Toll-like receptor 3 ligands induce CD80 expression in human podocytes via an NF-κB-dependent pathway

Michiko Shimada1,2, Takuji Ishimoto1, Pui Y. Lee3, Miguel A. Lanaspa1, Christopher J. Rivard1, Carlos A. Roncal-Jimenez1, David T. Wymer3, Hideaki Yamabe2, Peter W. Mathieson4, Moin A. Saleem4, Eduardo H. Garin5 and Richard J. Johnson1

1Division of Renal Diseases and Hypertension, University of Colorado Denver, Aurora, CO, USA, 2Division of Cardiology, Respiratory Medicine and Nephrology, Hirosaki University, Hirosaki, Japan, 3Department of Nephrology, Hypertension and Transplantation, University of Florida, Gainesville, FL, USA, 4Academic Renal Unit, University of Bristol, Bristol, UK and 5Division of Pediatric Nephrology, University of Florida, Gainesville, FL, USA

Correspondence and offprint requests to: Michiko Shimada; E-mail: mshimada@cc.hirosaki-u.ac.jp

Abstract
Background. Recent studies suggest that CD80 (also known as B7.1) is expressed on podocytes in minimal-change disease (MCD) and may have a role in mediating proteinuria. CD80 expression is known to be induced by Toll-like receptor (TLR) ligands in dendritic cells. We therefore evaluated the ability of TLR to induce CD80 in human cultured podocytes.

Methods. Conditionally immortalized human podocytes were evaluated for TLR expression. Based on high expression of TLR3, we evaluated the effect of polyinosinic-polycytidylic acid (polyIC), a TLR3 ligand, to induce CD80 expression in vitro.

Results. TLR1-6 and 9 messenger RNA (mRNA) were expressed in podocytes. Among TLR ligands 1–9, CD80 mRNA expression was significantly induced by polyIC and lipopolysaccharide (TLR4 ligand) with the greatest stimulation by polyIC (6.8 ± 0.7 times at 6 h, P < 0.001 versus control). PolyIC induced increased expression of Cathepsin L, decreased synaptopodin expression and resulted in actin reorganization which suggested a similar injury pattern as observed with lipopolysaccharide. PolyIC induced type I and type II interferon signaling, nuclear factor kappa B (NF-κB) activation and the induction of CD80 expression. Knockdown of CD80 protected against actin reorganization and reduced synaptopodin expression in response to polyIC. Dexamethasone, a corticosteroid commonly used to treat MCD, also blocked both basal and polyIC-stimulated CD80 expression, as did inhibition of NF-κB.

Conclusions. Activation of TLR3 on cultured human podocytes induces CD80 expression and phenotypic change via an NF-κB-dependent mechanism and is partially blocked by dexamethasone. These studies provide a mechanism by which viral infections may cause proteinuria.

Keywords: CD80; corticosteroids; minimal-change disease; Toll-like receptor

Introduction
Idiopathic minimal-change disease (MCD) is the most common nephrotic syndrome in children and adolescents. In 1974, Shalhoub [1] proposed that MCD is a T-cell disorder, based primarily on the lack of immune reactants in the glomeruli, the occasional association of the disease with Hodgkin’s lymphoma and the exquisite sensitivity to corticosteroids. Nevertheless, over the last 40 years, the specific etiology of MCD has remained elusive.

A potential clue for the etiology of MCD was provided by Reiser et al. [2] when they demonstrated that podocytes can express CD80 in various proteinuric conditions. CD80 is a costimulatory molecule required for T-cell activation and is primarily expressed by antigen-presenting cells (APC) as well as other cell types including podocytes and renal tubular cells.

The role of CD80 in nonimmune cells in the kidney is not well understood. An important observation was made by Reiser et al. [2] who showed that lipopolysaccharide (LPS), puromycin and some other stimuli could induce CD80 expression in cultured murine podocytes resulting in actin reorganization which suggested a similar injury pattern as observed with lipopolysaccharide. PolyIC induced type I and type II interferon signaling, nuclear factor kappa B (NF-κB) activation and the induction of CD80 expression. Knockdown of CD80 protected against actin reorganization and reduced synaptopodin expression in response to polyIC. Dexamethasone, a corticosteroid commonly used to treat MCD, also blocked both basal and polyIC-stimulated CD80 expression, as did inhibition of NF-κB.

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observation that LPS induces CD80 [2] raised the possibility that activation of Toll-like receptors (TLRs) could be involved since LPS binds TLR4. This is also consistent with the observation that MCD is commonly induced by viral infection.

We therefore hypothesized that other TLR ligands as well may similarly induce CD80 and podocyte phenotype change. Furthermore, since CD80 expression can be induced in podocytes independently of T cells [2], we determined if corticosteroids might act directly on the podocyte to inhibit CD80 expression in response to TLR ligands.

Methods

Cell culture

Immortalized human podocytes [5] were obtained from Dr Moin Saleem at the University of Bristol and were maintained in RPMI 1640 containing 10% fetal bovine serum (FBS), 1% insulin-transferrin-selenium-A supplement (Invitrogen, Carlsbad, CA), penicillin (100 U/mL) and streptomycin (100 μg/mL). Cells were grown at 33°C in 95% air, 5% CO2 and then converted to differentiated cells by incubating at 37°C for 10 days. Cells were kept in 1% FBS for 24 h and then treated with TLR1 ligand: Pam3CSK4 (0.5 μg/mL), TLR2 ligand: HKL1 (108 cells/mL), TLR3 ligand: polyIC (10–500 ng/mL), TLR4 ligand: LPS (20 μg/mL), TLR5 ligand: flagellin (10 μg/mL), TLR6 ligand: FSL1 (1 μg/mL), TLR7/8 ligand: R848 (5 μg/mL) and TLR9 ligand: CpG-DNA (10 μg/mL) (Invivogen, San Diego, CA) for designated hours. For the analysis of signaling pathways, podocytes were incubated with polyIC (500 ng/mL) with or without additional dexamethasone (10−7 to 10−8M), type 1 interferon (IFN) blocking peptide B18R (0.2 μg/mL) or nuclear factor kappa B (NF-kB) inhibitor pyrrolidine dithiocarbamate (PDTC) (20 μM). Cells were used between passages 13–25.

CD80 silenced cell lines by short hairpin RNA

Cultured podocytes were transfected using short hairpin RNA (shRNA) and Lipofectamine 2000 (Invitrogen) as described by the manufacturer. Lipid–DNA complexes are prepared as recommended protocols using growth media without serum. The Lipid–DNA complexes in solution were added to attached cells in low serum media without antibiotics and incubated overnight. Normal serum media with antibiotics were added 24 h posttransfection and media with puromycin was added after 48 h. Stable transfectants (clones) were picked using glass cloning cylinders and were subjected to a 10-fold dilution series, replated and a second colony selection performed to provide a clean clone for further analysis. Multiple clones were ultimately generated followed by validation of functional effectiveness of the specific vector.

Western blot analysis

Cell protein lysates were prepared from confluent cell cultures as described previously [6]. For nuclear and cytoplasmic extraction, NE-PER Nuclear and cytoplasmic extraction reagents (Thermo Scientific, Rockford, IL) were used according to the instruction. Sample protein content was determined by the BCA protein assay (Thermo Scientific). Fifty micrograms of total protein were loaded per lane for sodium dodecyl sulfate–polyacrylamide gel electrophoresis (10% w/vol) analysis and transferred to polyvinylidene difluoride membranes. Membranes were incubated with primary antibodies and visualized by using a horseradish peroxidase (HRP) secondary antibody (Cell Signaling, Danvers, MA) and the HRP Immunostar detection kit (Bio-Rad, Hercules, CA). Chemiluminescence was recorded with an Image Station 440CF, and results were analyzed with the 1D Image Software (Kodak Digital Science). Blots were also analyzed for glyceraldehyde 3-phosphate dehydrogenase (GAPDH), tubulin for cytosolic fraction and laminA/C for nuclear fraction as a loading control.

F-actin and p65 immunofluorescence

Podocytes were grown and differentiated on glass bottom culture dishes (MatTec, Ashland, MA). Cells were kept in 1% reduced serum for 24 h and stimulated with polyIC for designated hours. After stimulation, cells were fixed with 4% paraformaldehyde for 30 min, permeabilized with 0.1% Triton X-100 in phosphate-buffered saline (PBST) for 10 min and blocked with 5% milk in PBST (blocking buffer) for 1 h. For staining of p65, cells were incubated overnight with a rabbit polyclonal antibody (Cell Signaling), diluted 1:50 in blocking buffer and then incubated with an Alexa-Fluor 488 goat anti rabbit antibody for 2 h. For F-actin staining, the cells were incubated after fixation with an Alexa Fluor 488-conjugated phallolidin antibody (Molecular Probes, Carlsbad, CA) for 2 h. Samples were covered with antifading mounting medium with or without 4′,6-diamidino-2-phenylindole (Vector Laboratories, Burlingame, CA). Preparations were imaged with a 40× water-immersion objective by using a laser scanning confocal microscope (model LSM510; Zeiss).

RNA extraction, reverse transcription and quantitative reverse transcription–polymerase chain reaction

Total RNA was extracted using RNasy (Qiagen, Valencia, CA) and processed with DNasel (Qiagen). Each 1 μg of total RNA was reverse transcribed using iScript cDNA synthesis kit (Bio-Rad) according to the manufacturer’s protocol. Specific primers are shown in Table 1. Quantitative real-time polymerase chain reaction (PCR) was performed using i-Cycler (Bio-Rad). Each reaction was carried out in 20 μl total volume containing 10 μl of 2× IQ SYBR Green supermix (Bio-Rad), forward and backward primers at 500 nM and 2 μl of complementary DNA (cDNA). Amplification were performed for 1 cycle at 95°C for 3 min and 45 cycles at 95°C for 15 s and designated annealing temperature for 30 s and 72°C for 30 s. The results were normalized by GAPDH expression. As control, we used cDNA from nonstimulated human peripheral blood mononuclear cells (PBMC), also, we prepared cDNA from purchased total adult human kidney RNA (Bio-Rad). Quantitative reverse transcription–PCR for Cathepsin L was performed using an ABI PRISM 7700 Sequence Detector (PE Applied Biosystems, Foster City, CA) and the primers, Cathepsin L (Hs00377632_m1) and GAPDH, (Hs99999905_m1) were obtained from Applied Biosystems.

Statistical analysis

All data are presented as the mean ± S.D. Data graphics and statistical analyses were performed using Instat (version 3.0) and Prism 4 (both from GraphPad, San Diego, CA). Independent replicates for each data point (n) are identified in figure legends. P<0.05 was recognized as statistically significant.

Results

TLR expression in podocytes

Firstly, we determined if TLR1 through 10 messenger RNA (mRNA) transcripts were expressed in adult human kidneys and human podocytes using PBMC as a positive control. The values shown in Table 2 represent the relative expression compared to PBMC (setting the expression in PBMC = 100). All the values were normalized for GAPDH mRNA expression. Human kidney expressed TLR1-10 mRNA, whereas cultured podocytes expressed TLR1-6 and TLR-9 mRNA. The expression of TLR7, 8 and 10 in podocytes was minimal. TLR3 mRNA expression was the most prominently expressed in both the whole kidney and cultured podocytes. This pattern is similar to what we have also observed in isolated murine glomeruli (data not shown).

TLR 3 and 4 ligands induce CD80 expression in podocytes

To assess whether TLR signaling can activate CD80 expression, we stimulated podocytes with the ligands for TLR1 through 9 for 6 h. Among the various ligands studied, the TLR3 ligand polyIC and TLR4 ligand LPS significantly increased CD80 expression compared to...
CD80 is required for polyIC-induced podocyte injury

To further understand the role of CD80, stable CD80-silenced clones of podocytes were produced using the shRNA technique. Silencing was verified by reduced CD80 mRNA expression (Figure 3a). PolyIC treatment of CD80-silenced clones blocked the increase in CD80 protein expression (Figure 3b) and time dependently with the maximum expression after 24 h suggesting that polyIC-induced podocyte injury may involve a similar pathway as has been reported for LPS (Figure 2g) [7].

**Table 1.** Primers used for quantitative reverse transcription-PCR

<table>
<thead>
<tr>
<th>Gene type</th>
<th>Sequence</th>
<th>Size (bp)</th>
<th>Annealing temperature</th>
</tr>
</thead>
<tbody>
<tr>
<td>TLR1</td>
<td>5'-Forward ACCTCACACCTTGGACCTCCTGTC-3'</td>
<td>141</td>
<td>58</td>
</tr>
<tr>
<td></td>
<td>5'-Reverse AATGAGCAATGGGCACACACTAG-3'</td>
<td>77</td>
<td>59</td>
</tr>
<tr>
<td>TLR2</td>
<td>5'-Forward CACGGCTCTGGTGATGACATCC-3'</td>
<td>294</td>
<td>59</td>
</tr>
<tr>
<td></td>
<td>5'-Reverse TGGTCGAAGCTGGCCAAGAAG-3'</td>
<td>200</td>
<td>61</td>
</tr>
<tr>
<td>TLR3</td>
<td>5'-Forward CGTGCTATTGGCCACACACTTCC-3'</td>
<td>122</td>
<td>58</td>
</tr>
<tr>
<td>TLR4</td>
<td>5'-Forward TGGCCTTACTCTCAATGCTCCC-3'</td>
<td>135</td>
<td>57</td>
</tr>
<tr>
<td>TLR5</td>
<td>5'-Forward CTCCCTAATCTCTACATCTCC-3'</td>
<td>88</td>
<td>58</td>
</tr>
<tr>
<td>TLR6</td>
<td>5'-Forward AATTCCATGTCCTTCGAGCAAGAAG-3'</td>
<td>181</td>
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</tr>
<tr>
<td>TLR7</td>
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<td>108</td>
<td>58</td>
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<tr>
<td>TLR8</td>
<td>5'-Forward GCCACCCCAAATTGCAAGAAG-3'</td>
<td>78</td>
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<tr>
<td>TLR9</td>
<td>5'-Forward GAATCCAGTTCTCCGACGGAAATG-3'</td>
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<td>55</td>
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<tr>
<td>TLR10</td>
<td>5'-Forward CGGGGCTGATGAGGAGGAGGAG-3'</td>
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<tr>
<td>GAPDH</td>
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<td>54</td>
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<td>Mxi1</td>
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<tr>
<td>IFN-γ</td>
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<td>145</td>
<td>55</td>
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<tr>
<td>CXCL9</td>
<td>5'-Forward GAGATTCTCCCTGTGACCTGAGAAG-3'</td>
<td>79</td>
<td>53</td>
</tr>
<tr>
<td>Synaptopodin</td>
<td>5'-Reverse AATTCTGTTGAGTCTGAGAAG-3'</td>
<td>86</td>
<td>58</td>
</tr>
</tbody>
</table>

**Table 2.** TLR mRNA expression in human kidney and podocytes

<table>
<thead>
<tr>
<th>Gene type</th>
<th>PBMC</th>
<th>Total kidney</th>
<th>Podocyte</th>
</tr>
</thead>
<tbody>
<tr>
<td>TLR1</td>
<td>100 ± 6.5</td>
<td>15 ± 2.2</td>
<td>31 ± 6.8</td>
</tr>
<tr>
<td>TLR2</td>
<td>100 ± 12</td>
<td>10 ± 1.3</td>
<td>3.0 ± 1.1</td>
</tr>
<tr>
<td>TLR3</td>
<td>100 ± 12</td>
<td>2892 ± 320</td>
<td>2779 ± 383</td>
</tr>
<tr>
<td>TLR4</td>
<td>100 ± 7.1</td>
<td>60 ± 18</td>
<td>18 ± 11</td>
</tr>
<tr>
<td>TLR5</td>
<td>100 ± 19</td>
<td>139 ± 20</td>
<td>53 ± 25</td>
</tr>
<tr>
<td>TLR6</td>
<td>100 ± 11</td>
<td>5.3 ± 1.5</td>
<td>40 ± 22</td>
</tr>
<tr>
<td>TLR7</td>
<td>100 ± 17</td>
<td>6.4 ± 0.8</td>
<td>0.06 ± 0.06</td>
</tr>
<tr>
<td>TLR8</td>
<td>100 ± 9.6</td>
<td>1.3 ± 0.3</td>
<td>0.08 ± 0.1</td>
</tr>
<tr>
<td>TLR9</td>
<td>100 ± 35</td>
<td>29 ± 12</td>
<td>5.4 ± 3.5</td>
</tr>
<tr>
<td>TLR10</td>
<td>100 ± 23</td>
<td>8.1 ± 0.4</td>
<td>0.0 ± 0.06</td>
</tr>
</tbody>
</table>

*TLR1-10 mRNA expression normalized by GAPDH in adult human kidney and in podocytes using PBMC as positive controls. The values are expressed relative to PBMC, considering baseline expression in PBMC = 100.*

The effect of polyIC on podocytes

Since polyIC stimulation induced the highest levels of CD80 expression, we continued our studies utilizing polyIC as our stimulant for podocytes. TLR3 mRNA expression was significantly increased in podocytes after 6-h incubation with polyIC (500 ng/mL) (Figure 2a) suggesting polyIC act on podocytes via TLR. PolyIC also increased CD80 mRNA both dose dependently (Figure 2b) and time dependently with the maximum expression at 6 h (Figure 2c). CD80 protein expression in response to polyIC was confirmed by western blotting with densitometry (Figure 2d and e). PolyIC stimulation also resulted in a decrease in synaptopodin expression by quantitative PCR (data not shown) and western blotting (Figure 2d and f), suggesting podocyte injury. Additionally, polyIC increased Cathepsin L mRNA expression in podocytes after 24 h suggesting that polyIC-induced podocyte injury may involve a similar pathway as has been reported for LPS (Figure 2g) [7].
The effect of dexamethasone on CD80 expression and podocyte injury

Corticosteroids are commonly used to treat MCD [8], raising the question of whether corticosteroids might be able to interfere with TLR-stimulated CD80 expression. Incubation of human podocytes with dexamethasone suppressed both basal and polyIC-induced CD80 mRNA expression at 6 h (Figure 4a). Consistent with these findings, PolyIC-stimulated CD80 protein expression by western blot was suppressed by dexamethasone in a dose-dependent manner that was confirmed by densitometry (Figures 4b and c). Dexamethasone also increased synaptopodin expression in untreated podocytes and prevented the decrease of synaptopodin in response to polyIC (Figures 4b and d).

PolyIC induced type1 and type2 interferon

Activation of TLR3 is known to induce both type I and type II interferon-dependent signaling in dendritic cells [9–11]. Consistent with these findings, treatment of differentiated podocytes with polyIC (500 ng/mL) for 6 h resulted in activation of Type1 interferon signaling as shown by increased mRNA expression of the interferon-stimulated...
genes myxovirus resistance 1 (MX1; Figure 5a) and interferon-induced protein 44 (IFI44) (Figure 5b) [10, 12]. Type 2 IFN activation was also shown by increased expression of chemokine (C-X-C motif) ligand 9 (CXCL9) (Figure 5c) and IFN-γ (Figure 5d). However, blockade of type 1 viral interferon with the specific antagonist B18R (0.2 μg/mL) did not prevent the upregulation of CD80 (Figure 5e).
CD80 induction by polyIC was attenuated by NF-κB inhibition

Stimulation of TLR3 is also known to induce NF-κB activation [11]. To evaluate the activation of NF-κB, podocytes were stimulated with polyIC for 1 h and the translocation of the p65 subunit from the cytoplasm to the nucleus was demonstrated by immunofluorescence, documenting the activation of NF-κB (Figure 6a and b). Similarly, western blotting of cytosolic and nuclear extracts confirmed the translocation of p65 to the nucleus in polyIC-stimulated cells (Figure 6c and d). Furthermore, the NF-κB inhibitor PDTC significantly reduced the expression of CD80 following polyIC stimulation (Figure 6e), documenting that CD80 upregulation in podocytes is dependent on NF-κB activation.

PolyIC-induced actin reorganization in podocytes

Untreated differentiated podocytes demonstrate characteristic central stress fibers (Figure 7a). In contrast, polyIC stimulation for 20 h resulted in reorganization of the actin cytoskeleton with disruption of central stress fibers and increased F-actin at the cortical ring (Figure 7b). Addition of dexamethasone with polyIC partly inhibited the actin reorganization induced by polyIC (Figure 7c). Furthermore, CD80 silencing by shRNA largely prevented actin reorganization (Figure 7d and f), whereas this did not
Discussion

TLRs are expressed in various cell types, most notably in APC. TLRs sense the molecular patterns of microbial pathogens and play an important role in both the innate and adaptive immune responses [11]. When TLR ligands bind to the TLRs on APC, inflammatory responses are induced in association with increased expression of activated phenotype markers including CD80, CD86 and the major histocompatibility complex [11]. Emerging evidence suggests that TLRs have an important role in various kidney diseases [13–15]. Recent studies by Reiser et al. [2] suggest that TLR could have a role in inducing proteinuria in the LPS-injected mouse. This observation, coupled with our recent discovery that podocytes express CD80 in MCD [4], led us to evaluate the role of TLR in activating podocytes in vitro.

We found that TLR1-6 and -9 are expressed in human podocytes and that TLR3 expression was the highest relative to peripheral blood mononuclear cells and was also induced in response to polyIC (the TLR3 ligand). We also found a similar pattern of TLR3 expression in isolated podocytes following transfection with an irrelevant shRNA (Figure 7e).
murine glomeruli (data not shown), and this pattern is similar to that observed by Banas et al. [14]. Importantly, polyIC both dose- and time-dependently induced CD80 expression in podocytes in association with a decrease in synaptopodin and actin reorganization consistent with podocyte injury. Documentation that CD80 was critical for polyIC-mediated reduction in synaptopodin and actin reorganization was shown by the absence of polyIC effects in podocytes in which CD80 expression was knocked down with shRNA to CD80. Additionally, polyIC increased Cathepsin L mRNA expression in podocytes suggesting that polyIC induce podocyte injury via a mechanism similar to that reported with LPS [7]. New findings included observations that the signaling mechanism by which polyIC acts on podocytes involves both activation of type 1 and type 2 interferon pathways as well as NF-κB signaling. PolyIC could induce the translocation of p65 to the nucleus confirming NF-κB activation. PDTC (an NF-κB inhibitor) could partially ameliorate polyIC-induced CD80 expression.

Since NF-κB is a target for corticosteroid effects, we also tested the hypothesis that corticosteroids may act directly on the podocyte in this model system. Dexamethasone decreased both basal- and polyIC-stimulated CD80 expression in podocytes, in association with preservation of synaptopodin and actin organization. These studies suggest that corticosteroids, which are commonly used in MCD [8], may act in part via direct effects on the podocyte in addition to effects on T cells and is consistent with other studies showing that corticosteroids may have direct effects on this cell type [16].

In our study, we found a reduction in synaptopodin mRNA and protein in response to CD80 expression. Some authorities have reported that synaptopodin is not decreased in MCD but rather may be increased [17]. However, a study in childhood MCD did find low synaptopodin expression using quantitative immunostaining, with even lower levels present in focal and segmental glomerulosclerosis [18]. Importantly, the decrease in synaptopodin in our in vitro model can be viewed as a marker of podocyte injury in response to CD80 expression and may not have a pathogenic role in CD80-mediated proteinuria.

Our study found TLR3 to be expressed in podocytes. TLR3 expression has been observed in isolated murine glomeruli [14]. However, one report in human kidneys found TLR3 by immunostaining only in mesangial regions [19]. As immunostaining can be insensitive, it does not rule out the possibility of TLR3 being expressed by podocytes. Furthermore, the observation that TLR3 was increased following polyIC would be consistent with its expression being inducible under certain conditions. Importantly, our studies do not implicate TLR3 as the TLR involved in MCD but rather suggest a pathway by which TLR activation on podocytes may induce CD80 expression and phenotypic changes. It is possible that other TLR may be involved in the proteinuria of MCD. For example, Pawar et al. reported that injection of a TLR ligand for TLR2 could induce massive proteinuria in mice with lupus nephritis but not control mice [20]. It is also possible that under some conditions, different TLR may be upregulated and could mediate the increased glomerular permeability to proteins.

While we document that TLR binding can induce CD80, the CD80 expression induced by TLR3 was transient. We have hypothesized that a ‘second hit’ is required to maintain the expression of CD80 [21]. One possibility is by continued stimulation by the Th2 cytokine IL-13. IL-13 is overexpressed by T cells in MCD [22]. IL-13 overexpression can also induce an MCD-like nephrotic syndrome in rats [23]. Thus, our studies do not negate the potential
involvement of T cells in this disorder. Furthermore, there is increasing evidence that T-regulatory cells are defective in MCD [24], thus allowing continuous T-cell activation and stimulation that could maintain CD80 expression.

In conclusion, we demonstrate that activation of viral-associated TLR in podocytes can induce CD80 expression and podocyte shape change via an NF-xB-dependent pathway. Corticosteroids were able to block the upregulation of CD80. These studies may provide insight into the pathogenesis of MCD and provide new avenues for research.

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Conflict of interest statement. None declared.

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