Knockout of Toll-Like Receptor-2 Attenuates Both the Proinflammatory State of Diabetes and Incipient Diabetic Nephropathy

Sridevi Devaraj, Peter Tobias, Balakuntalam S. Kasinath, Rajendra Ramsamooj, Alaa Afify, Ishwarlal Jialal

Objective—Type 1 diabetes (T1DM) is a proinflammatory state and confers an increased risk for vascular complications. Toll-like receptors (TLR) could participate in diabetic vasculopathies. Whether TLR activation contributes to the proinflammatory state of T1DM and the pathogenesis of diabetic nephropathy remains unknown.

Methods and Results—We induced T1DM in TLR2 knockout mice (TLR2−/−) and wild-type littermates (C57BL/6J-WT) using streptozotocin (STZ). Fasting blood, peritoneal macrophages, and kidneys were obtained for flow cytometry, Western blot, microscopy, and cytokine assays at 6 and 14 weeks after induction of diabetes. Macrophage TLR2 expression and MyD88-dependent signaling were increased in diabetic mice (WT+STZ) compared with nondiabetic WT mice. These biomarkers were attenuated in diabetic TLR2−/− macrophages. WT+STZ mice showed increased kidney:body weight ratio due to cell hypertrophy, increased albuminuria, decreased kidney nephrin, podocin, and podocyte number and increased transforming growth factor-β and laminin compared with WT mice. Nephrin, podocin, and podocyte number and effacement were restored, and transforming growth factor-β and laminin levels were decreased in TLR2−/−+ STZ mice kidneys versus WT+STZ. Peritoneal and kidney macrophages were predominantly M1 phenotype in WT+STZ mice; this was attenuated in TLR2−/−+ STZ mice.

Conclusion—These data support a role for TLR2 in promoting inflammation and early changes of incipient diabetic nephropathy, in addition to albuminuria and podocyte loss. (Arterioscler Thromb Vasc Biol. 2011;31:00-00.)

Key Words: immune system ■ kidney ■ macrophages ■ microvascular complications ■ nephropathy

Type 1 diabetes (T1DM) is a proinflammatory state, as evidenced by increased levels of C-reactive protein, inflammatory cytokines, and nuclear factor-κB (NF-κB) activation1–3; these increases are further accentuated in T1DM patients with microvascular complications.4–7 Diabetic nephropathy (DN) is the leading cause for end stage renal disease in the United States, affecting 30% of T1DM patients.8–9 The mechanism of kidney injury in diabetes is multifactorial, and recent findings suggest an important role for activation of immunologic pathways.10 Studies support increased biomarkers of inflammation in diabetic kidneys.11–13

The emerging concept is that activation of the innate immune system and inflammation via Toll-like receptor (TLR) activation in the pathogenesis of T1DM and its complications are significant.14–18 Recent findings have shown increased TLR2/4 expression, signaling, ligands, and functional activation in T1DM subjects compared with controls,19,20 which is further accentuated in monocytes of T1DM with microvascular complications (mainly nephropathy).16 Overactivation of TLRs contributes to the pathogenesis of acute kidney injury, ischemic renal damage, and allograft rejection.21 Recently, Brown et al22 showed in a murine model of crescentic glomerulonephritis that administration of a synthetic TLR2 ligand (Pam3CSK4) significantly influenced disease severity through a TLR2-dependent mechanism. These data imply that selective targeting of TLR2 and the signaling pathways may have major clinical implications. However, at the present time, the role of TLR2 in diabetic vascular complications including DN is not known.

There are distinct changes in kidney structure and clearance function in early stages of diabetes preceding the appearance of pathological levels of albumin in urine. Thus, renal hypertrophy and onset of glomerular accumulation of extracellular matrix proteins in the form of thickening of glomerular basement membrane and mesangial matrix expansion due to an increase in laminin content are seen generally within days of onset of diabetes in rodent models.23 Thickening of the glomerular basement membrane is associated

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with transforming growth factor-β (TGF-β), and laminin expression can occur early in DN and may even precede albuminuria. Nephrin, a transmembrane receptor protein essential for maintaining the structure and function of the glomerular slit diaphragm, is significantly decreased in albuminuria. Nephrin, a transmembrane receptor protein associated with T1DM and ameliorates early abnormalities in DN is known to increase TLR2/4 expression in DN.

Methods

Animals

TLR2−/− mice (male, 8 to 10 weeks of age) generated on a C57BL/6J genetic background (wild-type [WT]) were purchased from the Jackson Laboratory (Bar Harbor, ME). Diabetes was induced by injecting multiple low doses of streptozotocin (STZ) (Sigma, 50 mg/kg body weight IP daily for 4 days), a widely accepted method for inducing diabetes in rodents.29,30 Diabetes was confirmed by elevated blood glucose levels in tail vein blood. Normal (normoglycemic) and diabetic mice were fed a diet with 5% (Ly6C, interleukin-6 [IL-6], CCR2) and M2 phenotype (CD206, etc) and decrease matrix degradation.27,28

Flow Cytometry

Surface TLR2 and TLR4 expression and markers of M1 phenotype (Ly6C, interleukin-6 [IL-6], CCR2) and M2 phenotype (CD206, CD163) in peritoneal and kidney macrophages was determined by flow cytometry as described previously.30,31

Nuclear/Cytoplasmic Extracts and NF-κB Transcription Factor Assays

Nuclear and cytoplasmic extracts were prepared from isolated macrophages and kidneys as described previously.32

Western Blotting

Western blot analysis was used to examine the downstream signaling events.

Small Interfering RNA Transfection Assays

THP-1 cells (American Type Culture Collection) were incubated in normoglycemic (LG: 5.5 mmol/L) and hyperglycemic (HG: 15 mmol/L glucose) conditions for 48 hours with specific TLR2 or scrambled small interfering RNA (siRNA) (Ambion) as described previously.33

Transmission Electron Microscopy of Diabetic Kidneys and Immunohistochemistry

Structural changes in diabetic kidneys were detected using transmission electron microscopy and following the CAP protocols of the University of California Davis Medical School as described previously.33,34

Statistical Analysis

Statistical analyses were performed using SAS. Data are expressed as mean±SD for parametric data and as median and interquartile range for nonparametric data. Following ANOVA, parametric data were analyzed using paired t-tests, and nonparametric data were analyzed using Wilcoxon signed rank tests. The level of significance was set at P<0.05. The Pearson and Spearman correlations were computed for variables of interest such as nephrin, podocin, laminin, WT-1 score, podocyte width, and microalbuminuria.

Results

Baseline characteristics of the mice are provided in the Table. There were no significant differences in body weight or lipid levels between the 4 groups of mice. Following STZ injection, as expected, there was significant elevation in glucose levels compared with WT or TLR2−/− mice (Table). TLR2 and TLR4 expression was examined in all the groups. Compared with nondiabetic WT mice, WT diabetic mice (WT+STZ) had increased expression of both TLR2 and TLR4 in macrophages, whereas TLR2−/− diabetic mice (TLR2−/−+STZ) had increased expression only of TLR4 (Figure 1a). The extremely low MFI for TLR2 in TLR2−/− mice compared with WT mice (WT:

### Table. Baseline Characteristics

<table>
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<tr>
<th></th>
<th>WT (n=5)</th>
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<th>WT+STZ (n=20)</th>
<th>TLR2−/−+STZ (n=22)</th>
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<td>91±4†</td>
<td>132±17*</td>
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<td>Microalbumin/creatinine ratio, μg/g (median)</td>
<td>7±5 (6)</td>
<td>9±7 (7)</td>
<td>89±29* (86)</td>
<td>28±17§ (26)</td>
</tr>
</tbody>
</table>

Data are presented as mean±SD. Blood glucose was obtained by measurements from the tail vein.

*P<0.001 vs WT and TLR2−/−.
†P<0.04 vs WT.
‡P<0.04 vs WT+STZ.
§P<0.015 vs WT+STZ.
TLR2 activates NF-κB via MyD88, resulting in transcription of proinflammatory genes. Infiltrating macrophage-derived products in the diabetic kidney induce inflammation and are involved in the subsequent development and progression of DN. However, it is not clear whether macrophage TLR2-mediated inflammation plays a role in this process. Therefore, levels of MyD88, IRAK-1 protein phosphorylation, Trif, IRF3, and NF-κB activity were examined in peritoneal macrophages of the mice. TLR2−/− + STZ mice showed significant reduction in MyD88 expression and phosphorylation of IRAK-1, whereas there was no significant abrogation of non-MyD88 dependent signaling proteins, such as Trif and IRF3 (Figure 1b), compared with the WT + STZ mice. As shown in Figure 1c, concomitant with the activation of MyD88-dependent signaling cascade, WT + STZ mouse macrophages had significantly increased nuclear NF-κB activity compared with WT and TLR2−/− mice. Furthermore, compared with WT + STZ mice, there was a significant decrease in STZ-induced NF-κB activity in the TLR2−/− + STZ mice (55% reduction, P<0.001). In addition, there was significant increase in MyD88 (50%) and phosphorylation of IRAK-1 (63%) activity in WT + STZ mice compared with WT mice (Figure 1b).

NF-κB activation leads to increased inflammatory gene expression. To study the functional significance of reduced NF-κB activation in diabetic TLR2−/− mice, we measured levels of proinflammatory chemokines and cytokines known to be activated by TLR induction. Figure 2 depicts serum levels of cytokines/chemokines. WT + STZ mice exhibited significantly increased levels of IL-1β, IL-6, KC/IL-8, IP-10, MCP-1, and TNF-α compared with nondiabetic WT mice (P<0.001), and all were significantly attenuated in TLR2−/− + STZ mice (P<0.001). TNF levels, though decreased in TKLR−/− + STZ mice compared with WT + STZ mice, did not reach statistical significance (P=0.071). We also examined IL-10 levels, which were not significantly altered in TLR2−/− + STZ compared with WT + STZ mice. In addition, serum amyloid P component levels were significantly increased in WT + STZ mice compared with nondiabetic WT mice, and this increase was significantly attenuated in the diabetic TLR2−/− mice (median serum amyloid P component in WT + STZ: 77 μg/mL; in TLR2−/− + STZ: 46±11 MFI/10⁵ cells; WT + STZ: 75±21 MFI/10⁵ cells; P<0.05).

**Figure 1.** a, Surface expression of TLR2 and TLR4. Peritoneal macrophages were obtained from WT (n=5), TLR2−/− (n=5), WT + STZ (n=20), and TLR2−/− + STZ (n=22) mice at 6 weeks, and surface expression of TLR2 and TLR4 was assessed by flow cytometry as described in Methods. Values are expressed as MFI/10,000 cells (mean±SD). *P<0.001 vs WT and TLR2−/−; †P<0.001 vs WT or WT + STZ; ‡P<0.05 vs WT. b, TLR2−MyD88 dependent signaling: MyD88, IRAK-1 phosphorylation, IRF3, Trif, and nuclear p65 protein levels were measured in peritoneal macrophage lysates from WT + STZ (n=20) and TLR2−/− + STZ mice (n=22) at 6 weeks using Western blot assay. Representative blots and densitometric ratios are depicted in the figure. Values are expressed as protein/β-actin ratio (mean±SD). *P<0.001 vs WT + STZ mice. Total IRAK-1 and β-actin were used as internal controls. c, Nuclear NF-κB activation in peritoneal macrophages of WT (n=5), TLR2−/− (n=5), WT + STZ (n=20), and TLR2−/− + STZ (n=22) mice at 6 weeks was determined by Trans-AM activity assay and normalized to nuclear protein as described in Methods. Values are expressed as ng/mg of protein (mean±SD). *P<0.005 vs WT and TLR2−/−; †P<0.001 vs WT + STZ mice.
mice: 28 μg/mL; P<0.001). Similar results were obtained for cytokine/chemokine release from peritoneal macrophages (Supplemental Figure I). TLR2−/−+STZ mouse macrophages released significantly lesser amounts of IL-1β (48% reduction, P<0.01), IL-6 (44% reduction), KC/IL-8 (64% reduction, P<0.001), IP-10 (66% reduction, P<0.001), and MCP-1 (66% reduction, P<0.001) compared with WT+STZ mice. The decrease in TNF levels was not significant.

Because all the TLRs except TLR3 activate the MyD88 pathway,19 we used TLR2 siRNA inhibition strategy in human THP-1 cells to confirm the dominant role of TLR2 knockout in decreasing MyD88 in the diabetic milieu. As reported previously,32 compared with normoglycemic conditions (5.5 mmol/L glucose), there were significant increase in MyD88 and its downstream signaling with HG (Supplemental Figure II). This was attenuated by siRNA to TLR2. Having shown that TLR2KO ameliorates the proinflammatory state of diabetes, we next focused on a microvascular complication, DN.

One of the cardinal manifestations of renal involvement in early stages of DN is renal hypertrophy, defined as an increase in the ratio of kidney to body weight. As expected, the kidney to body weight ratios were significantly higher in WT+STZ mice compared with WT and lower in TLR2−/−+STZ mice compared with WT+STZ mice at both 6 weeks (Table) and 14 weeks (WT+STZ: 112.45±12; TLR2−/−+STZ: 98±7; P<0.05). In addition, we examined protein:DNA ratios to confirm cell hypertrophy, rates of which were significantly higher in WT+STZ mice compared with WT and lower in TLR2−/−+STZ mice compared with WT+STZ mice at 14 weeks (WT+STZ: 102±39; TLR2−/−+STZ: 53±23; P<0.05). The 24-hour urinary microalbumin:creatinine ratios were significantly increased in WT-STZ mice compared with WT mice (median: 86 μg/mg creatinine versus 6 μg/mg creatinine; P<0.001); furthermore, the ratios were significantly decreased in the TLR2−/−+STZ mice (median: 26 μg/mg) compared with WT+STZ mice (median: 86 μg/mg; P=0.015). Thus, we established early DN by the accepted criterion of the AMDCC of a 10-fold increase in albuminuria in WT-STZ mice, which is attenuated in TLR2−/−+STZ mice.36

Because we recorded significantly higher levels of proinflammatory cytokines/chemokines and no absolute increase in number of kidney macrophages, we examined the phenotype of macrophages in peritoneum and kidney. As shown in Figure 3a, there was a significant increase in M1 phenotype of both kidney and peritoneal macrophages in WT-STZ mice, and this was attenuated in the TLR2−/−+STZ mice. Because there was decreased albuminuria in TLR2−/−+STZ mice compared with WT+STZ mice, we proceeded to examine podocyte morphology. Electron micrographs of the WT+STZ and TLR2−/−+STZ kidneys were examined using standard electron microscopy techniques. The diabetic group (WT+STZ mice) showed foot process effacement and widening of the podocyte villi at 14 weeks after induction of diabetes. This finding was more pronounced at the tips of the capillary loops. These changes were also observed in the
TLR2−/− + STZ mice kidneys, but to a lesser degree (Supplemental Figure III). The number of cells positive for WT-1 in 5 hpf was counted, and a WT-1 score was computed. There was significant reduction in WT-1 score in the WT + STZ mice, confirming podocyte loss compared with the WT and TLR2−/− nondiabetic mice, and this was restored in the TLR2−/− + STZ mice (WT-1 score in non-DM mice: 14 ± 0.5, TLR2−/− mice: 7 ± 0.4 cells; TLR2−/− + STZ mice: 4 ± 0.5 cells per 5 hpf; P < 0.05 compared with WT-STZ mice). Representative glomeruli are shown in Figure 3b. There was no change in glomerular volume between the groups. Thus, our EM and WT-1 staining fulfill a second criterion of AMDCC, ie, podocyte loss. When we examined immunohistochemical staining for mesangial proliferation or macrophage recruitment (using Mac-2 antibody), it was not significantly different between the WT + STZ and TLR2−/− + STZ mice.

To characterize molecular and signaling changes in kidneys as evidence for early kidney damage under diabetic conditions, nephrin, podocin, laminin, and TGF-β expression were measured in all the 4 groups of mice using immunoblot assays after 6 and 14 weeks of persistent diabetes. WT + STZ kidneys showed significantly reduced levels of nephrin (−50%) and podocin (−50%), whereas laminin (↑ 85%) and TGF-β (↑ 80%) expression were higher compared with WT mice at 6 and 14 weeks (Figure 4a and 4b). Levels of nephrin and podocin were restored in TLR2−/− + STZ mice to those of nondiabetic WT mice. TGF-β and laminin protein levels were decreased in TLR2−/− + STZ kidneys compared with WT + STZ kidneys, similar to nondiabetic WT mice. It is important to note that there was a significant correlation between microalbuminuria and podocin (r = −0.56, P = 0.03), podocyte width (r = 0.6, P = 0.03), and a trend to significance in the relationship between microalbuminuria and WT-1 score (r = −0.5, P = 0.08).

In addition, TLR2, MyD88, and IRAK-1 phosphorylation were increased in WT + STZ kidneys and attenuated in TLR2−/− + STZ mice (Figure 4a and 4b). There were no significant differences in the nephrin, podocin, laminin, and MyD88 protein expression in kidneys of nondiabetic WT and TLR2−/− mice (Figure 4a and 4b). Associated with MyD88-dependent signaling, WT + STZ kidneys had significantly increased nuclear NF-κB activity (7 ± 2 ng/mg protein) compared with those of nondiabetic WT (1.2 ± 0.2 ng/mg protein) and TLR2−/− mice (1 ± 0.2 ng/mg protein) (P < 0.002). Furthermore, compared with WT + STZ mice, there was a significant decrease in STZ-induced NF-κB activity in the kidneys of TLR2−/− + STZ mice (2 ± 0.5 ng/mg protein; 71% reduction; P < 0.001). Furthermore, we measured inflammation in the kidneys using multiplex assays. IL-1β, IL-6, KC/IL-8, MCP-1, IP-10, and TNF-α concentrations were significantly increased in WT + STZ mice compared with nondiabetic WT and TLR2−/− mice. There was a significant reduction in the concentration of these inflammatory mediators in TLR2−/− + STZ mice compared with WT + STZ mice (Figure 5) at 14 weeks.
Figure 5. Cytokine and chemokine concentrations in the kidney lysates of WT, TLR2−/−, WT+STZ, and TLR2−/−+STZ mice (n=11/group) was measured by multiplex assay at 14 weeks as described in Methods. Values are expressed as pg/mg of cell protein (mean±SD). *P<0.001 vs WT and TLR2−/−; †P<0.01 vs WT+STZ.

Discussion

We recently showed that there is increased TLR2 and TLR4 expression and activity in monocytes of T1DM patients compared with matched controls and that these are further accentuated in T1DM patients with microvascular complications, with 66% of the T1DM-microvascular patients having DN. Therefore, in this study, we focused on the contribution of TLR2 to the proinflammatory state of diabetes and a critical microvascular complication, namely, DN, because there is a paucity of data on the role of inflammation in DN. We provide novel evidence supporting these 2 key pathogenic processes using STZ-induced T1DM in WT and TLR2−/− mice. First, we showed attenuation of the increased inflammation via circulating cytokine/chemokine levels in the TLR2−/−+STZ mice compared with WT+STZ mice, despite the expression of other TLRs, including TLR4, being intact; then, using the sentinel inflammatory cell macrophage, we demonstrated inhibition of MyD88-dependent signaling, NF-κB activity, and proinflammatory mediators in diabetic TLR2−/− mice. Second, we identified early renal changes consistent with DN through increased kidney size, increased albuminuria, decreased nephrin and podocin expression, and increased TGF-β and laminin expression in WT+STZ mice at 6 and 14 weeks of diabetes. The increased albuminuria at 14 weeks or greater than 10-fold in WT+STZ mice compared with WT mice is a validating criterion for DN recommended by AMDCC.

Also, the increased podocyte foot process widening and effacement and decreased WT-1-positive cells (podocyte loss) is another accepted criterion of the AMDCC. Finally, we showed increased MyD88 signaling in WT+STZ kidneys coupled with increased IL-6 and MCP-1, which were significantly attenuated in TLR2−/−+STZ mice. These molecular changes were restored in TLR2−/−+STZ mice, pointing to a critical role for TLR2 in inducing a proinflammatory phenotype in T1DM and attendant nephropathy. We showed increased renal biomarkers of inflammation (IL-1, IL-6, MCP-1) implicated in DN that were attenuated in TLR2−/−+STZ mice. Using Mac2 antibody in IHC, we failed to see increased macrophage recruitment in the kidney. We are unsure whether TLR2KO will necessarily reduce macrophage recruitment in the kidney, because it has not been shown previously to persist by 2 groups in the renal ischemia reperfusion model and may be a function of the time course of DN with both TLR2 and TLR4. We clearly showed decreased biomarkers of inflammation implicated in the genesis of DN. In addition, we examined the phenotype of kidney and peritoneal macrophages and showed that there was polarization to the M1 phenotype. These exciting data indicate that macrophages infiltrating the kidney in DN are predominantly of the M1 proinflammatory phenotype and contribute to the increased inflammation seen in DN. Also, PAS/silver methanamine staining did not reveal mesangial proliferation, in spite of the increase in TGF-β and laminin.

Rodent models of DN are excellent tools by which to gain insight into the pathogenesis of the disease and to test new therapies. Renal and glomerular hypertrophy are key features of early T1DM and may occur within days of experimental diabetes. In nonobese diabetic mice, renal hypertrophy occurs 10 days after diabetes onset and reaches a plateau by 20 days. However, glomerular sizes did not differ in nonobese diabetic mice from controls in the early stages of diabetes except for partial thickening of basement membrane as revealed by electron microscopy. In agreement with the above studies, we did not see major changes in microalbuminuria at 6 weeks after the onset of T1DM, but there was significantly increased albuminuria at 14 weeks after diabetes onset (P=0.015). These observations are in accordance with the published literature showing that at ~16 weeks, these C57BL6 mice get significant albuminuria and also develop glomerulosclerosis. At the same time, the kidney:body weight and protein:DNA ratios were significantly higher in WT+STZ mice compared with WT and lower in TLR2−/−+STZ mice compared with WT+STZ mice (Table), indicating early DN. There were significant changes in TGF-β consistent with earlier studies using diabetic mice. Thus, enhancing extracellular matrix accumulation or inhibiting extracellular matrix degradation by TGF-β makes TGF-β a pivotal biomediator in mesangial expansion. Moreover, TGF-β has consistently been shown to be increased in renal parenchymal and infiltrating macrophages in the diabetic kidney of humans and experimental models. Our data are consistent with the above studies, and deficiency of TLR2 attenuates TGF-β expression in diabetic kidneys. Also, because TGF-β promotes mesangial expansion, we quantitated laminin as a readout. Both TGF-β and laminin were increased in WT+STZ mice compared with nondiabetic and TLR2−/−+STZ mice. Had we kept our mice for a longer duration, we anticipate that increased PAS positivity would have been evident.

Foot process effacement is a result of retraction, widening, and shortening of the processes of podocytes. Jefferson et al postulated that podocyte effacement could be caused by (1) changes in slit diaphragm–associated proteins, (2) interference with the GBM or podocyte–GBM interaction, (3) actin cytoskeleton abnormalities, or (4) alterations in the negative apical membrane domain of podocytes. Because proteinuria is a cardinal clinical characteristic of DN, many studies have focused on the changes in slit diaphragm–associated molecules in DN. Bonnet et al demonstrated a reduction in nephrin mRNA and protein expression in STZ-induced diabetic spon-
taneously hypertensive rats.\textsuperscript{50} In the present study, in conjunction with improvement in proteinuria, diabetic TLR2\textsuperscript{--/-} mice showed significant normalization of podocyte villi width and foot process effacement compared with WT+STZ mice; concomitantly, there was normalization of the slit membrane proteins nephrin and podocin and normalization of podocytes compared with WT+STZ mice. The significant inverse correlation in our study between the accepted clinical biomarker microalbuminuria and podocin protein levels and podocyte number in this diabetic model suggests that if confirmed in future studies, they could emerge as novel biomarkers of DN.

Slit diaphragm proteins have become increasingly important in signal transduction and in mediating downstream events in kidney diseases. Recently, nephrin and podocin, 2 slit membrane proteins, have drawn significant attention in the pathogenesis of DN.\textsuperscript{51} Menne et al\textsuperscript{52} showed that protein kinase C-\(\alpha\) activation may be an important regulator of nephrin expression and glomerular albumin permeability under diabetic conditions. Dasu et al\textsuperscript{52} demonstrated that hyperglycemia-induced TLR2 expression and activation are mediated by protein kinase C-\(\alpha\) in human monocytes. However, the question of whether TLR2 signaling is involved in mediating the effects of diabetes on the slit-membrane proteins is not known. Both nephrin and podocin serve a survival function for podocytes,\textsuperscript{10} and nephrin is a key regulator of podocyte signaling.\textsuperscript{53} The decrease in nephrin and podocin in WT+STZ kidneys could presage podocyte loss and were partially restored in TLR2\textsuperscript{--/-} + STZ mice, indicating the involvement of TLR2 in regulating slit membrane protein expression pivotal in podocyte activity. However, in addition, we show podocyte loss in WT+STZ mice. Also because it has been previously shown that AGE-low-density lipoprotein activates TLR4,\textsuperscript{47,54} this interaction with respect to TLR2 will be studied in the future.

The role of TLR2 has been examined in other disease states. Leemans et al\textsuperscript{10} found that TLR2 deficiency resulted in reduced renal dysfunction and tubular damage following ischemia/reperfusion and that this was associated with decreased macrophage numbers in the kidney, but only at day 3, and these differences were lost by day 10. Recent reports have established a protective role of TLR2 deficiency in mice; concomitantly, there was normalization of the slit membrane proteins nephrin and podocin and normalization of podocytes compared with WT+STZ mice. The significant inverse correlation in our study between the accepted clinical biomarker microalbuminuria and podocin protein levels and podocyte number in this diabetic model suggests that if confirmed in future studies, they could emerge as novel biomarkers of DN.

TLRs activate 2 types of downstream signaling pathways: MyD88-dependent and MyD88-independent pathways.\textsuperscript{18} TLR2 signals primarily through the MyD88-dependent pathway to induce inflammation. Here, we provide novel evidence, using the STZ-induced diabetes model, that TLR2 knockout results in significant decrease in diabetes-induced inflammation independent of TLR4, because TLR4\textsuperscript{’s} non-MyD88 dependent signaling proteins (Trif, IRF3) were unaltered. The decrease in inflammations (ie, release of proinflammatory cytokines and chemokines) was associated with a significant reduction in NF-\(\kappa\)B activity, MyD88, and phosphorylation of IRAK-1. Similar findings were found when TLR2 was knocked down using siRNA in cells under hyperglycemic conditions.\textsuperscript{32} It is worth mentioning that TLR2 deficiency in the diabetic milieu has a profound effect on MyD88-dependent signaling, even with increased TLR4 expression, and warrants further investigation. We have confirmed our in vivo finding using siRNA to TLR2 under hyperglycemic conditions and confirm the decrease in MyD88. Thus, abrogating inflammation in diabetes using TLR2 as a target appears to be a reasonable therapeutic strategy to alleviate inflammation and the associated DN.

In summary, we document 2 novel findings: that genetic deficiency of TLR2 (1) significantly abrogates the proinflammatory state of T1DM up to 14 weeks, despite the other TLRs, including TLR4, being intact; and (2) attenuates incipient DN at the cellular level, as evidenced by normalization of microalbuminuria and retention of podocyte number. In support of our data, Li et al recently documented increased TLR2 expression in both rat and human kidneys with DN.\textsuperscript{58} Also, in a previous report, we showed that after a short duration of 2 weeks, there were decreased biomarkers of inflammation in TLR2KO STZ mice and improvement in wound healing parameters.\textsuperscript{59} We are now extending our findings and show the durability of the amelioration of inflammation at 14 weeks in TLR2KO-STZ mice, with a benefit on a critical microvascular complication associated with increased morbidity and mortality, DN. Collectively, the published data and the present report support a pivotal role of TLR2 signaling to the proinflammatory state of T1DM and incipient nephropathy. Evidence from prospective trials has emphasized the role of inflammation in contributing to increased complications and cardiovascular events in diabetes. Therapeutic strategies targeted at decreasing TLR2 to abrogate inflammation in diabetes may eventually result in decreased complications, including nephropathy. In this context, we and others have shown that statins, peroxisome proliferator–activated receptor-\(\gamma\) agonists,\textsuperscript{32} and angiotensin receptor blockers\textsuperscript{60} decrease both TLR2 and TLR4 expression and signaling.

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Disclosures

None.

References

Innate immunity and the pathogenesis of type 1 diabetes.


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Supplemental Methods:

Animal Studies:

Blood glucose levels were measured in tail veins after five days and diabetes was established with a glucose level of >250 mg/dL on three consecutive days. Slow-release insulin pellets (Linshin Canada, Ontario, Quebec) were implanted subcutaneously to maintain the diabetic state of the animals to avoid severe morbidity and mortality from untreated diabetes. Non-diabetic C57BL/6J and TLR2-/- mice served as controls. All the animals had ad libitum access to food and water and were maintained in a sterile environment. Study animals were euthanized at six and 14 weeks after established diabetes after an overnight fast. Peritoneal macrophages, kidneys, and blood were collected for analysis. 24hr urines were collected from the mice using metabolic cages. Serum was used for measurement of glucose, cholesterol, triglycerides, and cytokines. Kidney to body weight ratios were calculated and expressed as % of control. Biochemical characteristics including urine microalbumin and creatinine were measured and ratios were computed using standard rodent assays in the Veterinary Pathology laboratories at UC Davis. The Institutional Animal Use and Care Committee at UC Davis approved all the studies.

Isolation of Peritoneal and Kidney Macrophages: Following euthanization, mice peritoneal macrophages were obtained after injecting the peritoneal cavity with 3 ml of warmed PBS, collecting the contents, centrifuging, followed by 2 washes with PBS. Kidney was minced into pieces and digested with collagenase and
DNase followed by isolation of kidney macrophages using positive magnetic separation (Miltenyi) as described (ref). F4/80 was used as the macrophage marker.

Flow Cytometry: Briefly, analysis of TLR expression was performed using anti-mouse TLR2 (Cat#: 51-9021, eBioscience) and anti-mouse TLR4 (Cat# 12-9041, eBioscience) with BD flow FACS array flow cytometer. The extremely low MFI for TLR2 in TLR2-/- mice was consistent with the background. 80-90% of the cells isolated were CD68 positive.

Phenotypic Characterization of Classical (M1) and Alternatively Activated Monocytes (M2): Phenotypic characterization of M1 and M2 cells from peritoneum and kidney was performed using 4-color flow cytometry as described previously (30,31). Macrophages were gated using F4/80 and M1 cells were characterized by positivity for Ly6C, IL-6 and CCR2 and M2 cells were characterized by positivity for CD206 (mannose receptor) and CD163. Percentage of classically versus alternatively monocytes was estimated. Fluorescently labeled macrophages were analyzed under the same instrument settings (to eliminate measurement bias) and all data were corrected for background fluorescence intensity obtained with isotype control antibodies (all positive cells were over the first quadrant in the BDFACS array) and were usually less than 10%. While high positive expression of Ly6C, IL-6, CCR2 was used for the M1 cells, these cells were also very low expressors of CD163 and CD 206 (less than 10%) and hence classified as “M1 phenotype”. For “M2 phenotype”, characterization by flow cytometry used the same criteria.
NFKb assays: Nuclear extracts were used to perform NF-kB (Cat# 40096) transcription factors activation assays (Active Motif, Carlsbad, CA). Intra and inter assays CV for these assays was < 7%.

Western Blotting: Western blot analysis was used to examine the downstream signaling events. Twenty µg of total protein was resolved, transferred, and probed with antibodies for Interleukin-1 receptor-associated kinase 1 (IRAK1), Myeloid differentiation factor 88 (MyD88), nuclear p65, interferon regulatory factor 3 (IRF3) (SantaCruz, Santacruz CA), TIR-domain-containing adapter-inducing interferon-β (Trif) (Abcam, Cambridge, MA), Laminin (Abnova, Walnut, CA), Nephrin, Podocin, Transforming growth factor-β (TGF-β) (Santa Cruz, Santacruz CA), and stripped membranes were further incubated with β-actin. Band intensities were determined using Image Quant Software (GE Healthcare Biosciences, Piscataway, NJ) and normalized to β-actin and presented as a ratio. Some of the blots shown consist of pooled samples from 3 mice/group/lane to minimize variability in expression patterns.

siRNA Transfection Assays: Following treatment, cells were collected for immunoblotting as described above. All the reagents used including cell culture media and antibodies were purchased as endotoxin free. Solutions were prepared in pyrogen free water and again tested for endotoxin levels using LAL assay. Endotoxin levels were consistently below 20 pg/ml in all experiments. Mannitol (9.5mM) was used as an osmotic control in all the experiments.
Methods for Transmission Electron Microscopy: Briefly, resected kidneys from all the four groups of mice were fixed in 4% paraformaldehyde fixative and paraffin blocks were prepared by routine histological procedures. A small portion of kidney tissue was removed from the paraffin blocks, placed in 100% xylene and left to de-paraffinize overnight. The tissue was then re-hydrated through descending concentrations of acetone to 0.1M phosphate (PO4) buffer then fixed in Karnovsky’s fixative for a minimum of 1 hour. Tissues were rinsed with 0.1M PO4 buffer and post-fixed for 2hrs in 1% buffered osmium tetraoxide. Dehydration was through ascending concentrations of acetone with three changes at 100%. Infiltration was accomplished with microwave assistance. Epon-Araldite resin in three ascending concentrations with 100% acetone were used. Finally three changes of resin with microwave assistance were done before transferring to capsules and polymerizing overnight. Survey sections were cut and stained for presence of glomeruli. Ultrathin sections were cut using a diamond knife. They were stained with uranyl acetate and lead citrate before viewing in a Phillips CM120 Biotwin (Hillsboro, OR). Micrographs were taken using a Gatan MegaScan, model 794/20, digital camera (Pleasanton, CA) at the Electron Microscopy Facility of UC Davis. Podocyte width was measured in a blinded fashion using NIH Image J software and expressed as arbitrary units (AU). Twenty five foot processes/section were assessed from ten sections of each kidney. Also, immunohistochemistry was done using antibodies to WT-1 (for the podocytes) and Mac-1 (for macrophages) and PAS (for mesangial proliferation) and WT-1 scoring was done by counting cells positive for the stain.
using 40X magnification in the glomerulus and an average of 5 different high power fields was computed.

**Supplemental Figure Legends:**

**Figure I:** Pro-inflammatory cytokines and chemokines release by peritoneal macrophages from WT (n=5), TLR2-/- (n=5), WT+STZ (n=20) and TLR2-/-+STZ (n=22) mice at 6 weeks was measured by Multiplex assay as described in Methods. Values are expressed as pg/mg cell protein (mean ± SD). *P<0.005 vs WT and TLR2-/-; †P<0.05 vs to WT or WT+STZ.

TLR2-/-+STZ mice macrophages released significantly lesser amounts of IL-1β (48% reduction, P<0.01), IL-6 (44% reduction), KC/IL-8 (64% reduction, P<0.001), IP-10 (66% reduction, P<0.001) and MCP-1 (66% reduction, P<0.001) compared to WT+STZ mice. Having shown that TLR2KO ameliorates the pro-inflammatory state of diabetes, we next focused on a microvascular complication, diabetic nephropathy.

**Figure II:**

Targeted deletion of TLR2 in THP-1 cells under HG conditions using gene specific siRNA: Monocytic THP-1 cells were incubated under low glucose (LG: 5.5mM) high glucose (HG: 15mM) and transfected with either scrambled control siRNA or TLR2 siRNA as described in Methods. MyD88, IRAK-1 phosphorylation, IRF3, Trif, and nuclear p65 protein levels were measured using Western blot assay. Experiments were repeated three times and a representative blot is provided. Total IRAK-1 and β-actin were used as internal controls.
LG+Sc-Low Glucose and Scrambled siRNA; LG+TLR2si-Low Glucose +TLR2siRNA; HG+Sc-High glucose of scrambled siRNA and HG+TLR2si-High Glucose +TLR2siRNA

Under HG conditions (15mM), TLR2 siRNA transfected cells exhibited significant reductions in the levels of MyD88, IRAK-1 phosphorylation, and NF-kB p65 dependent activity compared to scrambled control siRNA transfected cells. However, there were no changes observed in Trif and IRF-3 expression since they are non-MyD88 dependent.

**Figure III:**
Podocyte foot processes widening and effacement in diabetic wild type (WT) and TLR2-/- mice kidneys at 14 weeks were examined using TEM as described in methods (n=11/gr). Representative electron micrograph is depicted. Bar represents 0.5µ and black and red arrows point to the podocyte widening and effacement while the green arrows indicate normal podocyte.

When comparing these groups with regard to podocyte villous thickness, the WT+STZ kidneys showed thicker podocyte villi compared to the TLR2-/-+STZ group. There was a statistically significant difference in podocyte width between the WT+STZ and TLR2-/-+STZ mice (136 ± 21 vs. 89 ± 22 AU; P < 0.001).
Online Supplemental Figure I

- **WT**
- **TLR2**
- **WT+STZ**
- **TLR2**

Cytokine/Chemokine (pg/mg protein)

- IL-1β
- IL-6
- KC/IL-8
- IP-10
- MCP-1
- TNF-α

* and † indicate significant differences.
Online Supplemental Figure II

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