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Global toll-like receptor 4 knockout results in decreased renal inflammation, fibrosis and podocytopathy

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ABSTRACT

Background and purpose: Type 1 diabetes mellitus (T1DM) is a pro-inflammatory state with increased toll-like receptor (TLR) activity. Inflammation is crucial in diabetic nephropathy (DN). We tested the effect of global deficiency of TLR4 on renal inflammation, fibrosis and podocytopathy using control (C) and streptozotocin (STZ) induced diabetic wildtype (WT) and TLR4-knockout (TLR4KO) mice.
Methods: Following STZ treatment, mice were euthanized at 17 weeks and plasma and kidneys collected.
Results: Compared to C, STZ-WT mice had significantly increased macrophage and TLR4 immunostaining in kidney, significant increases in MyD88, Interferon Regulatory Factor-3, NFKappaB activity, TNF-Alpha, IL-6, and MCP-1; all these were significantly decreased in the STZ-TLR4KO compared to STZ-WT mice. Compared to C, there were significant increases in fibrosis markers (collagen 4, and transforming growth factor-beta) in STZ-WT which were significantly decreased in the STZ-TLR4KO versus STZ-WT. Podocyte numbers and podocin were decreased in the STZ-WT versus C and increased in the STZ-TLR4KO mice.
Conclusion: Global genetic deficiency of TLR4 also ameliorates renal inflammation, fibrosis and podocytopathy and could be important in DN.

1. Introduction

In addition to its attendant morbidity and mortality, diabetic nephropathy (DN) is a major risk factor for cardiovascular disease in type 1 diabetes mellitus (T1DM) (Duca, Sippl, & Snell-Bergeon, 2013). T1DM is a pro-inflammatory state as evidenced by both cellular and circulating biomarkers of inflammation (Devaraj, Dasu, & Jialal, 2010; Devaraj, Dasu, Rockwood, et al., 2008; Devaraj, Glaser, Griffen, et al., 2006; Jialal & Kaur, 2012). We have previously shown that in patients with T1DM there is increased expression and activity of the pattern recognition receptors, the toll-like receptors (TLR) 2 and 4, on monocytes, which appeared to be further accentuated in T1DM patients with microvascular disease, predominantly nephropathy (Devaraj, Jialal, Yun, et al., 2011; Devaraj et al., 2008). Furthermore, studies support a pivotal role for inflammatory processes in the pathogenesis of DN (Mudaliar, Pollock, & Panchapakesan, 2014; Navarro-González, Mora-Fernández, Muros de Fuentes, et al., 2011; Wada & Makino, 2013). In a recent review, it has been suggested that chronic inflammation plays a vital role in renal fibrosis (Kanasaki, Taduri, & Koya, 2013). We have previously demonstrated that a global knockout of TLR2 reduces not only plasma and macrophage biomarkers of inflammation but renal inflammation and features of DN in diabetic mice (Devaraj, Tobias, Kasinath, et al., 2011). Furthermore, we have shown that genetic deficiency of TLR4 (TLR4KO) reduces systemic and macrophage inflammation in T1DM (Devaraj, Tobias, & Jialal, 2011). However Lin et al failed to show any changes of DN in TLR4KO diabetic mice after 12 weeks (Lin, Yiu, Wu, et al., 2012) and had to inflict a second injury of uninephrectomy to recapitulate features consistent with DN. Hence in this study we tested the effect of a global deficiency of TLR4 on renal inflammation, fibrosis and podocytopathy with a major focus on fibrosis.

2. Materials and methods

2.1. Animal details

TLR4 deficient mice (male, 8–10 weeks) generated on C57BL/6 J genetic background were obtained from Dr. Akira as reported

There are no potential conflicts of interest related to this article, and the authors have nothing to disclose.

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Table 1
Biochemical characteristics.

	WT (N = 8)	STZ-WT (N = 8)	STZ-TLR4-/- (N = 7)	ANOVA
Body weight (g)	35.1 ± 3	33 ± 4	36 ± 4	p = 0.34
Blood glucose (mg/dL)	92 ± 38	316 ± 20	301 ± 29	p = 0.0001
Cholesterol (mg/dL)	109 ± 25	103 ± 30	85 ± 40	p = 0.49
Plasma triglycerides (mg/dL)	27(24,47)	26(14,61)	16(12,39)	p = 0.8
Plasma creatinine (mg/dL)	0.066 ± 0.01	0.059 ± 0.031	0.157 ± 0.2	p = 0.31

Data are expressed as mean ± S.D except for trigycerides which are median (25^{th}, 75^{th} percentile).

previously (Hoshino, Takeuchi, Kawai, et al., 1999). Wild type (WT) C57BL/6 J (male, 8–10 weeks age) were purchased from Jackson Lab (Bar Harbor, ME). T1DM was induced in mice by injecting low doses of streptozotocin (STZ; Sigma, St. Louis, MO, 60 mg/kg body weight, I/P daily for four consecutive days) as reported previously (Devaraj, Tobias, & Jialal, 2011; Devaraj, Tobias, Kasinath, et al., 2011; Leiter, 1982). Blood glucose levels were measured in tail veins of STZ injected WT mice, STZ injected TLR4 KO mice and WT mice after five days, and the mice with glucose level of >250 mg/dL on 3 consecutive days were considered diabetic. To avoid decompensation and weight loss and mimic human T1DM over the 17 weeks, we implanted subcutaneous slow release insulin pellets (Linshin Canada, Ontario, Quebec) as reported previously (Devaraj, Tobias, Kasinath, et al., 2011). The control group included non-diabetic WT mice. Previously we have shown no differences between the WT and TLR4KO non-diabetic mice and hence only used WT mice as the non-diabetic controls (Devaraj, Tobias, & Jialal, 2011). All the diabetic and control group mice were fed standard chow diet and had ad libitum access to food and water. Study animals were euthanized at 17 weeks after established diabetes. Blood and kidney tissues were collected, snap frozen, and stored at −80 °C. Plasma glucose, creatinine, triglycerides and cholesterol were assayed in the Veterinary Pathology Laboratory, UC Davis. The animal protocol was approved by the IACUC, University of California at Davis.

2.2. Immunohistochemistry (IHC)

All IHC studies were performed at Baylor College of Medicine. For the IHC studies, either snap frozen kidneys or formalin-fixed sections were used. The latter were deparaffinized and boiled for 10 minutes in 10 mM sodium citrate buffer (pH 6.0). Sections were then incubated with 10% normal horse serum followed by 60-minute incubation with primary antibodies; goat anti-mouse-TLR4 antibody (Santa Cruz Biotechnology, Santa Cruz, CA), rabbit anti-mouse WT-1 polyclonal antibody (Invitrogen, Carlsbad, CA), a rat anti-mouse CD68 antibody (AbD Serotec Inc., Oxford, UK), or an appropriate isotype negative control. Sections were exposed to H_2O_2 for 5 min and then incubated with biotinylated anti-rat IgG (BD Biosciences Pharmingen, San Diego, CA) or anti-rabbit IgG (BD Biosciences Pharmingen, San Diego, CA) or anti-goat IgG respectively (Vector Laboratories Inc., Burlingame, CA). A Vector stain ABC kit (Vector Laboratories Inc., Burlingame, CA) was applied to the tissue followed by DAB solution (DAKO). The slides were counterstained with haematoxylin. Immunostaining for alpha-smooth muscle actin (α-SMA) was performed on formalin-fixed paraffin sections using Dako ARK™, Peroxidase for Mouse Primary Antibodies (DAKO) according to the manufacturer's instructions. Also, immunofluorescence studies using anti-Laminin antibody was performed on acetone-fixed frozen sections after blocking with 10% normal horse serum at 4 °C overnight. For detection, sections were incubated with an FITC-conjugated anti-rabbit antibody for 1 hour. Sections were then mounted in ProLong® Gold antifade reagent (Invitrogen, Carlsbad, CA). Collagen in the kidney sections was stained using Picrosirius red. All sections were analyzed using 20× high power lenses in a Nikon microscope. Quantification of immunostaining was assessed for all of the images using ImageJ and expressed as percentage of positive area stained.

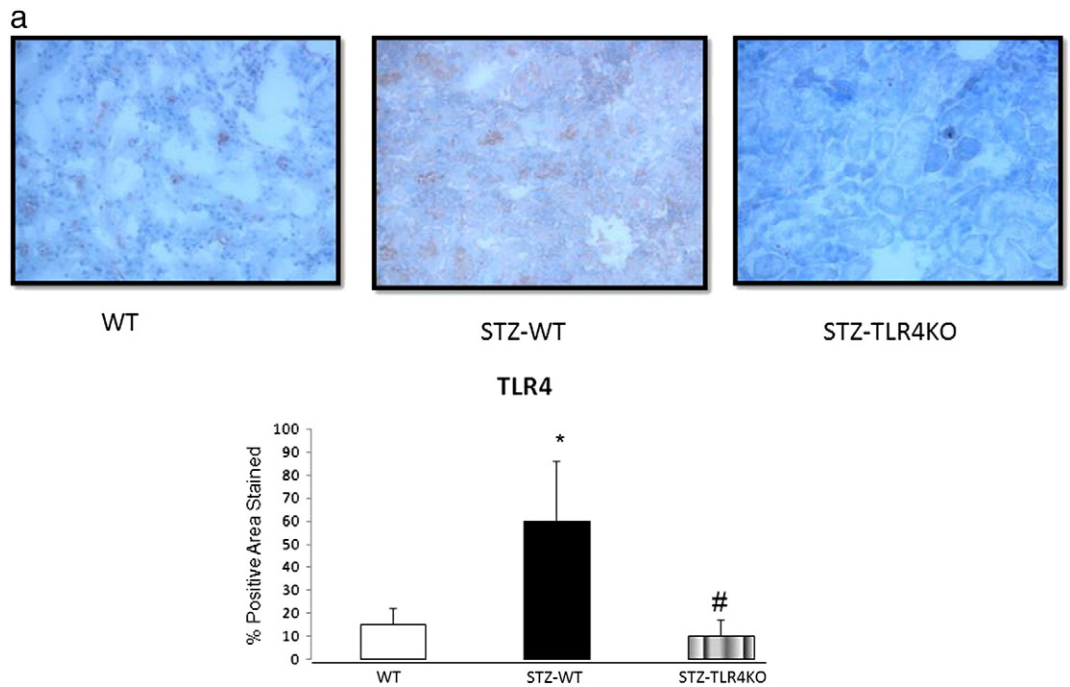

Fig. 1. a: Top: Representative sections of kidney were stained with goat anti-mouse-TLR-4 polyclonal antibody (Invitrogen, Carlsbad, CA) which stains TLR-4, mainly in tubules as seen by the red staining. Bottom: %positive area stained: *p < 0.05 compared to WT and #p < 0.01 compared to STZ. b: Top: Representative western blotting of kidney lysates using rabbit anti-mouse-MyD88 polyclonal antibody (Invitrogen, Carlsbad, CA). Bottom: Densitometric ratio vs. actin from n = 4 experiments. *p < 0.05 compared to WT and #p < 0.01 compared to STZ. c: Top: Representative western blotting of kidney lysates using a rat pp65 antibody (Santa Cruz Biotechnology, Santa Cruz, CA) as described in Methods. Bottom: Densitometric ratio vs. actin from n = 4 experiments. *p < 0.01 compared to WT and #p < 0.05 compared to STZ.