Nuclear Factor Erythroid 2-Related Factor 2 Drives Podocyte-Specific Expression of Peroxisome Proliferator-Activated Receptor γ Essential for Resistance to Crescentic GN

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ABSTRACT
Necrotizing and crescentic rapidly progressive GN (RPGN) is a life-threatening syndrome characterized by a rapid loss of renal function. Evidence suggests that podocyte expression of the transcription factor peroxisome proliferator-activated receptor γ (PPARγ) may prevent podocyte injury, but the function of glomerular PPARγ in acute, severe inflammatory GN is unknown. Here, we observed marked loss of PPARγ abundance and transcriptional activity in glomerular podocytes in experimental RPGN. Blunted expression of PPARγ in podocyte nuclei was also found in kidneys from patients diagnosed with crescentic GN. Podocyte-specific Pparγ gene targeting accentuated glomerular damage, with increased urinary loss of albumin and severe kidney failure. Furthermore, a PPARγ gain-of-function approach achieved by systemic administration of thiazolidinedione (TZD) failed to prevent severe RPGN in mice with podocyte-specific Pparγ gene deficiency. In nuclear factor erythroid 2-related factor 2 (NRF2)–deficient mice, loss of podocyte PPARγ was observed at baseline. NRF2 deficiency markedly aggravated the course of RPGN, an effect that was partially prevented by TZD administration. Furthermore, delayed administration of TZD, initiated after the onset of RPGN, still alleviated the severity of experimental RPGN. These findings establish a requirement for the NRF2–PPARγ cascade in podocytes, and we suggest that these transcription factors have a role in augmenting the tolerance of glomeruli to severe immune-complex mediated injury. The NRF2–PPARγ pathway may be a therapeutic target for RPGN.


Necrotizing and crescentic rapidly progressive glomerulonephritis (RPGN) is a heterogeneous condition characterized by rapidly declining kidney function. Left untreated, patients with RPGN often require long-term RRT.

Characteristic histologic features of RPGN include an irreversible loss of podocyte quiescence, aggravated endothelial injury, and the development of cellular crescents, all of which eventually lead to glomerular obsolescence. During crescent formation in mouse

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models of RPGN, podocytes assume a migratory phenotype, attaching to the parietal basement membrane where they proliferate for a limited period of time.\textsuperscript{1–3} Recent data have confirmed that podocytes also contribute to crescent formation in man,\textsuperscript{4,5} although there is a great deal of heterogeneity in those affected, suggesting that in some cases local regulatory mechanisms fail to maintain a quiescent podocyte phenotype. Hence, modulation of podocyte phenotype may help the glomerulus to withstand inflammatory stress and to prevent, or arrest, the destructive process of crescent formation.

Peroxisome proliferator-activated receptor \(\gamma\) (PPAR\(\gamma\)) belongs to a group of nuclear receptors whose endogenous ligands include free fatty acids and eicosanoids.\textsuperscript{6,7} Podocytes constitutively express \(Ppar\gamma\) mRNA \textit{in vitro} and this is reduced in models of glomerular inflammation.\textsuperscript{8} Furthermore, pioglitazone, a clinically used thiazolidinedione (TZD) and pharmacological agonist of PPAR\(\gamma\), increases both \(Ppar\gamma\) mRNA and activity in cultured podocytes.\textsuperscript{8} Systemic PPAR\(\gamma\) stimulation is also effective in preventing podocyte injury in models of glomerular inflammation\textsuperscript{9} and rat models of diabetic nephropathy.\textsuperscript{10–14} Interestingly, in patients with diabetic nephropathy, TZDs have antiproteinuric effects.\textsuperscript{15,16} There are probably multiple mechanisms explaining the renoprotection conferred by TZDs, including a reduction in inflammation.

Figure 1. Glomerular PPAR\(\gamma\) expression is decreased in NTN. (A) Western blot analysis of PPAR\(\gamma\) expression in glomeruli isolated from NTS-challenged mice (NTS) and non-injected mice (control). Tubulin is used as loading control. (B) Quantification of Western blot bands for PPAR\(\gamma\) normalized to tubulin band intensity (means of six mice per group, of two independent experiments). (C) PPAR\(\gamma\) activity was determined by RT-PCR analysis of the relative abundance of Cd36 as PPAR\(\gamma\) target gene. Relative Cd36 mRNA expression in glomerular extracts from control or NTS-challenged mice (means of five mice per group, of two independent experiments). (D) Representative images of PPAR\(\gamma\) expression (red) and podocalyxin (green) by immunofluorescence on kidney sections from control mice (control) or NTS-challenged mice (NTS) 10 days after the first injection of nephrotoxic serum. Images are representative of at least six mice per condition. Scale bar, 50 \(\mu\)m. **\(P<0.01\) versus control mice.
and fibrosis, suppression of the reninangiotensin system and antiapoptotic effects. It is also probable that these effects are mediated through a number of cell types including resident glomerular cells and immune cells.

To date, there has been no study of the glomerular PPARγ pathway in acute, severe inflammatory GN. This is likely due to the major focus being on suppression of those aspects of the immune system mediating injury rather than those promoting tissue tolerance to injury. Accordingly, most current therapeutic approaches to RPGN target the immune system. The current study aimed to evaluate the proof of principle that TZD administration could treat potentially lethal experimental RPGN.

RESULTS

Glomerular PPARγ Expression Is Decreased in Nephrotoxic Nephritis

In mice exposed to nephrotoxic nephritis (NTN) immunoblot analysis showed a 65% decrease in PPARγ glomerular expression in nephritic mice at day 10 compared with baseline (Figure 1A and B). Cd36 mRNA expression—a measure of PPARγ transcriptional activity was consistently reduced in glomerular extracts from NTN mice compared with controls, suggesting blunted PPARγ transcriptional activity in this model (Figure 1C). Interestingly, the loss of most glomerular PPARγ immunofluorescence in nephritic glomeruli was found in podocytes (Figure 1D).

Podocyte-Specific Deletion of PPARγ Does Not Modify Kidney Structure and Function

To determine the role of the podocyte PPARγ pathway in NTN, we generated mice with a podocyte-specific deletion of Pparγ. Confirmation of the deletion was shown by RT-PCR with a significant reduction in Pparγ mRNA level in isolated podocytes of podocin−Cre PPARγ lox/lox (Pod−PPARγ lox) mice compared with control animals (Pod−PPARγ wild-type [WT]) (Figure 2A). The purity of the primary podocyte culture was validated by nephrin and podocin immunostaining as previously described (data not shown). Similarly, double immunofluorescence staining revealed a marked decrease in PPARγ expression in glomeruli from Pod−PPARγ lox mice (Figure 2B). Adult Pod−PPARγ lox mice showed no abnormalities in glomerular morphology, urinary albumin excretion or renal function as estimated by BUN levels (Figure 2C–E).
affect systemic mouse anti-sheep IgG humoral response (Figure 4D).

Effects of Podocyte PPARγ Pathway Inhibition on Kidney Inflammation
Nephritic mice exhibited an accumulation of inflammatory cells (F4/80 and CD3 positive) in the renal interstitium and around glomeruli. There was also a significant upregulation of monocyte chemoattractant protein 1 (Mcp1) and IL-6 (Il6) mRNA in the glomeruli of these mice. Surprisingly, podocyte-specific PPARγ deletion alone induced a significant increase in infiltrating cells and Mcp1 and Il6 mRNA in the renal cortex during RPGN (Figure 4E–I).

Podocyte-Specific Deletion of PPARγ or Rosiglitazone Treatment Does Not Modify Proliferative and Migratory Podocytes In Vitro
To determine the role of the podocyte PPARγ we performed primary cultures of podocytes to assess their proliferation and migration, both hallmarks of podocyte dedifferentiation and crescent formation. Podocyte outgrowth area and K67 mRNA expression—as a measure of cell proliferation—were no different in glomeruli isolated from Pod-PPARγ lox mice, those taken from Pod-PPARγ WT mice and those from Pod-PPARγ WT and treated with rosiglitazone in vitro (Figure 4A–C). Moreover, neither PPARγ activation nor podocyte-specific deletion of PPARγ had any effect on podocyte motility (Figure 5D and E).

Podocyte-Specific Pparγ Deletion Mice Are More Susceptible to Doxorubicin Nephropathy
Although PPARγ did not modulate podocyte proliferation or migration, we hypothesized that this pathway may prevent podocyte death. To this end, we challenged Pod-PPARγ lox and Pod-PPARγ WT mice with doxorubicin (DXR). DXR nephropathy is a model of toxic podocyte injury and death and is characterized by proteinuria and glomerular scarring. Baseline proteinuria did not differ between Pod-PPARγ lox and Pod-PPARγ WT mice. However, the DXR-induced proteinuria was significantly increased in the Pod-PPARγ lox mice compared with controls (Figure 6A). Similarly, Pod-PPARγ lox displayed more glomerular podocyte–parietal epithelial cell bridges and FSGS than controls (Figure 6B and C).

Decrease in PPARγ Activity in Nuclear Factor Erythroid 2-Related Factor 2–Deficient Podocytes
The nuclear factor erythroid 2-related factor 2 (NRF2 or NFE2L2) plays a vital role in cytoprotection against oxidative and electrophilic stress as well as in suppression of inflammation.24 Nyr2−/− (NRF2 knockout [KO]) mice have reduced PPARγ expression.25–27 Hence, we assessed PPARγ expression and activity in glomeruli and podocytes from NRF2 KO mice. First, young adult NRF2 KO mice displayed no abnormalities in kidney structure (including glomerular ultrastructure) and function (Figure 7A–D). However, Cd36 mRNA and PPARγ protein (Figure 7E and F) were reduced in glomerular extracts from NRF2 KO mice, suggesting reduced PPARγ transcriptional activity compared with NRF2 WT glomeruli.
Figure 4. Podocyte-specific deletion of PPARγ accentuates inflammatory cells infiltration in NTN. (A) Representative pictures showing immunostaining for sheep IgG and mouse IgG in renal cortex from Pod-PPARγ WT and Pod-PPARγ lox mice after NTS injection on day 10 and from untreated normal Pod-PPARγ WT mice (control) Scale bar, 20 μm. (B, C) Quantitative image analysis of immunofluorescent staining for glomerular mouse IgG (B) and sheep IgG deposition (C) 10 days after NTS injection. (D) Titers of mouse IgG to sheep IgG measured in serial dilutions of plasma from Pod-PPARγ WT or Pod-PPARγ lox mice immunized with sheep NTS and from
Pod-PPARγ lox and Pod-PPARγ WT mice. Podocyte-specific PPARγ deletion was associated with reduced NRF2 expression and activity (as reflected by NAD(P)H quinone oxidoreductase 1 [Nqo1] and glutathione S-transferase [Gstm1] mRNA levels) compared with controls (Figure 8A and B). In addition, Pparγ gene deficiency decreased NRF2 nuclear translocation, as shown by decreased NRF2 fluorescence and nuclear accumulation in primary cultures of podocytes (Figure 8C).

Overall, these data suggest that the PPARγ pathway is altered in glomeruli from NRF2 KO mice and vice versa.

**NRF2 Deficiency Aggravates RPGN in Mice in a PPARγ-Dependent Fashion**

Given the reciprocal regulation of the NRF2 and PPARγ pathways in podocytes, and based on our earlier experiments, we exposed NRF2-deficient mice to NTS, hypothesizing that they would demonstrate a similar kidney phenotype to Pod-PPARγ lox mice.

As expected, NRF2 WT mice exhibited crescent formation and renal dysfunction. Interestingly, NRF2 KO mice developed more aggressive GN compared with controls as shown by a two-fold higher incidence of crescent formation (49.9±4.0% versus 23.9±11.7%, *P*<0.001) (Figure 9A), and nearly three times as many glomeruli with necrotizing lesions (30.5±2.6% versus 12.6±1.7%, *P*<0.001) than NRF2 WT animals. These histologic differences were associated with more severe functional renal impairment in NRF2 KO mice than in control animals (Figure 9B and C). NRF2 KO mice also showed extensive podocyte foot process fusion and glomerular basement membrane (GBM) thickening (Figure 9D). These data suggest that NRF2 deficiency markedly aggravates experimental nephritis and that NRF2 KO mice have a similar renal phenotype to Pod-PPARγ lox mice in this model.

Of note, NRF2 KO animals showed a similar humoral immune response to sheep NTS as did NRF2 WT mice, as demonstrated by measurements of mouse and sheep IgG in non-immunized control mice (means of five mice per group, of two independent experiments). (E) Immunostaining for CD3" and F4/80" cells (scale bar, 20 μm) and (F, G) quantification by image analysis of CD3" and F4/80" infiltrates in renal cortex from nonimmunized controls and NTS-injected Pod-PPARγ WT or Pod-PPARγ lox mice at day 10 after NTS injection (means of eight mice per group, of two independent experiments). (H and I) mRNA expression of Mcp1 and Il6 was determined by RT-PCR analysis in renal cortex tissue from groups of mice as in (A) (means of eight mice per group, of two independent experiments). *P*<0.05; **P*<0.01; ***P*<0.005 versus control mice.

Notably, constitutive nuclear NRF2 expression was observed in podocytes from normal NRF2 WT mice at baseline (Figure 7G).

**The NRF2 Pathway Is Decreased in PPARγ-Deficient Podocytes**

To analyze the cross-talk between NRF2 and PPARγ, we investigated NRF2 abundance and activity in podocytes from experimental nephritis and that NRF2 KO mice have a similar kidney phenotype to Pod-PPARγ lox mice.

Figures 5. Loss-of-function approach by podocyte-specific deletion of Pparγ or gain-of-function with rosiglitazone treatment do not modify podocyte proliferation and migration in vitro. (A, B) Representative pictures and quantification of podocyte proliferation involving decapsulated glomeruli from Pod-PPARγ lox mice or podocytes treated with rosiglitazone (means of 11 mice per group, of three independent experiments). Podocyte outgrowth area was assessed after 4 days, Scale bars, 20 μm. (C) RT-PCR analysis of the relative abundance of ki67 in primary podocyte cultures treated either with or without rosiglitazone (10 μM) for 16 hours, or from Pod-PPARγ lox mice (means of five mice per group, of two independent experiments). (D, E) Representative images and quantification of wound assay showing the migration of podocytes incubated either with or without rosiglitazone for 12 hours, or from Pod-PPARγ lox mice (means of 12 mice per group, of three independent experiments). Migration was assessed over a period of 12 hours. Scale bars, 100 μm.


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kidney cortex (Figure 9E–G) as well as assessment of mouse anti-sheep IgG circulating IgG titers (Figure 9H).

PPARY Gain of Function Approach with Pioglitazone Improves Glomerular Structure and Function in Nephrotoxic Nephritis in NRF2-Competent and NRF2-Deficient Mice

To establish the extent to which the increased severity of NTN caused by NRF2 deficiency could be linked to altered PPARγ activity, we administered pioglitazone to NTS-treated NRF2 KO mice and WT controls. Pioglitazone administration reduced glomerular injury (Figure 10A and B), proteinuria (not shown), and BUN (Figure 10C).

Pioglitazone Anti-Nephritis Effects Are Blunted in Mice with Podocyte-Selective Deletion of PPARγ

To determine the extent to which pioglitazone prevented glomerular damage through stimulation of PPARγ on podocytes we treated nephritic Pod-PPARγlox mice with either vehicle or pioglitazone. Pioglitazone administration in Pod-PPARγ WT mice significantly prevented the histologic damage characteristic of NTN (Figure 11A and B) and functional glomerular impairment (Figures 11C and D). By contrast, pioglitazone administration proved less effective in Pod-PPARγlox mice as demonstrated by a non-significant alleviation of glomerular damage, albuminuria and BUN (Figure 11A–D), although there was a trend for proteinuria to be lower (Figure 11C). Altogether, these data suggest that pioglitazone administration has limited efficacy in protecting from RPGN in the absence of a functional PPARγ system in podocytes.

PPARY Gain of Function Approach with Pioglitazone Treatment Improves Glomerular Structure and Function in NTN

Our data so far suggest that NTN is associated with reduced PPARγ expression and activity and that this may be pathogenic in disease progression. Hence, we went on to test whether pharmacological stimulation of the PPARγ pathway improved renal injury. We administered a clinically available TZD, pioglitazone, on day 4 after infusion of NTS. This time-point was chosen as it is clinically relevant, associated with peaks in both albuminuria and serum creatinine. This regimen was compared with the effects of vehicle alone and with the administration of pioglitazone 6 hours before the first infusion of NTS.

Ten days after NTS injection, these mice developed significant ascites, hypoalbuminemia, and albuminuria characteristic of the nephrotic syndrome. Pioglitazone administration, given before and after NTS, was associated with a lower incidence and severity of ascites and less albuminuria than in vehicle-treated NTN mice (Figure 12A and B). Furthermore, whereas vehicle only-treated mice (NTN) developed rapid and
life-threatening renal failure, mice treated with pioglitazone, before and after NTS, had BUN levels within the normal range (Figure 12B). The functional protection conferred by pioglitazone administration was associated with marked alleviation of histologic damage as measured using Masson-trichrome staining of renal cortex (Figure 12C and D).

We sought to determine if the kidneys from the Pod-PPARγ WT and Pod-PPARγ lox mice showed a similar humoral response and glomerular staining pattern to NTS treatment. Ten days after the induction of NTN, sera from the Pod-PPARγ WT and Pod-PPARγ lox mice displayed similar titers of mouse IgG to anti-sheep IgG (Figure 12E) and the kidneys from both groups showed similar amounts of mouse anti-sheep IgG antibodies in their glomeruli (Figure 12F–H). Additionally, the anti-GBM antibodies were observed to bind exclusively to the glomerular capillary wall in a linear pattern (Figure 12F). Finally, pioglitazone treatment significantly reduced both the number of infiltrating F4/80 and CD3 positive cells around glomeruli (Figure 13A–C) as well as Mcp1 and IL6 mRNA expression (Figure 13D and E).

Figure 7. PPARγ abundance and activity is decreased in NRF2 KO podocytes in vitro and in vivo. (A) Representative transmission electron micrographs of glomeruli from 10-week-old NRF2 WT and NRF2 KO male mice at baseline. (B) Masson trichrome-stained kidney sections of NRF2 WT and NRF2 KO 10-week-old mice at baseline. Scale bars, 20 μm. (C, D) Urinary albumin excretion rate (C) and BUN concentration (D) at baseline of NRF2 WT and NRF2 KO 10-week-old male mice. (E) PPARγ activity determined by RT-PCR analysis of the relative abundance of Cd36 mRNA in primary podocyte cultures from NRF2 WT or NRF2 KO mice. (F) PPARγ protein expression in primary podocyte cultures (F) from NRF2 WT or NRF2 KO mice. (G) Representative image of NRF2 (red) and podocalyxin (green) expression by immunofluorescence on kidney sections from NRF2 WT and NRF2 KO mice at baseline. Scale bars, 20 μm. Values are means of six mice per group, of two independent experiments. *P<0.05; **P<0.01; ***P<0.001 versus NRF2 WT mice.

Loss of PPARγ in Podocytes and Crescents in Patients Diagnosed for RPGN

As in mice, constitutive PPARγ nuclear expression was found in podocytes from normal human glomeruli (Figure 14A). By
contrast, immunoreactive PPARγ podocyte expression was widely blunted in kidney biopsies from individuals diagnosed with crescentic RPGN (Figure 14B–D). In these patients, nuclear PPARγ is seen in glomerular endocapillary cells as well as in an extracapillary pattern in glomerular epithelial cells: podocytes and parietal epithelial cells. Importantly, in cells forming the crescent, nuclear PPARγ expression is abolished in favor of cytoplasmic expression implying fewer PPARγ in cells forming the crescent.

**DISCUSSION**

In the current study we have shown for the first time that, in a model of NTS-induced RPGN, mice with a podocyte-specific deletion of Pparγ are more susceptible to crescent formation and renal failure than controls. These novel data support PPARγ activation in podocytes as a potentially disease-limiting pathway in a severe form of extracapillary GN. Mirroring this loss of function approach, a gain of function approach—by means of PPARγ receptor activation—conferred similarly significant histopathological and functional glomerular protection (despite an untouched anti-GBM humoral response). Importantly, from a clinical perspective, delayed administration (“therapeutic dosing”) of a PPARγ receptor activator was as effective as the preventive dosing strategy.

There have been a number of studies that have investigated the PPARγ system in relation to the kidney. One of the first of these from more than 10 years ago showed that preventive administration of a TZD reduced proteinuria and crescent formation in a model of anti-GBM disease in rats. Another,
Figure 9. NRF2-deficient mice develop more severe glomerulonephritis than normal littermates although with similar anti-sheep IgG humoral response. (A) Representative pictures of Masson trichrome-stained kidney sections—crescent outlined in yellow. Scale bars, 20 μm (B) Albuminuria and (C) BUN concentrations in NTS-challenged NRF2 KO and NRF2 WT mice (means of 15–17 mice per group, of four independent experiments). (D) Representative transmission electron micrographs of glomeruli from NRF2 WT mice at day 10 after NTS injection. Nephritic NRF2 KO mice display glomerular capillary endotheliosis and more widespread podocyte foot process.
Although it is considered that PPARγ agonism primarily influences the natural history of disease through anti-inflammatory actions on endothelial and myeloid cells,33–35 our data suggest an alternative pathway that focuses on the podocyte. Podocyte-specific abrogation of PPARγ exacerbated RPGN-related renal injury, illustrating the crucial local homeostatic role for this transcription factor. In this model, humoral and cellular immune responses were not targeted. Notably, although PPARγ may control cell proliferation and apoptosis, migration and invasion in cells other than podocytes,36–38 PPARγ deficiency did not directly influence podocyte proliferation and migration in vitro but significantly accentuated periglomerular infiltrates of T cells and macrophages as well as MCP1 and IL6 mRNA abundance in the cortex of nephritic mice. These two cytokines were used as measures of renal inflammation as they are involved in several diseases where crescentic change is seen.39–49 It is somewhat surprising that the deletion of a single pathway (PPARγ) in a single cell type (the podocyte) could promote such an impressive increase (approximately 30%) in both monocyte chemoattractant protein 1 and IL-6 mRNA levels, as well as adding to severe local damage, but this goes to highlight the importance of the podocyte PPARγ system in this disease process. We cannot exclude PPARγ-dependent paracrine effects of the diseased podocytes on surrounding inflammatory cells such as limitation of endogenous danger signals released by injured podocytes; a question that will require further studies beyond the scope of the present work.

To investigate whether an intact PPARγ pathway is needed for podocyte survival, we induced the DXR model of FSGS in mice lacking a functional podocyte PPARγ pathway. Evidence from animal models and in vitro studies suggests that injury inherent within or directed to the podocyte is a central pathogenic factor to FSGS.50 Again, Pparγ gene deletion in podocytes alone was sufficient to significantly accentuate albuminuria and sclerosis. These data suggest that PPARγ may critically sustain podocyte survival and function upon inflammatory (RPGN) or toxic (FSGS) injury.

which deleted PPARγ in the hematopoietic compartment (leaving the renal PPARγ system intact) showed these animals to have moderately increased susceptibility to experimental NTN in terms of histologic damage although there was no impact on proteinuria and no detail was provided about kidney function.29 Although evidence has since accumulated that PPARγ may influence podocyte survival in vitro and in vivo,8,9,12,30 its activity was also expected to exert anti-angiogenic effects.31,32 a potentially major detrimental effect in the context of RPGN, which is characterized by marked endothelial cell injury. Hence, no further investigation of this major pathway has been conducted in this form of extracapillary glomerulonephritis.

effacement than their NRF2 WT counterparts. **P<0.01; ***P<0.001 versus NTS-injected NRF2 WT mice. (E) Representative photomicrographs after immunofluorescent staining for mouse IgG and sheep IgG in renal cortex from nonimmunized (control) mice and from NTS-injected NRF2 WT and NRF2 KO mice. (F, G) Quantitative image analysis of immunofluorescence staining for glomerular mouse IgG (F) and sheep IgG deposition (G) 10 days after nephrotoxic serum injection. (H) Titers of mouse IgG to sheep IgG measured in plasma from controls and NTS-injected NRF2 WT or NRF2 KO (means of five mice per group, of two independent experiments).
To address the question as to whether the beneficial effects of TZD administration in NTN are mediated in part through actions on the podocyte we treated animals with podocyte-speciﬁc PPARγ deﬁciency with a regimen that was effective in WT animals. Surprisingly, TZD treatment was signiﬁcantly less beneﬁcial in Pod-PPARγ lox mice compared with controls, suggesting that a signiﬁcant part of the beneﬁcial action of TZD administration is mediated through the podocyte PPARγ pathway (as well as complementary anti-inﬂammatory actions on immune cells).28,29 These ﬁndings highlight the important homeostatic mechanisms of podocytes in managing inﬂammatory stress.

Our study also investigated the cross-talk between the podocyte NRF2 and PPARγ pathways in vitro and in vivo. Whereas the mechanism whereby NRF2 induces PPARγ activity is known,26,27 the converse pathway remains elusive. The current data suggest that in the context of severe immune complex-mediated podocyte injury, NRF2-driven PPARγ induction has an essential protective role in glomerular oxidant injury as TZD administration could overcome part of the deleterious effect of complete NRF2 deﬁciency.

In summary, our study demonstrates the pivotal role of the local PPARγ system in maintaining podocyte quiescence and orchestrating the global glomerular tolerance to a severe immune complex-mediated disease. PPARγ was found to be a downstream effector of the NRF2 pathway, unveiling the critical protective role of both NRF2 activity and PPARγ. We also provide proof of principle that delayed PPARγ agonism could display therapeutic actions on glomerular function and structure in a severe model of RPGN.

CONCISE METHODS

Animals

We generated mice with a podocyte-speciﬁc deletion of Pparγ by using the Nphs2-Cre recombinase or podocin-Cre mouse (Pod-Cre), which expresses Cre-recombinase exclusively in podocytes starting from the capillary loop stage during glomerular development.20 Podocyte-speciﬁc disruption of Pparγ alleles was generated by crossing podocin-Cre-positive mice with the B6.129S6-Pparγtm1.1Mgn/Mmeh strain obtained from the Mutant Mouse Regional Resource Center at the University of Missouri (stock number 012035-MU) (herein named PPARγ lox mice).31 on a C57BL6/J background. Their littermates with no deletion of Pparγ alleles in any cells are considered as controls. Direct comparison was made between littermates’ age-matched controls.
Figure 12. Pioglitazone treatment improves glomerular structure and function in NTN. (A) Urinary albumin excretion rates in noninjected mice (control) and at day 4 and 10 after NTS in NTS-challenged mice (NTS) or NTS-challenged mice treated with pioglitazone started either at the same time as NTS (NTS+Pio) or in a curative protocol, started 4 days later (NTS+Pio delayed). (B) BUN concentration in groups of mice as in (A). (C) Representative images of Masson trichrome-stained kidney sections from groups of mice as in (A)—crescent.
Pioglitazone treatment decreases renal leukocytes infiltration in NTN. (A) Immunostaining for CD3+ and F4/80+ cells in renal cortex from nontreated mice (control), NTS-challenged mice (NTS) and NTS-challenged mice with pioglitazone treatment at same time (NTS+Pio). Scale bar, 20µm. (B, C) Quantification of CD3+ area or F4/80+ area to total area ratio (means of eight mice per group, of two independent experiments). (D, E) mRNA expression of mcp1 and il6 determined by RT-PCR analysis in renal cortex tissue from groups of mice as in (A). *P<0.05, **P<0.01; ***P<0.001 versus unchallenged control mice and #P<0.05 versus NTS-challenged mice.

Figure 13.
After 4 days of primary culture, podocytes were trypsinized then plated into m-Dish 35 mm high with Culture-Insert (Ibidi). Ibidi wounding inserts were used for cell migration studies. The coverage of the 500-μm gap was assessed after 12 hours of culture and podocyte migration area was quantified using ImageJ software. The effect of rosiglitazone (10 μM) on differentiated podocytes was tested during 16 hours. After stimulation, podocytes were scraped in Phosphosafe buffer for protein extraction or in RLT buffer for total RNA extraction.

Histology
Kidneys were harvested and fixed in 4% formalin. Paraffin-embedded sections (5-μm thick) were stained by Masson's trichrome to evaluate kidney morphology and determine proportion of crescentic glomeruli by a blinded examination on at least 50 glomeruli per section.

Immunohistochemistry and Immunofluorescence
Deparaffinized kidney sections were incubated for 30 minutes at 95°C in the target retrieval solution (S1699; Dako), then in peroxidase blocking reagent (S2001; Dako), blocked in PBS containing 5% BSA and immunostained against PPARγ (Abcam, Inc.), Podocalyxin (R&D Systems), CD3 (Dako), or F4/80 (AbD Serotec). For PPARγ, CD3, F4/80, specific staining was revealed using Histofine reagents (Nichirei Biosciences), which contained anti-rabbit or antirat immune-peroxidase polymer for mouse tissue sections. NRF2 or PPARγ primary antibody was followed by a secondary rabbit anti-goat IgG Cyanin3 and podocalyxin primary antibody was followed by a secondary donkey anti-goat IgG AF488-conjugated antibody (Invitrogen). Podocyte culture cells were immunostained against WT1 (Abcam, Inc.) and NRF2 (Abcam, Inc.). The nuclei were stained using DAPI.

For sheep IgG and mouse IgG staining, 5-μm kidney cryosections were fixed in cold acetone for 10 minutes, then washed in PBS and blocked with 5% BSA-PBS for 1 hour at room temperature. Kidney sections were then incubated with donkey anti-mouse IgG AF594-conjugated antibody (Invitrogen) and donkey anti-sheep IgG. Images were obtained on an AxioImager Z1 microscope apotome with AxioCam camera (Carl Zeiss).

Measurement of the Murine Anti-Sheep IgG Immune Response
Serum mouse anti-sheep IgG levels were measured by ELISA. Ninety-six-well plates were coated overnight at 4°C with 100 μg/ml of sheep IgG (Sigma-Aldrich). After blocking with 3% BSA, undiluted or diluted serum samples were incubated for 1 hour at 37°C. After washing, peroxidase-coupled anti-mouse IgG (Fc-specific) (Rockland Immunochemicals) and peroxidase substrate were added. Optical density was read in a microquant with Gen5 software (Bio-Tek).

Human Tissues
Formalin-fixed, paraffin-embedded renal tissue specimens were obtained from the Hôpital Européen Georges Pompidou, Assistance Publique-Hôpitaux de Paris, Paris, France. Human tissue was used after approval from, and following the guidelines of, the local ethics committee (IRB00003888, FWA00005831). Renal biopsy specimens with sufficient tissue for immunohistochemical evaluation after the completion of diagnostic workup were included.

Transmission Electron Microscopy Procedure
Small pieces of renal cortex were fixed in 4% glutaraldehyde, postfixed in 1% osmium tetroxide and embedded in epoxy resin. Ultrathin sections were counterstained with uranyl acetate and examined in a JEOL 1011 transmission electron microscope with Digital Micrograph software for acquisition.

Western Blot Analysis
After extraction from glomeruli or podocytes with lysis buffer, proteins were quantified using a BCA protein assay kit (iNtRON Biotechnology). Samples were resolved on 4%–12% Bis-Tris Criterion XT gels (Bio-Rad) then transferred to a polyvinylidene difluoride membrane. Membranes were incubated with the appropriate

Figure 14. Loss of nuclear PPARγ in podocytes and crescents in patients diagnosed for RPGN. Representative images of immunostaining for PPARγ in sections of kidney biopsies from random control patient (CT) or 3 subjects diagnosed with RPGN (MPA and GPA). Scale bars, 100 μm. (A, B, C and D) higher magnification of left panel (red square). Selective nuclear PPARγ (black arrows) is seen in glomerular endocapillary cells as well as with an extracapillary pattern in glomerular epithelial cells: podocytes and parietal epithelial cells. PPARγ expression is abolished in the nuclei of cells forming the crescent in favor of cytoplasmic expression (yellow arrows), whereas endocapillary staining is retained.
primary antibodies: rabbit anti-PPARγ (Abcam, Inc.), rabbit anti- NRF2 (Abcam, Inc.). Protein loading was monitored by using the rat anti-tubulin antibody (Abcam, Inc.). Secondary antibodies were donkey anti-rabbit horseradish peroxidase (GE Healthcare Life Sciences). Antigens were revealed by enhanced chemiluminescence (Supersignal West Pico; Pierce) and detected on a LAS-4000 imaging system (Fuji). Densitometric analysis with ImageJ software was used for quantification.

Real-Time PCR
Total RNA extraction of mice glomeruli was performed using an RNeasy Minikit (Qiagen) and reverse transcribed into cDNA using the Quantitect Reverse Transcription kit (Qiagen) according to the manufacturer’s protocol. Both cDNA and standard were amplified in Maxima SYBR Green/Rox qPCR mix (Fermentas) on an ABI PRISM thermocycler. The comparative method of relative quantification (2-ΔΔCT) was used to calculate the expression level of each target gene, normalized to glyceraldehyde-3-phosphate dehydrogenase. The oligonucleotide sequences are available upon request. The data are presented as the fold change in gene expression.

Statistical Analyses
All values are expressed as means±SEM. Statistical analyses were calculated using GraphPad Prism software (GraphPad Software, La Jolla, CA). Comparison between two groups was performed using Mann–Whitney t test. Comparison between multiple groups was performed by using one-way ANOVA followed by Tukey post-test. Values of P<0.05 were considered significant.

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DISCLOSURES
The authors declare no competing financial interests.

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