitors including lithium and 4-benzyl-2-methyl-1,2,4-thiadiazolidine-3,5-dione (TDZD-8).

**Results**

**LPS-induced podocyte dysfunction is associated with \(\text{NFkB}\) activation and GSK3\(\beta\) overactivity**

LPS causes injury via toll-like receptors (TLR) in a variety of mammalian cell types, including podocytes.\(^29\) Consistent with previous report,\(^30\) LPS induced the expression of
TLR-4 in differentiated murine podocytes (Figure 1A) and elicited prominent injury in a dose and time dependent fashion, characterized by disruption of the phalloidin labeled filamentous actin network and diminished expression of synaptopodin (Figure 1). This was associated with overactivity of the redox sensitive GSK3β, measured by the repressed inhibitory phosphorylation of GSK3β, and NFκB activation, marked by RelA/p65 nuclear translocation and phosphorylation at multiple amino acid residues, including serine 276, serine 467 and serine 536 (Figure 1). Subsequently, expression of NFκB dependent molecules was markedly triggered by LPS in a dose and time dependent manner. These include the antiapoptotic/prosurvival factor Bcl-xL and immune/inflammatory mediators, like B7-1 and cathepsin L, which play a pivotal role in podocyte injury by disrupting podocyte cytoskeleton. Actin cytoskeleton disorganization has been centrally implicated in podocyte dysfunction and is associated with podocyte hypermotility. As assessed by a traditional cell migration assay (Figure 2), LPS injured podocytes demonstrated a strikingly accelerated closure of the gap between the leading edges of the migrating podocyte sheets, suggesting an enhanced podocyte motility.

Broad range inhibition of NFκB abrogates cellular dysfunction but potentiates apoptosis in LPS injured podocytes

NFκB pathway has been shown to mediate the LPS induced injury. In our study, PDTC and TPCK, broad range inhibitors of NFκB, attenuated the LPS induced podocyte injury, as evidenced by prevented loss of synaptopodin, reinstated actin cytoskeleton integrity (Figure 3A and 3C), improved podocyte shape and a correction of podocyte hypermotility, as measured by the cell migration assay (Figure 2). This was associated with reduced phosphorylation of RelA/p65 at all examined residues, intercepted NFκB nuclear translocation and diminished expression of all NFκB target molecules, including MCP-1, B7-1, cathepsin L and Bcl-xL (Figure 3A). Despite the above protective effects, PDTC and TPCK treatments, however, aggravated the LPS induced apoptosis, as probed by TdT-mediated dUTP nick-end labeling (TUNEL) staining and confirmed by immunoblot (Figure 3A) and immunocytochemistry analysis for cleaved caspase 3 (Figure 3C). This prodeath effect coincided with the diminished expression of the NFκB dependent prosurvival Bcl-xL (Figure 3A).

Blockade of GSK3β suppresses NFκB p65 phosphorylation selectively at serine 467 in podocyte

GSK3β is an indispensable element for NFκB activation and directs the expression of a selective array of NFκB target molecules implicated in immune/inflammatory responses and kidney injury. Indeed, lithium chloride and TDZD-8, specific inhibitors of GSK3β, induced the inhibitory phosphorylation of GSK3β, indicative of an effective GSK3β inhibition, and diminished expression of the LPS elicited phosphorylation of RelA/p65 at serine 467 (Figure 3B). In stark contrast, the LPS elicited phosphorylation of RelA/p65 at other residues was barely affected, including serine 536 and serine 276. Following GSK3β inhibition by lithium or TDZD-8, the LPS induced expression of NFκB target podocytopathic mediators, like MCP-1, cathepsin L and B7-1, was drastically reduced. Accordingly, podocyte injury was attenuated, characterized by replenished synaptopodin expression (Figure 3B and 3C), reinstated integrity of actin cytoskeleton (Figure 3C),
normalized podocyte morphology (Figure 2A), and a correction of podocyte hypermotility (Figure 2). However, the LPS provoked expression of the NFκB dependent prosurvival Bcl-xL was seemingly unaffected by GSK3β inhibition (Figure 3A). In agreement, apoptosis, detected by TUNEL or cleaved caspase 3 staining, was not potentiated, but slightly attenuated by lithium or TDZD-8 treatment (Figure 3C).

GSK3β fine tunes NFκB RelA/p65 phosphorylation at serine 467

GSK3β targets multiple downstream pathways, including the β-catenin and the TSC2/mTOR pathways25–27. GSK3β inhibition by lithium or TDZD-8 at high doses (Supplementary Figure 1A) did induce β-catenin activation, as measured by nuclear translocation of β-catenin. However, low doses of lithium or TDZD-8, as used in this study, were sufficient to suppress RelA/p65 serine 467 phosphorylation without activating β-catenin (Figure 3B), suggesting that β-catenin is less likely involved. Moreover, lithium or TDZD-8 treatment indeed suppressed the LPS acivated TSC2/mTOR signaling in podocytes, as shown by diminished phosphorylation of the mTOR substrate p70S6K (Supplementary Figure 1B). However, the TSC2/mTOR pathway was unlikely responsible for GSK3β regulation of RelA/p65 phosphorylation at serine 467, because specific inhibition of the TSC2/mTOR pathway by rapamycin (Supplementary Figure 1C) did not inhibit NFκB, but instead amplified the LPS induced NFκB response in a dose dependent fashion.

To explore a possible direct causal relationship between GSK3β and NFκB p65 phosphorylation at serine 467, the activity of GSK3β was manipulated in mouse podocytes by forced expression of vectors encoding the hemagglutinin (HA) conjugated wild type (WT) GSK3β, kinase dead (KD) or constitutively active (S9A) mutant of GSK3β with a satisfactory transfection efficiency of over 70%, reflected by immunoblot analysis (Figure 4A) and immunostaining for HA (Figure 4B). Ectopic expression of KD blunted NFκB p65 phosphorylation selectively at serine 467, abolished the expression of B7-1, cathepsin L and MCP-1, prevented synaptopodin loss and injury in LPS stimulated podocytes, mimicking the effect of lithium or TDZD-8. Conversely, GSK3β overactivity induced by forced expression of S9A reinforced RelA/p65 phosphorylation selectively at serine 467, elevated the expression of B7-1, cathepsin L and MCP-1, promoted synaptopodin depletion and podocyte injury following LPS treatment (Figure 4A). No difference in phosphorylation of NFκB p65 at other residues or in nuclear β-catenin expression was noted among podocytes expressing different vectors. Recent studies28, 35 of human RelA/p65 proved that serine 468 of human RelA/p65, which is equivalent to serine 467 of mouse RelA/p65 by sequence homology, is situated in a GSK3β consensus motif and could be directly phosphorylated by GSK3β both in vivo and in vitro. To further understand the mechanistic essence of the GSK3β regulated murine RelA/p65 phosphorylation at serine 467, the amino acid sequences of murine RelA/p65 (NCBI accession number: NP_033071) were subjected to computational active site analysis (http://kinasephos2.mbc.nctu.edu.tw/). In silico analysis deduced that serine 467 of murine RelA/p65 resides in the consensus motifs for phosphorylation by GSK3β with a prediction score of 0.998869, indicating a high-confidence match to GSK3β phosphorylation motif and suggesting RelA/p65 as a putative cognate substrate of GSK3β (Figure 4C).
To further examine the functionality of the GSK3β regulated RelA/p65 serine 467 phosphorylation in controlling the physical interaction between NFκB and the target genes, podocytes expressing different vectors were injured with LPS in the presence or absence of GSK3β inhibitors and subjected to chromatin immunoprecipitation (ChIP) assay followed by quantitative real time PCR. Shown in Figure 4D–4G, bioinformatics analysis revealed that murine B7-1, cathepsin L, MCP-1 and Bcl-xL genes all contain multiple putative NFκB cis elements in their promoter regions. LPS injury prominently up regulated the recruitment of NFκB to all of these target genes. Ectopic expression of the KD mutant diminished the association of NFκB with B7-1, cathepsin L and MCP-1 genes, reminiscent of the effect of lithium or TDZD-8. Conversely, forced expression of the S9A mutant reinforced this binding. Of note, the LPS elicited NFκB binding to Bcl-xL gene seemed unaffected in podocytes with different GSK3β activity.

The GSK3β target serine 467 in NFκB RelA/p65 is essential for podocyte expression of NFκB dependent podocytopathic mediators

To determine the role of serine 467 of NFκB RelA/p65 in controlling expression of podocytopathic mediators in podocytes, native RelA/p65 in murine podocytes was knocked down by RNA interference and reconstituted with ectopic expression of HA conjugated wild-type human RelA/p65 (HA-hRelA wt) or mutant human RelA/p65 (HA-hRelA S468A), in which the serine 468, equivalent to serine 467 of mouse RelA by sequence homology, is replaced by alanine (Figure 5). RelA/p65 silencing abrogated the LPS induced expression of all NFκB dependent molecules, including both podocytopathic mediators, like MCP-1, cathepsin L and B7-1, and the prosurvival Bcl-xL, and potentiated podocyte apoptosis, as indicated by amplified caspase 3 cleavage. These effects were largely counteracted by reconstitution of RelA/p65-silenced podocytes with wild-type human RelA/p65. In contrast, in RelA/p65-silenced podocytes reconstituted with mutant human RelA/p65, the LPS induced expression of Bcl-xL rather than the podocytopathic mediators was selectively reinstated.

Fine tuning of NFκB activity by GSK3β in LPS induced experimental podocytopathy

To define the role of GSK3β regulated NFκB activity in podocyte injury in vivo, we employed the mouse model of LPS induced proteinuria and podocytopathy. LPS injury caused overt albuminuria at 24 h (Figure 6A). This coincided with the ultrastructural lesions of glomerular podocytes on electron microscopy (Figure 6D), characterized by podocyte foot process effacement and microvillous transformation. Moreover, acute LPS injury also caused occasional cellular apoptosis at the periphery of glomerular tufts in WT-1 positive cells, indicative of podocyte apoptosis as demonstrated by TUNEL staining (Figure 6E). Immunoblot analysis of homogenates of glomeruli isolated from kidneys using magnetic beads (Figure 6C) demonstrated that LPS injury elicited GSK3β overactivity, marked by the reduced inhibitory phosphorylation of GSK3β, and triggered NFκB activation, reflected by phosphorylation of RelA/p65 at multiple sites, associated with induced expression of both podocytopathic and podocyte protective NFκB dependent molecules in glomeruli, including MCP-1, B7-1, cathepsin L and Bcl-xL (Figure 7A). Confocal microscopy of fluorescent immunohistochemistry staining (Figure 7C) showed that NFκB target molecules, like MCP-1, B7-1, and Bcl-xL, were largely expressed in glomerular cells positive for podocyte...
specific markers, including synaptopodin and WT-1, consistent with a pattern of podocyte distribution. Lithium or TDZD-8 treatment counteracted GSK3β overactivity, overrode RelA/p65 phosphorylation specifically at serine 467, but had little effect on RelA/p65 phosphorylation at other sites or on nuclear translocation of β-catenin in glomeruli. Consequently, lithium or TDZD-8 therapy attenuated the LPS induced glomerular expression of B7-1, cathepsin L and MCP-1, but largely preserved the prosurvival Bcl-xL in the glomerulus (Figure 7A and 7C). This was associated with preserved glomerular synaptopodin, remarkable attenuation of podocyte foot process effacement on electron microscopy, fewer apoptotic podocytes in glomeruli (Figure 6B, 6D and 6E) and improvement in albuminuria by 67% and 61% respectively for lithium and TDZD-8 therapy.

In contrast, TPCK treatment completely blocked the NFκB pathway, diminished the LPS elicited RelA/p65 phosphorylation at all sites and abolished glomerular expression of all NFκB dependent molecules, including podocytopathic mediators and prosurvival Bcl-xL, (Figure 7B and 7C). Consequently, TPCK improved albuminuria by a much lesser extent (33%). Despite a slight improvement in podocyte foot process effacement and a minimal restoration of glomerular expression of synaptopodin, TPCK therapy evidently aggravated glomerular cell apoptosis in LPS injured mice (Figure 6).

**Fine tuning of NFκB activity by GSK3β in adriamycin induced podocyte injury**

To test if the effect of GSK3β on fine tuning NFκB activity could be generalized to other types of podocytopathy, *in vitro* (Figure 8) and *in vivo* (Figure 9) models of ADR induced podocyte injury were adopted. ADR injury of cultured podocytes elicited the activity of GSK3β and NFκB, marked by reduced inhibitory phosphorylation of GSK3β and increased RelA/p65 phosphorylation (Figure 8A). This was associated with significant podocyte injury and apoptosis, as shown by actin cytoskeleton disorganization, TUNEL staining (Figure 8B) and caspase 3 cleavage (Figure 8A), concomitant with induced expression of prosurvival Bcl-xL and podocytopathic mediators like MCP-1, B7-1 and cathepsin L (Figure 8A and 8B). Both NFκB inhibition by TPCK and GSK3β inhibition by TDZD-8 mitigated the ADR induced expression of MCP-1, B7-1 and cathepsin L and attenuated actin cytoskeleton disorganization (Figure 8). However, TPCK blocked NFκB nuclear translocation and potentiated apoptosis, while TDZD-8 barely affected NFκB nuclear translocation but instead attenuated podocyte apoptosis (Figure 8A and 8B).

In murine models of ADR nephropathy, ADR injured mice started to develop massive proteinuria on day 4 (Figure 9A and 9B) and had evident podocytopathy, marked by extensive podocyte foot process effacement as shown by electron microscopy (Figure 9A and 9D), and glomerular podocyte apoptosis, indicated by dual positivity for both TUNEL and WT-1 on fluorescent immunohistochemistry staining (Figure 9E and 9F). This was associated with prominent NFκB phosphorylation and activation in the glomerulus and induced glomerular expression of podocytopathic mediators and prosurvival Bcl-xL (Figure 9G). Confocal microscopy of dual color immunohistochemistry staining indicated that the prosurvival Bcl-xL and podocytopathic mediators, like MCP-1 and B7-1, were mainly detected in glomerular cells positive for podocyte specific markers, such as synaptopodin and WT-1, suggesting a pattern of podocyte distribution (Figure 9H). Inhibition of GSK3β by TDZD-8 substantially prevented the ADR induced albuminuria on day 4 and on day 7...
respectively by 64% and 73% (Figure 9A and 9B). This was associated with remarkable attenuation of podocyte injury, including foot process effacement (Figure 9C and 9D) and apoptosis (Figure 9E and 9F). Mechanistically, TDZD-8 therapy suppressed RelA/p65 activation selectively at serine 467, but had little effect on RelA/p65 phosphorylation at other sites or on nuclear translocation of β-catenin in the glomeruli (Figure 9G). Consequently, TDZD-8 treatment reduced glomerular expression of podocytopathic mediators, but largely preserved glomerular expression of the prosurvival Bcl-xL (Figure 9G and 9H). Lithium therapy likewise induced a remarkable proteinuria remission (Figure 9A and 9B) in ADR injured mice and reduced glomerular expression of podocytopathic mediators (Figure 9G). In contrast, TPCK completly blocked the NFκB activity (Figure 9G), repressed glomerular expression of all NFκB target molecules, including the prosurvival Bcl-xL (Figure 9G and 9H), slightly ameliorated podocyte foot process effacement (Figure 9C and 9D), but substantially exacerbated podocyte apoptosis (Figure 9E and 9F), resulting in an improvement of albuminuria to a much lesser extent (on day 4 and day 7 respectively by 36% and 43%).

Discussion

NFκB acts as a double-edged sword in the pathogenesis of podocytopathy. On one hand, NFκB directs the transcription of a multitude of injurious mediators involved in podocyte disease, most of which are related to inflammatory response and immune reaction, including proinflammatory cytokines (e.g. MCP-1) and immune regulatory cathepsin L and B7-1, which also have been implicated in podocyte cytoskeleton disorganization.29, 36, 37 On the other hand, NFκB is a quintessential survival factor, that is essential for cellular response to stress or injury for self-protection.38–40 Therefore, it is conceivable that overall inhibition of NFκB would certainly protect against podocyte injury through blocking inflammatory responses but also sensitize podocytes to death. In support of this view, podocyte-specific knockout of NFκB essential modulator (NEMO) intercepted NFκB proximal signaling in podocytes and did ameliorate podocyte injury, lessen proteinuria and improve glomerular histology in mice with nephrotoxic nephritis.9 However, the absolute number of podocytes per glomerulus was not improved but further reduced in the knockout mice, suggesting that broad range NFκB inhibition might predispose podocytes to death despite some protective effects. Glucocorticoids, a typical broad range inhibitor of NFκB, have been proven to be a valuable treatment for a variety of glomerular diseases. The repressed NFκB-dependent antiapoptotic/prosurvival activity subsequent to glucocorticoids therapy, however, has been largely compensated and masked by the potent anti-apoptotic signaling of glucocorticoid receptor in glomerular cells.41, 42 Nevertheless, in extrarenal organs, like the skin and muscular system where glucocorticoid receptor signaling barely confers anti-apoptotic effects, glucocorticoid therapy could induce severe atrophic changes.43–45 Thus, it is imperative to develop a novel therapy that fine tunes NFκB pathway to eliminate the detrimental activities but preserve the self protective actions.

A plethora of studies recently suggest that fine tuning of NFκB response could be achieved by controlling the post-translational modifications of the transcription activation domain of the RelA/p65.46, 47 For instance, serine 468 of human RelA/p65 might be crucial for dictating the immune and inflammatory response of NFκB. Mattioli et al found that T-cell
costimulation, which selectively induces p65 phosphorylation at S468 in human peripheral T lymphocytes, elicited the expression of a specific set of Th1 cytokines, including IL-2 and INF-γ. Conversely, these cytokines were not induced by CD43 stimulation, which failed to phosphorylate S468 of p65. Our recent study indicated that human p65 S468 is required for the induced expression of proinflammatory genes like MCP-1, RANTES, and IL-8 in renal tubular epithelial cells, but not required for anti-inflammatory or antiapoptotic/prosurvival genes such as IkBα or Bcl-2. Likewise, in this study, we found that serine 467 of murine RelA/p65, which is equivalent to serine 468 in human RelA/p65 by sequence homology, is essential for podocyte expression of NFκB dependent podocytopathic mediators but not for the prosurvival Bcl-xL in glomerular podocytes (Figure 5).

How phosphorylation of RelA/p65 serine 467(8) is regulated remains uncertain, but converging evidence suggests that GSK3β likely plays a pivotal role. GSK3β is a well-conserved, ubiquitously expressed serine/threonine protein kinase and has recently been identified as an essential element for NFκB activation. Genetic disruption of GSK3β abolished NFκB activation and NFκB-mediated inflammatory responses to TNF-α or IL-1β. GSK3β seems to regulate NFκB activity directly rather than through other downstream transducers, like β-catenin, as shown in this study. Of note, there is a functional redundancy of GSK3α and GSK3β in controlling β-catenin signaling, but not for controlling NFκB activity. High-dose inhibition is required to suppress both GSK3α and GSK3β in order to achieve an appreciable change in β-catenin levels. This explains why low-dose inhibition of GSK3β is sufficient to suppress RelA/p65 serine 467 phosphorylation without activating β-catenin in this study. TSC2/mTOR is another target pathway of GSK3β and consistent with other work, inhibition of GSK3β by lithium or TDZD-8 indeed suppressed the mTOR activity. However, blocking the mTOR pathway has been shown to enhance rather than inhibit NFκB activity by this and other studies, thus ruling out the involvement of TSC2/mTOR in GSK3β regulation of NFκB. Recent in-depth research indicated that NFκB RelA/p65 physically interacts with GSK3β. Serine 468 of human RelA/p65 was found to lie within a GSK3β consensus motif and could be directly phosphorylated by GSK3β both in vivo and in vitro. Congruously in this study, GSK3β determined the phosphorylation of serine 467 of murine RelA/65 and selectively directed the expression of podocytopathic mediators in podocyte injury. Of note, GSK3β has been shown to be involved in kidney injury via regulating a number of other cellular events, such as mitochondrial permeability transition, cytoskeletal remodeling and cell proliferation. Therefore, inhibition of NFκB may be one of the multiple mechanisms accounting for the renoprotective effects of GSK3β blockade.

In summary, GSK3β fine tunes NFκB activation in podocytes by controlling RelA/p65 phosphorylation selectively at serine 467 and thereby directs the transcription of selective podocytopathic mediators, including MCP-1, cathepsin L and B7-1. Our findings suggest that the GSK3β dictated fine tuning of NFκB might serve as a novel therapeutic target for podocytopathy.
Methods

Cell culture and treatments
Conditionally immortalized mouse podocytes were provided by Dr. Shankland and cultured under permissive conditions as described previously.\(^5^8\) Podocytes differentiated under nonpermissive conditions and were pretreated with PDTC (2.5µM; Sigma, St. Louis, MO, USA), TPCK (1µM; Sigma), lithium chloride (10mM; Sigma) or different doses of TDZD-8 (1µM, 5µM; Sigma) for 20 minutes and then stimulated with LPS (20µg/ml, serotype: *E. coli* 0111:B4; Sigma) or ADR (Doxorubicin hydrochloride, 0.25 µg/ml, Sigma) for 24 h or indicated time.

Transient transfection and RNA interference
The eukaryotic expression vectors encoding the WT GSK3β or S9A or KD mutant of GSK3β were provided by Dr Johnson (Birmingham, AL) and those encoding the HA-hRelA/p65 wt and HA-hRelA/p65 S468A were used previously.\(^2^8\) The murine RelA/p65-specific siRNA duplex was provided by Santa Cruz Biotechnology (Santa Cruz, CA, USA) together with a scrambled siRNA control. Podocyte transfection was performed using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) as described before.\(^5^9, ^6^0\) In brief, podocytes were cultured under permissive conditions at 50% to 70% confluence in the absence of antibiotics. The DNA or siRNA oligomers-Lipofectamine 2000 complexes were prepared and applied to proliferating podocytes for transfection. The ratio of Lipofectamine 2000 to vectors or siRNA oligomers was optimized by a series of pilot experiments for each study until the best transfection efficiency or the best gene-silencing efficiency was achieved. After transfection, the cells were cultured under nonpermissive conditions in normal growth medium for 48 h before transfection efficiency or gene-silencing efficiency was assessed by immunocytochemistry staining or immunoblot analysis for target molecules. The cells were then subjected to LPS or vehicle treatments.

Cell Migration assay
Confluent monolayers of differentiated podocytes were scraped with a 10µl pipette followed by different treatment and visualized at 24 h using an inverted microscope. Phase contrast micrographs were obtained at 0 h and 24 h after scratching. Data were expressed as the percent area closure, which represents the percent difference in wound area after 24 hours and were analyzed using the ImageJ version 1.48 (NIH, Bethesda, MD) image processing program. The percentage of cell migration area was calculated as:

\[
\frac{(\text{Area}_{0\text{hour}} - \text{Area}_{24\text{hour}})}{\text{Area}_{0\text{hour}}} \times 100.
\]

ChIP assay and quantitative real time PCR
The *in situ* interaction between NFκB and promoter regions of its target genes in podocytes was examined by ChIP assay as described before by using a commercially available kit (Upstate Biotechnology, Charlottesville, VA, USA).\(^6^1\) The precipitated DNA was prepared for quantitative real time PCR as described before.\(^2^8\)
Animal studies

Animal studies were approved by the institution’s Animal Care and Use Committee and they conformed to the United States Department of Agriculture regulations and the National Institutes of Health guidelines for humane care and use of laboratory animals.

Murine models of LPS-induced proteinuria and podocytopathy—Male Balb/c mice aged 8 weeks were randomized to each of the following treatments. Lithium (40mg/Kg), TDZD-8 (5mg/Kg), TPCK (5mg/Kg) or saline (100µl) was given via i.p. injection 1 hour before i.p. injection of saline (100ul) or LPS (200µg; serotype: E. coli 0111:B4, Sigma). Mice were followed for 24 h before they were sacrificed and kidneys resected for further investigation. Urine was collected before and 24 h after LPS injection. Six mice were randomly assigned to each group.

Murine models of ADR-induced proteinuria and podocytopathy—Male BALB/c mice aged 8 weeks were randomly assigned to the following treatments. Lithium (40mg/Kg), TDZD-8 (5mg/Kg), TPCK (5mg/Kg) or saline was given via i.p. injection on day 0. ADR (10 mg/Kg) or an equal volume of vehicle was given as a tail vein injection 6 hour later. Spot urine was collected on post injury days 0, 4 and 7. Mice were sacrificed on day 7. Six mice were randomly assigned to each group for each observed time point. Urine albumin levels were measured using mouse albumin ELISA quantitation kit (Bethyl Laboratories, Montgomery, TX). Urine creatinine was determined by a creatinine assay kit (BioAssay Systems, Hayward, CA, USA).

Glomerular isolation

Glomerular isolation was carried out according to the method described preciously. In brief, mice were anesthetized and kidney perfused with 5ml of PBS containing \(8\times10^7\) Dynabeads M-450 (Dynal Biotech ASA, Oslo, Norway). Kidneys were then cut into 1mm\(^3\) pieces and digested in collagenase A. The tissue was then pressed gently through a cell strainer and glomeruli containing Dynabeads were gathered using a magnetic particle concentrator.

Western immunoblot analysis

Cultured cells were lysed and isolated renal glomeruli were homogenized in RIPA buffer supplemented with protease inhibitors. Nuclear fractions of cultured cells or isolated glomeruli were prepared by using the NE-PER kit (Thermo Scientific, Rockford, Illinois, USA). Samples were subjected to immunoblot analysis as previously described. The antibodies against GSK3\(\beta\), p-GSK3\(\beta\), synaptopodin, cathepsin L, GAPDH were purchased from Santa Cruz Biotechnology and those against p-RelA/p65 serine 467, p-RelA/p65 serine 536, p-RelA/p65 serine 276, cleaved caspase 3, \(\beta\)-catenin, histone H3 were purchased from Cell Signaling (Beverly, MA, USA). The antibodies against B7-1 and MCP-1 was purchased from R&D Systems (Minneapolis, Minnesota, USA).
Transmission electron microscopy

The kidney cortical specimens were cut into small pieces (1mm$^3$), fixed with 2.5% glutaraldehyde in 0.1M sodium cacodylate buffer, pH 7.4, postfixed with 1% (wt/vol) osmium tetroxide, and embedded in Epon 812 (Polysciences, Warrington, PA, USA). Transmission electron micrographs were obtained using a Zeiss EM-10 electron microscope operated at 60 kV with absolute magnifications of 1400 or 1800.

Immunofluorescent staining and TUNEL staining

Cultured cells or frozen cryostat sections were fixed and stained with primary antibodies and followed by applying the Alexa Fluor–conjugated secondary antibodies (Invitrogen). As a negative control, the primary antibodies were replaced by preimmune IgG from the same species; no staining occurred. Apoptotic cell death in cell cultures or kidney sections was detected by using the TUNEL kit (Roche Molecular Biochemicals, Mannheim, Germany) as described previously. Laser scanning confocal fluorescence microscopy was performed with a Zeiss LSM710 Meta confocal microscope (Carl Zeiss AG, Cologne, Germany).

Statistical analyses

All data are expressed as mean±s.d. Unless otherwise indicated, all experimental observations were repeated six times. Statistical analysis of the data from multiple groups was performed by analysis of variance followed by Student-Newman-Keuls tests. Data from two groups were compared by Student's-t test. $P<0.05$ was considered significant.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

ACKNOWLEDGEMENTS

This work was partially supported by the US National Institutes of Health grant R01DK092485, the Natural Science Foundation of China 81270136/H0111, and the International Society of Nephrology (ISN) Sister Renal Center Trio Program. The authors are indebted to Drs. Xu and Wang for their technical assistance. H.B. is an ISN Fellow and recipient of the ISN fellowship.

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Figure 1. LPS injury triggers NFκB activation, elicits GSK3β overactivity and induces injury in cultured murine podocytes

(A) Differentiated immortalized murine podocytes in culture were injured with varying doses of LPS (0, 1, 10, 20, 50 µg/ml) for 24 hours. Cell lysates were prepared for immunoblot analysis for indicated molecules, including TLR-4, phosphorylated GSK3β, phosphorylated RelA/p65 at S467, S536 and S276 residues, GAPDH and NFκB target molecules, such as B7-1, cathepsin L, and Bcl-xL; (B) Podocytes were treated with LPS (20 µg/ml) and harvested at indicated time. Cell lysates were processed for immunoblot analysis for indicated molecules; (C) Podocytes were treated with LPS (20 µg/ml) or equal volume of phosphate-buffered saline (PBS) for 24 h and fixed for fluorescent staining. Representative micrographs of laser scanning confocal fluorescence microscopy show nuclear translocation of NFκB RelA/p65, expression of B7-1 and synaptopodin (synpo), and phallolidin staining for F-actin in PBS or LPS treated podocytes. Bar=10µM. GAPDH, glyceraldehyde-3-
phosphate dehydrogenase; GSK3β, Glycogen synthase kinase 3β; LPS, lipopolysaccharides; PBS, phosphate-buffered saline; TLR-4, toll like receptor-4.
Differentiated immortalized murine podocytes in culture were pretreated with selective GSK3β inhibitors like lithium chloride (10mM) and TDZD-8 (5µM), or broad range inhibitors of NFκB, such as PDTC (2.5µM) and TPCK (1µM), for 20 minutes and then injured with LPS (20µg/ml), or an equal volume of phosphate-buffered saline (PBS). Scratch wound was made immediately after LPS or PBS treatment. (A) Phase contrast micrographs were taken immediately after wounding (0 h) and after migration for 24 h (Bar=40µM). Cell morphology at 24 h was taken under high-power fields. LPS injury resulted in marked podocyte shrinkage and this effect was abrogated by indicated treatments (Bar=10µM). (B) Quantification by computerized morphometric analysis of the cell migration area following the indicated treatments. #P<0.05 versus other groups (n=6). PDTC, Pyrrolidine dithiocarbamate; TDZD-8, 4-benzyl-2-methyl-1,2,4-thiadiazolidine-3,5-dione; TPCK, N-p-Tosyl-L-phenylalanine chloromethyl ketone.
Figure 3. Broad range inhibition of NFκB and inhibition of GSK3β exert distinct effects on NFκB activation and expression of NFκB target genes in LPS injured podocytes

(A) Differentiated immortalized murine podocytes were pretreated with PDTC (2.5µM) or TPCK (1µM), broad range inhibitors of NFκB, for 20 minutes and then stimulated with LPS (20µg/ml) for 24 h. Immunoblot analysis for indicated molecules were carried out on total cell lysates or conditioned medium. (B) Podocytes were pretreated with GSK3β inhibitor lithium chloride (10mM) or different doses of TDZD-8 (1µM, 5µM), a highly selective small molecule inhibitor of GSK3β, for 20 minutes and then injured by LPS (20µg/ml) for 24 hours. Total cell lysates and conditioned medium were prepared for
immunoblot analysis for indicated molecules. Nuclear protein fractions were prepared from cells and subjected to immunoblot analysis for nuclear β-catenin (n-β-catenin) or for nuclear protein histone (n-Histone H3), which served as loading controls. (C) Podocytes were pretreated with lithium chloride (10mM), TDZD-8 (5µM), PDTC (2.5µM), TPCK (1µM) or an equal volume of phosphate-buffered saline (PBS) for 20 minutes and then injured with LPS (20µg/ml) for 24 hours. Cells were fixed and subjected to TUNEL staining and immunofluorescence staining for indicated molecules. Representative micrographs of laser scanning confocal fluorescence microscopy indicate that both NFκB inhibition by PDTC or TPCK and GSK3β inhibition by lithium or TDZD-8 mitigated the LPS induced expression of B7-1 and cathepsin L and attenuated podocyte cytoskeleton disorganization. In contrast, broad range inhibition of NFκB by PDTC or TPCK in LPS injured podocytes blocked NFκB nuclear translocation and potentiated apoptosis, as measured by TUNEL staining and immunostating for cleaved caspase 3, whereas GSK3β inhibition by lithium and TDZD-8 barely affected NFκB nuclear translocation and did not potentiate apoptosis. Bar=10µM. PDTC, Pyrrolidine dithiocarbamate; TDZD-8, 4-benzyl-2-methyl-1,2,4-thiadiazolidine-3,5-dione; TPCK, N-p-Tosyl-L-phenylalanine chloromethyl ketone; TUNEL, Terminal deoxynucleotidyl transferase dUTP nick end labeling.
Figure 4. GSK3β fine tunes NFκB RelA/p65 phosphorylation at serine 467 and dictates the expression of selective NFκB target molecules involved in podocyte injury

Podocytes were subjected to liposome-mediated transient transfection with vectors encoding the hemagglutinin (HA)-conjugated wild type (WT), kinase dead (KD) mutant, or constitutively active (S9A) mutant of GSK3β. Cells were treated with LPS (20µg/ml) for 24 h after transfection. (A) Whole cell lysates and conditioned media were harvested and analyzed for indicated molecules by immunoblot analysis. Nuclear protein fractions were prepared from cells and subjected to immunoblot analysis for nuclear β-catenin (n-β-catenin) or for nuclear protein histone (n-Histone H3), which served as loading controls. (B)
Fluorescence immunocytochemistry staining of HA demonstrates that the transfection efficiency was >70%. Bar=10µM. (C) In silico analysis demonstrated that serine 467 of murine RelA/p65 resides in the consensus motifs for phosphorylation by GSK3β, suggesting RelA/p65 as a cognate substrate for GSK3β. (D–G) After transfection, cells were pretreated with lithium chloride (10mM), TDZD-8 (5µM) or an equal volume of vehicle for 20 minutes and then injured with LPS (20µg/ml) for 24 h. Bioinformatic analysis of mouse B7-1 (GenBank accession number: U33063.1), cathepsin L (Ctsl, GenBank accession number: L06427.1), MCP-1 (GenBank accession number: U12470.1) and Bcl-xL (GenBank accession number: U78030.1) gene indicated that multiple putative NFκB cis elements exit in their promoter regions. Chromatin immunoprecipitation (ChIP) assay was performed using anti-RelA/p65 antibody. The amount of B7-1, Cathepsin L (Ctsl), MCP-1 and Bcl-xL DNA that coprecipitated with transcription factor RelA/p65 was estimated by PCR followed by agarose gel electrophoresis and quantified by quantitative real time PCR, the results of which were expressed as fold induction over the value in EV transfected control cells. ns, not significant; #P<0.05 versus EV transfected control cells; *P<0.05, †P<0.01 versus EV transfected cells injured by LPS (n=3). ChIP assay revealed that ectopic expression of KD in podocytes abolished the recruitment of NFκB to B7-1, cathepsin L and MCP-1 genes in podocytes, mimicking the effect of lithium or TDZD-8. Conversely, GSK3β overactivity induced by forced expression of S9A promoted NFκB binding to B7-1, cathepsin L and MCP-1 genes in podocytes following LPS treatment. The LPS provoked NFκB interaction with Bcl-xL gene was barely affected by GSK3β activities. Bold letters indicate the NFκB cis elements. P1 and P2 indicate the primer pair encompassing the NFκB cis elements. EV, empty vector; HA, hemagglutinin.
Figure 5. The GSK3β target serine 467 in NFκB RelA/p65 is essential for podocyte expression of NFκB dependent podocytopathic mediators

Podocytes were transfected with scrambled (siControl) or murine RelA/p65-specific siRNA (siRelA) in the presence of empty vector (EV) or expression vectors encoding hemagglutinin (HA)-conjugated wild-type human RelA/p65 (HA-hRelA wt) or mutant human RelA/p65 (HA-hRelA S468A), in which the serine 468 (equivalent to serine 467 of mouse RelA by sequence homology) is replaced by alanine. Cells were treated with LPS (20µg/ml) for 24 h after transfection. Whole cell lysates and conditioned media were harvested 24 hours after LPS injury and analyzed for indicated molecules by immunoblot analysis. High efficiency of RelA/p65 silencing and ectopic expression was achieved as demonstrated by a drastic inhibition of native RelA/p65 expression and abundant expression of the HA-conjugated hRelA/p65 in cultured podocytes. RelA/p65 silencing abrogated the
LPS induced expression of all NFκB dependent molecules, including both podocytopathic mediators, like MCP-1, cathepsin L and B7-1, and prosurvival factor Bcl-xL, and potentiated podocyte apoptosis, measured by amplified expression of cleaved caspase 3. These effects were overridden by reconstitution of RelA/p65-silenced podocytes with wild-type human RelA/p65. In contrast, in RelA/p65-silenced podocytes reconstituted with mutant human RelA/p65, in which the serine 468 (equivalent to serine 467 of mouse RelA by sequence homology) is replaced by alanine, the LPS induced expression of Bcl-xL rather than the podocytopathic mediators was specifically reinstated, implying that serine 467 in NFκB RelA/p65 is essential for expression of NFκB dependent podocytopathic mediators.
Figure 6. GSK3\(\beta\) inhibition and broad range inhibition of NF\(\kappa\)B exert distinct effects on proteinuria and podocyte injury in LPS injured mice

Mice were pretreated with saline, TPCK (5mg/Kg, i.p.), lithium chloride (40mg/Kg, i.p.), or TDZD-8 (5mg/Kg, i.p.) and 1 hour later treated with saline or LPS (200µg, i.p.). Mice were followed for 24 h. (A) Urine was collected and processed for urine albumin ELISA assay adjusted for urine creatinine concentrations. Inhibition of GSK3\(\beta\) by lithium and TDZD-8 prevented the LPS induced albuminuria respectively by 67% and 61% at 24h. Broad range inhibition of NF\(\kappa\)B by TPCK prevented the LPS induced albuminuria by only 33%

#\(P<0.05\) versus other groups; *\(P<0.05\) versus Group LPS+TPCK (n=6). (B) Homogenates of isolated glomeruli were subjected to immunoblot analysis for synaptopodin. (C) Representative micrographs showing glomeruli isolated from kidneys by the magnetic beads based approach. Bar=100µm. (D) Electron microscopy of podocyte injuries in kidney glomeruli. LPS injury caused marked foot process effacement (black arrow heads) and microvillous transformation on electron microscopy. GSK3\(\beta\) inhibition by lithium or TDZD-8 significantly, while TPCK treatment slightly improved foot process effacement. Morphometric analysis of the number of foot processes per µm glomerular basement membrane (GBM) by electron microscopy (right panel). Bar=1µM; #\(P<0.05\) versus other
groups; *P<0.05 versus Group LPS+TPCK (n=6). (E) Representative micrographs of dual color fluorescence staining of kidney sections for TUNEL (green) and WT-1 (red). Absolute counting of the numbers of podocytes positive for both TUNEL and WT-1 (white arrow heads) as the means of 20 glomeruli (right panel). Bar=50µM; #P<0.05 versus other groups; *P<0.05 versus Group LPS+TPCK (n=6).
Figure 7. Fine tuning of NFκB activity by GSK3β in LPS induced experimental podocytopathy
Mice were treated as described in Figure 6. (A and B) Homogenates of glomeruli isolated from mouse kidneys were prepared and subjected to immunoblot analysis for indicated molecules. Nuclear protein fractions were prepared from isolated glomeruli and subjected to immunoblot analysis for nuclear β-catenin (n-β-catenin) or for nuclear protein histone (n-Histone H3), which served as loading controls. (C) Representative micrographs of laser scanning confocal microscopy of fluorescent immunohistochemistry staining demonstrate an enhanced glomerular expression of B7-1, MCP-1, and Bcl-xL in kidney specimens from
LPS injured mice. Note that the staining of B7-1, MCP-1 and Bcl-xL were largely detected in glomerular cells positive for podocyte specific markers, like synaptopodin and WT-1, suggesting a pattern of podocyte distribution. In the enlarged view of the boxed area, B7-1 was mainly found to colocalize with synaptopodin, MCP-1 mostly detected in cytoplasm of WT-1 positive glomerular cells, and Bcl-xL largely found to be external to synaptopodin, all indicative of a pattern of podocyte distribution. Lithium or TDZD-8 therapy attenuated the LPS induced glomerular expression of B7-1 and MCP-1, but barely affected the upregulated expression of Bcl-xL. TPCK treatment abolished the LPS elicited glomerular expression of all NFκB target molecules, including both podocytopathic ones, like MCP-1 and B7-1, and the prosurvival Bcl-xL. Bar=50µM.
Figure 8. Broad range inhibition of NFκB and GSK3β inhibition exert distinct effects on NFκB activation and the expression of NFκB target genes in adriamycin injured podocytes
Podocytes were pretreated with TPCK(1µM) or TDZD(5µM) for 20 minutes and then injured with adriamycin (ADR, 0.25µg/ml) or vehicle for 24 h. (A) Whole cell lysates and conditioned media were harvested 24 h after ADR injury and subjected to immunoblot analysis for indicated molecules (n=6). Nuclear protein fractions were prepared from cells and subjected to immunoblot analysis for nuclear β-catenin (n-β-catenin) or for nuclear protein histone (n-Histone H3), which served as loading controls. (B) Cells were fixed and processed for fluorescent immunocytochemistry staining and phalloidin or TUNEL staining. Representative micrographs of laser scanning confocal fluorescence microscopy indicate that both NFκB inhibition by TPCK and GSK3β inhibition by TDZD-8 mitigated the ADR induced expression of B7-1 and cathepsin L and attenuated actin cytoskeleton disorganization. However, TPCK blocked NFκB nuclear translocation and promoted apoptosis, whereas TDZD-8 treatment barely affected NFκB nuclear translocation and largely preserved the expression of prosurvival Bcl-xL, resulting in much less apoptosis as measured by TUNEL staining and caspase 3 cleavage. Bar=10µM. TDZD-8, 4-benzyl-2-methyl-1,2,4-thiadiazolidine-3,5-dione; TPCK, N-p-Tosyl-L-phenylalanine chloromethyl ketone.
Figure 9. Fine tuning of NFκB activity by GSK3β in experimental adriamycin nephropathy
Mice were injured with a single i.v. injection of adriamycin (ADR, 10mg/kg, i.v.) or equal volume of vehicle 6 hours after of i.p. pretreatment with TPCK (5mg/Kg), lithium chloride (40mg/Kg), TDZD-8 (5mg/Kg) or saline, and were followed for indicated days. (A) Urine was collected at the indicated time points and was subjected to SDS-PAGE followed by Coomassie brilliant blue staining. Bovine serum albumin (BSA, 40 µg) served as standard control. Urine samples (1.5 µL) collected on the indicated days from each group were loaded. (B) Quantification of urine albumin levels adjusted for urine creatinine.
concentrations. Inhibition of GSK3β by lithium and TDZD-8 prevented the ADR induced albuminuria respectively by 59% and 64% on day 4 and by 66% and 73% on day 7. Broad range inhibition of NFκB by TPCK prevented the LPS induced albuminuria by only 36% and 43% on day 4 and day 7. #P<0.05 versus other groups; *P<0.05 versus Group ADR +TPCK (n=6). (C) Electron microscopy of kidney specimens procured from animals on Day 7. Podocyte injury featured by extensive foot process effacement (black arrow head) was evident in ADR-injured kidneys. GSK3β inhibition by TDZD-8 markedly, while TPCK treatment minimally improved podocyte foot process effacement; Bar=1µM. (D) Absolute count of the number of foot processes per unit length of glomerular basement membrane (GBM) on electron micrographs of kidney specimens. #P<0.05 versus other groups; *P<0.05 versus Group ADR+TPCK (n=6). (E) Representative micrographs of dual color fluorescent staining of kidney sections for TUNEL (green) and WT-1 (red). Bar=50µM; F. Absolute count of the numbers of glomerular cells positive for both TUNEL and WT-1 (white arrow heads) as the means of 20 glomeruli. #P<0.05 versus other groups; *P<0.05 versus Group ADR+TPCK (n=6). (G) Glomeruli were isolated by the magnetic beads based approach from kidneys from differently treated animals and were homogenized for immunoblot analysis for indicated molecules. Nuclear protein fractions were prepared from cells and subjected to immunoblot analysis for nuclear β-catenin (n-β-catenin) or for nuclear protein histone (n-Histone H3), which served as loading controls. (H) Frozen sections of kidney tissues procured from animals on day 7 were subjected to dual color immunofluorescence staining for synaptopodin, WT-1, B7-1, MCP-1 and Bcl-xL. Representative micrographs of laser scanning confocal microscopy of fluorescent immunohistochemistry staining demonstrate an enhanced glomerular expression of B7-1, MCP-1, and Bcl-xL in kidney specimens from ADR injured mice. Bar=50µM.