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Vascular Endothelial Growth Factor-A_{165b} Restores Normal Glomerular Water Permeability in a Diphtheria-Toxin Mouse Model of Glomerular Injury

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Keywords

Glomerulus · Permeability · Diphtheria-toxin · Podocyte · Vascular endothelial growth factor-A_{165b}

Abstract

Background/Aims: Genetic cell ablation using the human diphtheria toxin receptor (hDTR) is a new strategy used for analysing cellular function. Diphtheria toxin (DT) is a cytotoxic protein that leaves mouse cells relatively unaffected, but upon binding to hDTR it ultimately leads to cell death. We used a podocyte-specific hDTR expressing (Pod-DTR) mouse to assess the anti-permeability and cyto-protective effects of the splice isoform vascular endothelial growth factor (VEGF-A_{165b}). **Methods:** The Pod-DTR mouse was crossed with a mouse that over-expressed VEGF-A_{165b} specifically in the podocytes (Neph-VEGF-A_{165b}). Wild type (WT), Pod-DTR, Neph-VEGF-A_{165b} and Pod-DTR X Neph-VEGF-A_{165b} mice were treated with several doses of DT (1, 5, 100, and 1,000 ng/g bodyweight). Urine was collected and the glomerular water permeability (L_pA/V_i) was measured ex vivo after 14 days. Structural analysis and podocyte marker expression were also assessed. **Results:** Pod-DTR mice developed an increased glomerular L_pA/V_i 14 days after administration of DT

(all doses), which was prevented when the mice over-expressed VEGF-A_{165b}. No major structural abnormalities, podocyte ablation or albuminuria was observed in Pod-DTR mice, indicating this to be a mild model of podocyte disease. However, a change in expression and localisation of nephrin within the podocytes was observed, indicating disruption of the slit diaphragm in the Pod-DTR mice. This was prevented in the Pod-DTR X Neph-VEGF-A_{165b} mice. **Conclusion:** Although only a mild model of podocyte injury, over-expression of the anti-permeability VEGF-A_{165b} isoform in the podocytes of Pod-DTR mice had a protective effect. Therefore, this study further highlights the therapeutic potential of VEGF-A_{165b} in glomerular disease.

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Introduction

Podocytes, glomerular endothelial cells (GENCs) and mesangial cells function together to form and maintain the glomerular filtration barrier (GFB), which serves to filter water and small solutes from the blood into Bowman's space while restricting the passage of large nega-

tively charged plasma proteins, such as albumin [1]. Injury to any of these cells types can result in renal dysfunction. Podocyte damage is well documented to play a key role in glomerular pathologies; rearrangement of the proteins that maintain the slit diaphragm can result in retraction/effacement of the podocyte foot processes, ultimately causing proteinuria [2]. Podocyte-targeted glomerular injury is problematic as podocytes lack the ability to proliferate in response to neighbouring cell loss.

Genetic cell ablation using the human diphtheria toxin receptor (hDTR) is a relatively new strategy for analysing cellular function: the use of a cytotoxic gene under the control of a cell/tissue specific promoter expressed in transgenic mice [3]. Diphtheria toxin (DT) is a cytotoxic protein that is secreted by the bacterium *Corynebacterium diphtheriae*. Upon binding of the larger B subunit of DT to the hDTR, on the cell surface, the toxin is internalised within acidic endosomes. The acidic environment allows the smaller A subunit to dissociate and translocate to the cytosol where it becomes enzymatically active and ADP-ribosylates elongation factor-1. This ultimately inactivates protein synthesis and results in cell death [4].

While most other transgenic models of podocyte injury cause rapid and irreversible destruction of podocytes (e.g., the Thy1.1 model; anti-Thy1.1 is injected in to mice expressing ectopic Thy1.1 in the podocytes) [5], the hDTR system allows ablation of podocytes in vivo in a dose-dependent manner [6, 7]. Murine cells are approximately 1,000-fold less susceptible to DT than human cells due to an alternative region of amino acids in the extracellular domain of murine DTR [4]. Wild-type (WT) mice receiving a single dose of 50 ng/g bodyweight (bw) DT have been reported to have no adverse effects [4]. However, more recent evidence states that an adverse effect to DT in WT mice may be strain dependent, as certain strains developed transient albuminuria in response to multiple doses of DT [8].

Murine models that express hDTR on the podocytes have previously been used to study glomerular disease in rats [7] and mice [6]. Furthermore, a mouse model that expressed DT within the podocytes has also been described [9]. All 3 models provide strong evidence for podocyte depletion resulting in glomerulosclerosis and proteinuria in a dose-dependent manner. In our study, we used a mouse model that expressed podocyte-specific hDTR using a fragment of the nephrin promoter. Upon intraperitoneal injection of DT, mice have been reported to develop proteinuria and glomerulosclerosis [10].

Vascular endothelial growth factor A (VEGF-A) has been implicated in several types of kidney pathology, in-

cluding chronic renal failure [11, 12]. Alternative splicing of exon 8 of the VEGF-A gene gives rise to 2 functionally different isoform families – the pro-angiogenic VEGF-A_{xxx} family (main renal isoform: VEGF-A₁₆₅) and the anti-angiogenic VEGF-A_{xxx}b family (main renal isoform: VEGF-A₁₆₅b) [1]. VEGF-A₁₆₅b has been suggested to comprise around 45% of the total VEGF-A protein in the human adult renal cortex [13]. This could explain the VEGF-A paradox in the kidney; a vast amount of VEGF-A is expressed by the podocytes, signalling to VEGFR-2 on the GEnCs; however, endothelial cell proliferation in the mature kidney is low. VEGF-A₁₆₅b reduces GEnC permeability, inhibits cell migration and is cyto-protective to podocytes and GEnCs in culture, as well as in mouse models of diabetic nephropathy [13–15]. Furthermore, VEGF-A₁₆₅b significantly reduces the phosphorylation of VEGFR-2 in HUVECs, compared to VEGF-A₁₆₅ [16], explaining its anti-angiogenic and anti-permeability properties. The Neph-VEGF-A₁₆₅b mouse over-expresses human VEGF-A₁₆₅b under a nephrin promoter in a constitutive manner [17]. While over-expression of the pro-angiogenic isoform of VEGF-A (VEGF-A₁₆₄) in mouse podocytes causes a glomerular disease phenotype [18, 19], the Neph-VEGF-A₁₆₅b mice are viable through adulthood and present only with a phenotype of reduced glomerular water permeability likely to be due to reduced endothelial fenestral density [17].

This study aimed to determine the cyto-protective properties of VEGF-A₁₆₅b over-expression in an established model of podocyte injury. A double transgenic mouse model was generated, which expressed both hDTR and human VEGF-A₁₆₅b in the podocytes. We propose that podocyte-expressed VEGF-A₁₆₅b could prevent disruption of the GFB in the Pod-DTR model, and therefore protect normal renal function.

Methods

Animals

All experiments were conducted in accordance with UK legislation and were approved by the University of Bristol research Ethics Committee. All experiments and procedures were approved by the UK Home office in accordance with the Animals (Scientific Procedures) Act 1986, and the Guide for the Care and Use of Laboratory Animals was followed.

The Pod-DTR mice were a gift from Neil Turner, University of Edinburgh, UK. The hDTR is expressed under a fragment of the nephrin promoter, resulting in podocyte-specific expression. The Neph-VEGF-A₁₆₅b mice were generated and bred in-house [17]. Human VEGF-A₁₆₅b is expressed under a nephrin promoter.

The breeding strategy involved crossing Pod-DTR^{+/-} mice with Neph-VEGF-A₁₆₅b^{+/-} mice, both on a c57BL/6 background, to generate litters with the 4 experimental groups required: wild-type (WT), Pod-DTR^{+/-}, Neph-VEGF-A₁₆₅b^{+/-}, and Pod-DTR^{+/-} X Neph-VEGF-A₁₆₅b^{+/-} mice. Both transgenes were kept heterozygous throughout and were identified by PCR analysis of genomic DNA using the following primer pairs: Pod-DTR; 5', 5'-GGTG GTGCTGAAGCTCTTTC-3'; 3', 5'-GCTTGTGGCTTGGAGGA TAA-3'. Neph-VEGF-A₁₆₅b; 5', 5'-TCAGCGCAGCTACTGCC ATC-3'; 3', 5'-GTGCTGGCCTTGGTGAGGTT-3'. A 218-bp fragment was amplified when the Pod-DTR transgene was present, and a 200-bp fragment when the Neph-VEGF-A₁₆₅b transgene was present.

DT Toxicity

Precautions were taken to reduce the risk of DT toxicity to animal handlers. All handlers had previously been immunised with DTP (diphtheria, tetanus and pertussis) vaccine. DT (Sigma Aldrich) was prepared under a fume hood with full human protection, and special care was taken during administration of DT to animals. Upon completion of procedures, all surfaces were wiped down with 1 M NaOH to inactivate any DT that may have spilled. A previous study did not find any active DT in the urine or faeces from rats administered up to 500 ng/g bw DT [7], and therefore no special precautions were necessary here.

DT Experimental Protocol

Mice (3–5 per experimental groups) received a single intraperitoneal injection of DT diluted in PBS (or vehicle control) at 6 weeks of age. Mice were culled by a schedule 1 method 14 days later. Metabolic cages were used to collect urine the day before DT administration, and then on days 1, 3, 5, 7, 10, and 14 post-DT administration. Mice were inspected daily and if signs of illness developed, they were culled before the 14-day time point. Four doses of DT were used; 1, 5, 100, and 1,000 ng/g bw DT. No obvious gender differences were observed and therefore both male and female mice were included in the study.

Albumin and Creatinine Assays

Urine albumin measurements were assayed using a mouse-specific albumin ELISA Quantitation Set (Bethyl Laboratories). Urine creatinine was assayed using an Enzymatic Creatinine Assay (Thermo Scientific). Both assays were run with 3 technical repeats.

Oncometric Assay for Glomerular Water Permeability

Upon culling mice on day 14, kidneys were immediately removed and placed in ice cold PBS; glomeruli were isolated from one pole using a set of sieves. Glomeruli were kept in 1% Bovine Serum Albumin (BSA; Sigma Aldrich) in mammalian Ringer solution for no more than 4 h. During the isolation, the concentration of plasma proteins within the glomerular capillaries equilibrates with the surrounding 1% BSA-Ringer solution. Single glomeruli were immobilised onto a micropipette tip using suction, within a micro-slide positioned under a microscope. The glomerulus is firstly perfused with 1% BSA, which is then switched to 8% BSA. The change in oncotic pressure results in fluid efflux from the glomerular capillaries, thereby changing the glomerular volume. The initial rate of volume change is used to estimate the glomerular water permeability normalised to glomerular volume. A more detailed methodology was previously published by

Salmon et al. [20]. The glomerular water permeability (L_pA/V_i) was measured in 4–6 glomeruli per mouse, from 3 to 5 mice per group.

Immunohistochemistry and Immunofluorescence

For immunohistochemistry studies, fresh renal cortex was fixed in 4% PFA and subsequently paraffin-embedded. Sections that were 5 μ m thick were air-dried for 2 h prior to fixing with 4% PFA for 20 min. Antigen retrieval required heating slides in 0.01 M sodium citrate buffer (pH 6.0) for 12 min. Endogenous peroxidase activity was blocked with 3% (w/v) hydrogen peroxide and non-specific IgG binding was then blocked with 1% BSA and 5% normal horse serum in PBS-Triton-X (0.05%) for 1 h. Primary antibody (human HB-EGF polyclonal Ab; R&D systems), or an IgG control, was applied to sections overnight. The next day, sections were incubated with a biotinylated secondary antibody (donkey anti-goat IgG; Vector Labs), followed by avidin-biotinylated enzyme complex buffer (Vector Labs), and then DAB substrate (Vector Labs) until sections changed colour from purple to brown. Sections were counter-stained with haematoxylin.

For immunofluorescence (IF) studies, kidney cortex was snap frozen in optimum cutting temperature embedding compound and sectioned using a cryostat. Sections, 5 μ m thick, were then fixed with 4% PFA for 20 min. Sections were blocked with 3% BSA and 5% normal goat serum before incubating with primary antibody (anti-nephrin; Acris) overnight. The next morning, sections were incubated with an Alexa Fluor secondary antibody (goat anti-guinea pig; Life Technologies).

IHC and IF were performed on 3 sections of kidney cortex (technical repeats) per mouse, from 3 mice per group (biological repeats). Between 3 and 8 glomeruli were analysed from each technical repeat. Images were taken at 400 \times magnification.

Western Blotting

Protein was extracted from sieved glomeruli using NP-40 lysis buffer (Fisher Scientific) and subjected to polyacrylamide gel electrophoresis (PAGE) on an SDS-polyacrylamide gel electrophoresis gel (BIO-RAD). After blotting, the membrane was probed with guinea pig polyclonal anti-nephrin (Acris) and rabbit polyclonal anti-podocin (Santa Cruz Biotechnology) overnight. The next day the bands were detected using fluorescent secondary antibodies (goat anti-guinea pig and goat anti-rabbit; Li-Cor Odyssey) and then imaged on a Li-Cor Odyssey.

Protein was extracted from 3 mice per group, and 3 technical repeats were performed. Intensities were calculated using ImageJ.

Transferase dUTP Nick End Labelling Assay

PFA-fixed sections of kidney cortex were subjected to a fluorescent terminal deoxynucleotidyl transferase dUTP nick end labelling assay (Promega) to detect levels of DNA fragmentation resulting from apoptotic signalling cascades in the glomerulus. A positive DNase-treated control was used. Experiments were performed on sections from 3 mice per group, with 3 technical repeats. Between 3 and 5 glomeruli were analysed per repeat. Images were taken at 400 \times magnification.

Electron Microscopy

The ultra-structural phenotype was determined by fixing 1 mm³ diced kidney cortex in 2.5% glutaraldehyde, post-fixing in 1% osmium tetroxide, followed by embedding in Araldite (Agar

Scientific). Sections were cut at 50–100 nm and stained with 3% aqueous uranyl acetate and Reynolds' lead citrate solution. Once imaged, measurements of the GFB were taken at random points using Adobe Photoshop. EM was carried out on the kidney cortex of 3 mice per group, 3 glomeruli per mouse.

Statistical Analysis

GraphPad Prism was used to conduct the statistical analysis. Values are expressed as mean \pm SE of the mean (SEM). Fold change was calculated for uACR measurements by normalising the value for each individual animal at each time point to the baseline uACR for that particular animal, measured before experiments began. An unpaired Student *t* test was used for statistical analysis of datasets involving 2 groups. A one-way analysis of variance was used for statistical analysis of datasets with 3 or more groups, with a Bonferroni post hoc test for comparison between pairs. Statistical significance was set at $p < 0.05$.

Results

hDTR Is Expressed on the Podocytes of Pod-DTR Mice

Human DTR was detectable by immunohistochemistry in the glomeruli of Pod-DTR but not in WT mice (Fig. 1a). No hDTR was seen in the cells of the Bowman's capsule. Therefore, we confirmed that the construct used to generate the Pod-DTR mouse strain (nephrin promoter-driven hDTR) resulted in the expression of hDTR on the podocytes at 6 weeks of age. Neph-VEGF-A₁₆₅b mice were confirmed to express glomerular human VEGF-A₁₆₅b as previously described [17].

DT Increases Glomerular Water Permeability in Pod-DTR Glomeruli, Which Is Rescued by Podocyte Expression of VEGF-A₁₆₅b

In one of our previous studies [14, 20], we have shown that measurement of glomerular permeability to water is an accurate and sensitive way to determine glomerular functional integrity. All mice were culled on day 14 post-DT/PBS administration, and glomeruli were isolated for L_pA/V_i measurements *ex vivo*. These were conducted on coded glomeruli by an observer blinded to the origin of the glomeruli studied. Pod-DTR mice treated with 1 ng/g bw DT had a significantly increased glomerular L_pA/V_i 14 days later (0.72 ± 0.14 mm Hg⁻¹ min⁻¹), compared to WT littermate controls (0.38 ± 0.045 mm Hg⁻¹ min⁻¹; $p < 0.05$; Fig. 1b). This was also the case when Pod-DTR mice were treated with 5 and 1,000 ng/g DT ($p < 0.05$; Fig. 1c, d). However, when Pod-DTR mice also over-expressed VEGF-A₁₆₅b in the podocytes (Pod-DTR X Neph-VEGF-A₁₆₅b), glomerular L_pA/V_i was rescued back to values observed in the DT-treated WT and Neph-VEGF-A₁₆₅b

controls groups ($p < 0.01$; Fig. 1b–d). Furthermore, there was no significant increase in glomerular L_pA/V_i observed between vehicle-treated Pod-DTR and DT-treated WT glomeruli ($p < 0.05$; Fig. 1d), indicating that the increases in glomerular L_pA/V_i observed in the Pod-DTR mice were due to the administration of DT.

We can therefore conclude that the Pod-DTR model does develop increased glomerular L_pA/V_i in response to DT, although not in a dose-dependent manner. This rise in permeability is rescued when the podocytes over-express VEGF-A₁₆₅b.

DT Administration Does Not Result in an Albuminuric Phenotype in Pod-DTR Mice

Data from previous characterization of this model suggested a starting dose of 1 ng/g DT [10]. When this was injected intraperitoneally into WT, Pod-DTR, Neph-VEGF-A₁₆₅b and Pod-DTR X Neph-VEGF-A₁₆₅b mice, no increases in uACR were observed in any of the groups on days 3, 5, 7, 10, and 14 post-DT administration ($p = ns$; Fig. 2a). Therefore, higher doses of DT were administered; 5, 100, and 1,000 ng/g. None of the doses used resulted in an increased uACR ($p = ns$; Fig. 2b–d). At the highest dose of DT, another group was added as an additional control: Pod-DTR mice were administered vehicle and not DT, as the mice may be sensitive to DT at such a high dose. However, no increases in uACR were observed in the DT-treated groups compared to vehicle-treated controls ($p = ns$; Fig. 2d). Therefore, the Pod-DTR model did not develop albuminuria in response to a range of doses of DT.

DT Administration Did Not Result in Any Structural Abnormalities in Pod-DTR Mice

We examined the structure of Pod-DTR mouse glomeruli using light microscopy to determine whether treatment with a single dose of DT measurably altered the glomerular structure. As shown in Figure 3ai and ii, treatment of Pod-DTR and WT littermates with DT (1,000 ng/g bodyweight) did not result in any detectable structural abnormalities, such as mesangial matrix expansion and glomerulosclerosis, 14 days later.

Furthermore, administration of DT did not result in any detectable changes to the glomerular ultra-structure, as assessed by EM (Fig. 3aiii, iv). There were no significant changes in the GBM thickness ($p = ns$; Fig. 3bi), endothelial fenestration number per μ m length ($p = ns$; Fig. 3bii), or in the podocyte slit width ($p = ns$; Fig. 3biii), 14 days after administration of 1,000 ng/g bw DT to Pod-DTR mice and WT littermates.

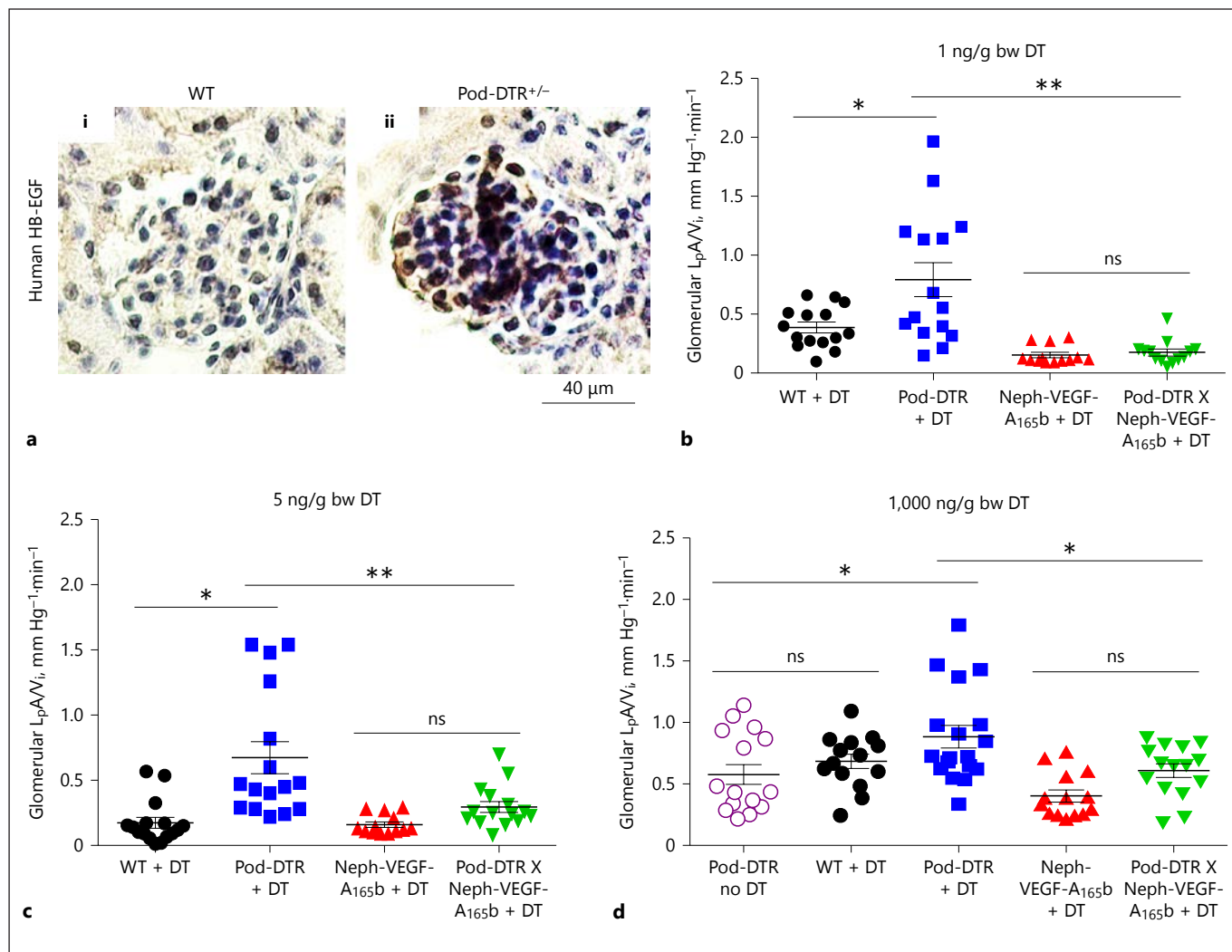


Fig. 1. Podocyte-specific expression of human diphtheria toxin receptor (hDTR) results in an increased glomerular water permeability, which is prevented in Pod-DTR X Neph-VEGF- A_{165b} mice. **a** hDTR (HB-EGF) protein was detected in glomeruli of transgenic Pod-DTR (**aii**) but not wild-type (WT) mice (**ai**). **b-d** Glomerular water permeability (L_pA/V_i) was elevated 14 days after a single dose of 1, 5, and 1,000 ng/g bw DT in Pod-DTR mice compared to

DT-treated WT and untreated Pod-DTR controls (* $p < 0.05$). Increased L_pA/V_i was significantly rescued in Pod-DTR X Neph-VEGF- A_{165b} mice at all doses of DT (* $p < 0.05$, ** $p < 0.01$; $n = 3-5$ mice, 13-24 glomeruli; 1 way analysis of variance [ANOVA] with Bonferroni comparison between pairs was used to analyse the data).

DT Administration Did Not Result in Podocyte Loss in the Pod-DTR Mouse

A transferase dUTP nick end labelling assay did not detect any increases in cell apoptosis in Pod-DTR glomeruli 14 days after mice were treated DT (1,000 ng/g), when compared to DT-treated WT littermate controls ($p = ns$; Fig. 4). This therefore confirmed that DT administration in this model of Pod-DTR mice did not cause podocyte cell death, in contrast with the published literature on similar cell ablation models [6, 7].

DT Results in Altered Nephryn Staining and Expression in Pod-DTR Glomeruli, Which Is Partially Rescued by VEGF- A_{165b}

Kidney sections were stained for nephryn, a podocyte marker, to assess podocyte and glomerular integrity. Pod-DTR mice treated with 5 and 1,000 ng/g DT had a marked reduction in glomerular nephryn staining 14 days later in comparison to DT-treated WT and vehicle-treated Pod-DTR controls ($p < 0.05$; Fig. 5a), expressed as a percentage glomerular area in Figure 5b. When Pod-DTR X Neph-

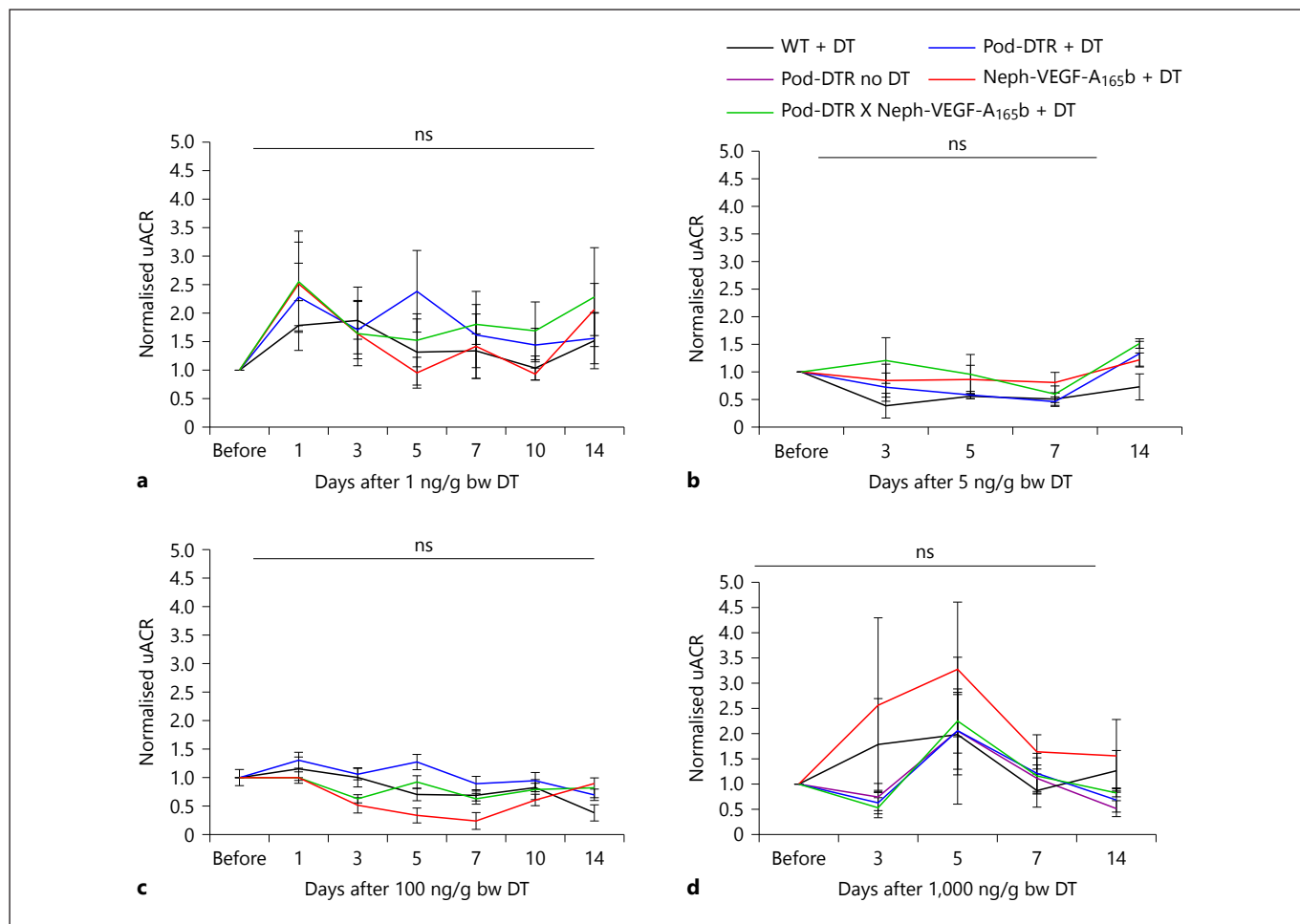


Fig. 2. Pod-DTR mice do not become albuminuric with varying doses of DT. The urinary albumin: creatinine ratio (uACR) was measured at various time points after DT administration and then normalised to baseline uACR in the 4 groups of mice; WT, Pod-DTR, Neph-VEGF-A₁₆₅b, and Pod-DTR X Neph-VEGF-A₁₆₅b. No increases in uACR were observed in Pod-DTR mice at any dose

of DT; 1 ng (**a**), 5 ng (**b**), 100 ng (**c**) and 1,000 ng/g bw (**d**), up to 14 weeks post DT treatment, when compared to DT-treated WT, Neph-VEGF-A₁₆₅b, Pod-DTR X Neph-VEGF-A₁₆₅b and untreated Pod-DTR littermate controls ($n = 3-5$ mice, $p = ns$; 1 way analysis of variance [ANOVA] with Bonferroni comparison between pairs).

VEGF-A₁₆₅b mice received the same dose of DT, the percentage glomerular area stained with nephrin was significantly increased, in comparison with Pod-DTR mice, towards that measured in DT-treated WT mice. Therefore, over-expression of VEGF-A₁₆₅b in the podocytes of Pod-DTR mice prevented the DT-induced reduction in nephrin staining seen in the Pod-DTR mice at both doses ($p < 0.05$; Fig. 5a, b).

The renal cortex protein expression of 2 key podocyte markers, nephrin and podocin, was evaluated by WB in all 4 groups of mice treated with 5 and 1,000 ng/g DT. There was a significant reduction of nephrin in Pod-DTR mice and rescue of the expression in Pod-DTR X Neph-

VEGF-A₁₆₅b mice, correlating with the immunostaining ($p < 0.05$; Fig. 5c). There was no evidence of a consistent reduction in podocin proteins in Pod-DTR mice in comparison to DT-treated WT controls ($p = ns$; Fig. 5c).

Discussion

The Pod-DTR mouse was confirmed to express hDTR within the glomeruli and was successfully crossed with the Neph-VEGF-A₁₆₅b mouse to generate a double transgenic that expressed both hDTR and human VEGF-A₁₆₅b on/within the podocyte. Pod-DTR mice treated with

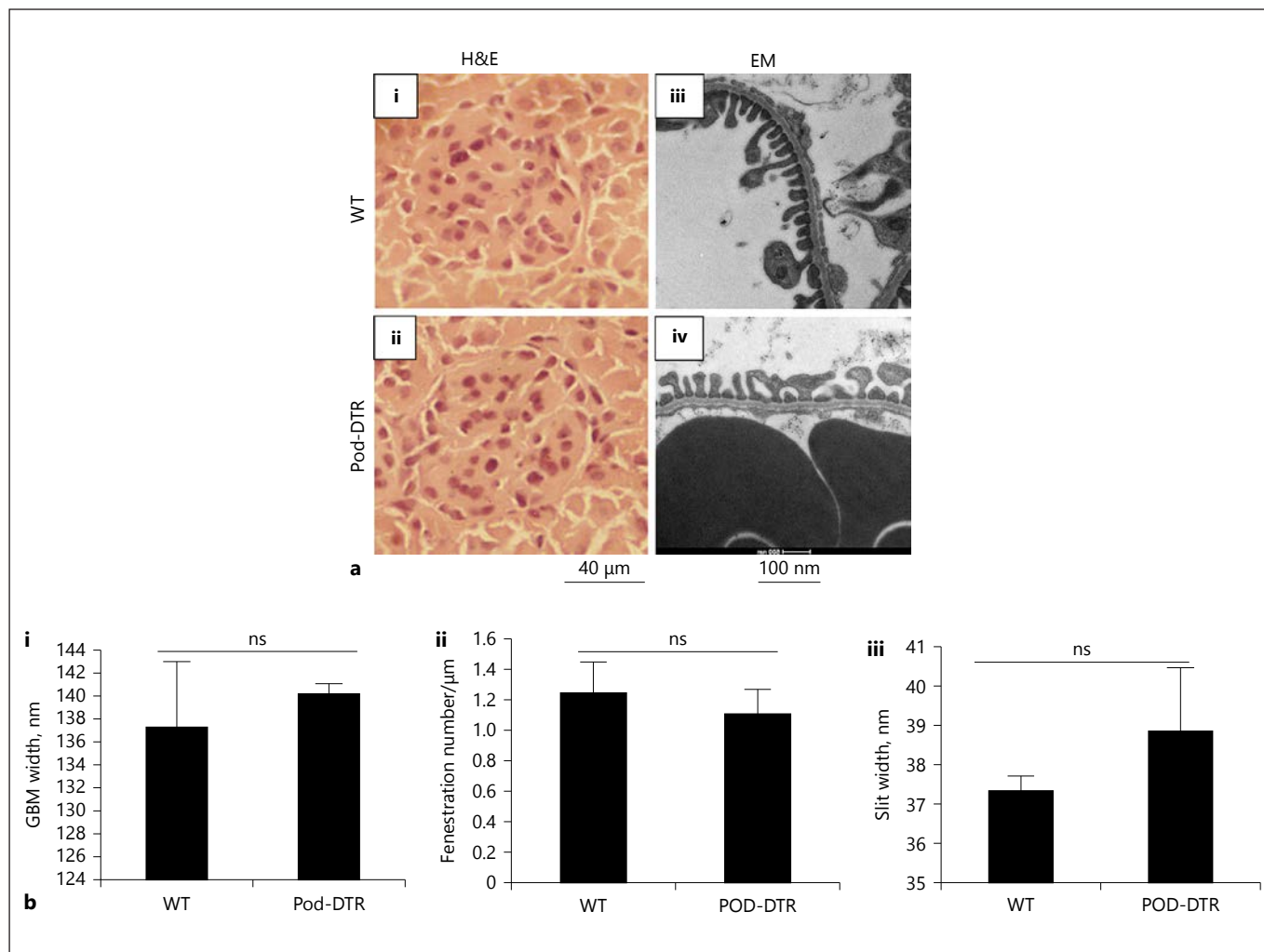


Fig. 3. Pod-DTR mice did not develop any glomerular structural abnormalities. **a** Haematoxylin and Eosin (H&E) staining did not detect any glomerular structural abnormalities in Pod-DTR mice treated with up to 1,000 ng/g bw DT after 14 days compared with DT-treated WT controls ($n = 3$ mice, 10–12 glomeruli, images taken at 400 \times magnification). **b** Electron microscopy of the GBM in

Pod-DTR mice treated with 1,000 ng/g bodyweight DT after 14 days did not show any changes in GBM width (**bi**), endothelial fenestrae number (**bii**) or podocyte slit width (**biii**), in comparison to DT-treated WT controls ($n = 3$ mice, 9 glomeruli, $p = ns$, unpaired t test).

varying doses of DT did not become albuminuric after 2 weeks, or develop any detectable glomerular structural/ultra-structural abnormalities. However, there was evidence of impaired podocyte function as DT did result in a significant increase in glomerular water permeability (L_pA/V_i) in Pod-DTR mice, although not in a dose-dependent manner. This phenotype was accompanied by a 40–50% reduction in nephrin staining intensity, as assessed by IF and Western blot, in Pod-DTR mice, indicative of potential podocyte injury possibly related to the rearrangement of the proteins of the slit diaphragm, specifically nephrin. The total expression of podocyte-specific

protein podocin remained unchanged in DT-treated Pod-DTR glomeruli, and no increase in podocyte apoptosis was observed, leading us to conclude that DT is not resulting in podocyte ablation. Over-expression of human VEGF- A_{165b} within the podocytes of Pod-DTR mice resulted in the prevention of the increased glomerular L_pA/V_i and the alterations in nephrin staining, indicating that VEGF- A_{165b} has a cyto-protective effect on the podocytes in response to DT.

This colony of Pod-DTR mice were previously characterised and a single dose of 1 ng/g bw DT was stated as the optimal dose to induce transient albuminuria 7–14 days

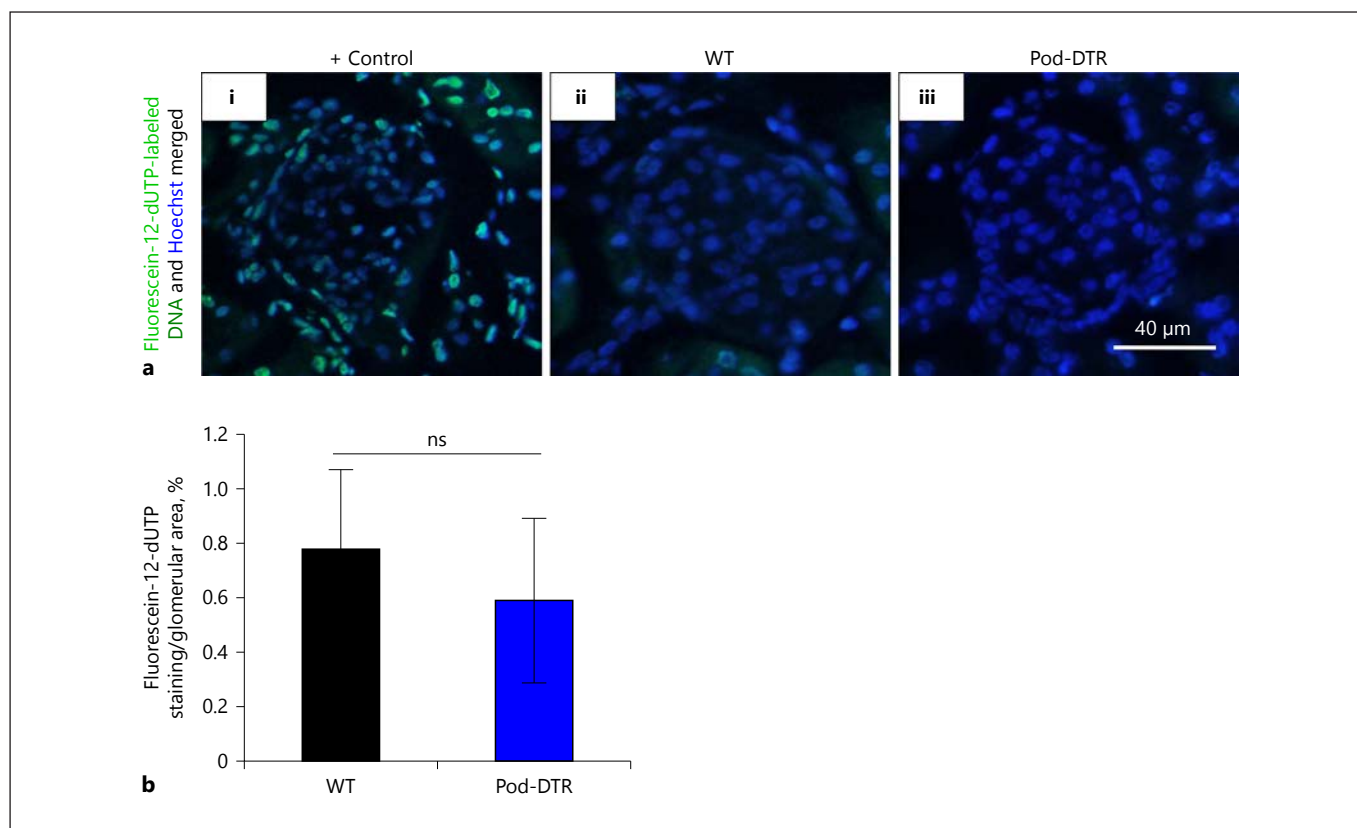


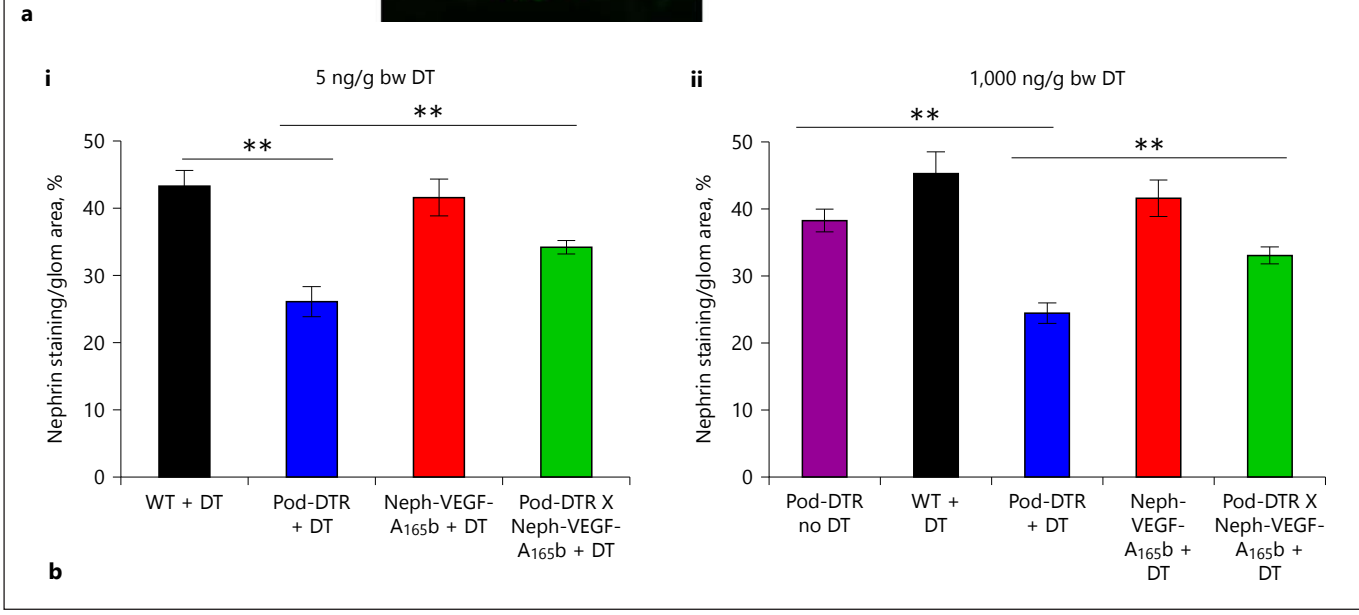
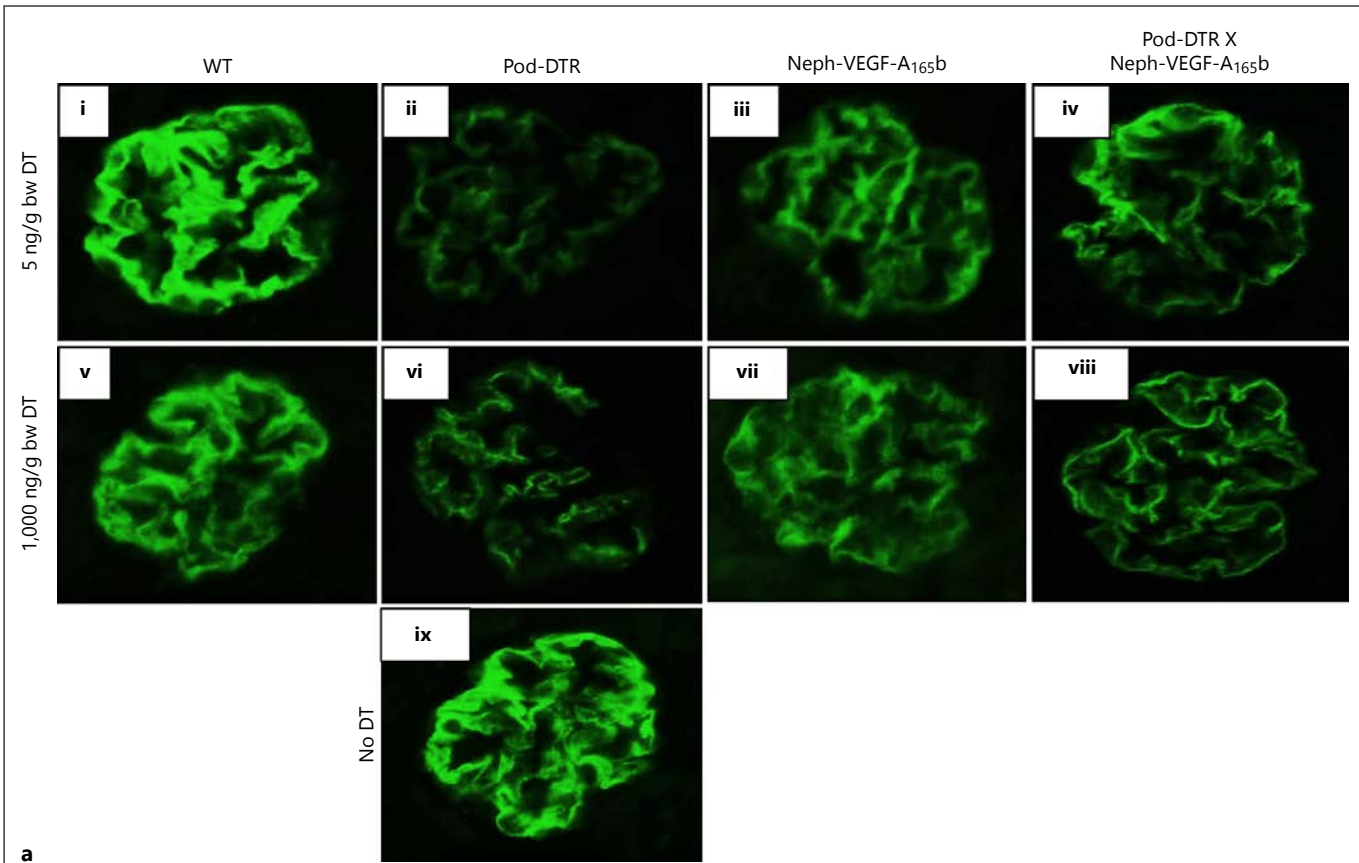
Fig. 4. Pod-DTR mice treated with DT did not exhibit podocyte loss. **a** TUNEL staining showed no increased cell apoptosis in glomeruli from Pod-DTR mice 14 days after treatment with 1,000

ng/g bw DT, in comparison to DT-treated WT littermate controls (summarised in **b**). DNase treatment served as a positive control ($n = 3$ mice, $p = ns$, unpaired t test).

later [10]. Studies using similar podocyte expressing hDTR models found an optimal dose of 25 ng/g bw DT was appropriate to induce transient proteinuria 7 days later [6, 7]. Within the current study, we found that even with a dose of 1,000 ng/g bw DT, no albuminuria was observed within the first 2 weeks post-administration. Generally, few mouse strains develop high levels of proteinuria with it being widely accepted that C57BL/6 mice are very resistant to high levels of proteinuria and renal disease [21]. However, the problem lies that many genetically altered mice are currently on a C57BL/6 background, including the mice used within this study.

This is the first study to measure the glomerular L_pA/V_i in a podocyte-specific injury model of glomerular disease. This accurate and reproducible assay, although not widely used due to its technical difficulties, is a robust method of measuring the isolated glomerular ultra-filtration coefficient independent of circulating and haemodynamic factors [20, 22]. Recent studies have correlated an increased glomerular L_pA/V_i to albuminuria

[15], which has been found to be a more sensitive assay to changes in GFB function than measuring the uACR [14]. Although the Pod-DTR mice do not become albuminuric in response to DT, there is a significant increase in glomerular L_pA/V_i at all doses of DT administered. Therefore, our study confirms that this oncometric glomerular permeability assay is a more sensitive technique for detecting subtle changes in glomerular function. At this stage, it appeared reasonable to assume that DT was causing a low amount of podocyte ablation in the Pod-DTR mice to account for this phenotype. However, when we measured cell apoptosis within the glomerulus it became evident that DT-treated Pod-DTR mice had no increase in podocyte apoptosis. This was further supported by the lack of reduction in expression of podocyte-specific proteins podocin, in DT-treated Pod-DTR glomeruli, and no detectable podocyte structural abnormalities. When looking at the arrangement of nephrin staining within the glomeruli of Pod-DTR mice, there did appear to be changes in the intensity of nephrin ex-



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(For figure 5c and legend see next page.)

pression; reducing in response to DT in Pod-DTR glomeruli, and a slight reduction in the expression of the protein by Western blot at 5 ng/g but not 1,000 ng/g. This suggests a rearrangement of nephrin within the podocyte in response to DT, resulting in the dysfunction of

the slit diaphragm and thus increased glomerular water permeability phenotype. Indeed, the interaction between nephrin and Neph1 in the slit diaphragm has previously been reported to alter glomerular permeability [23].

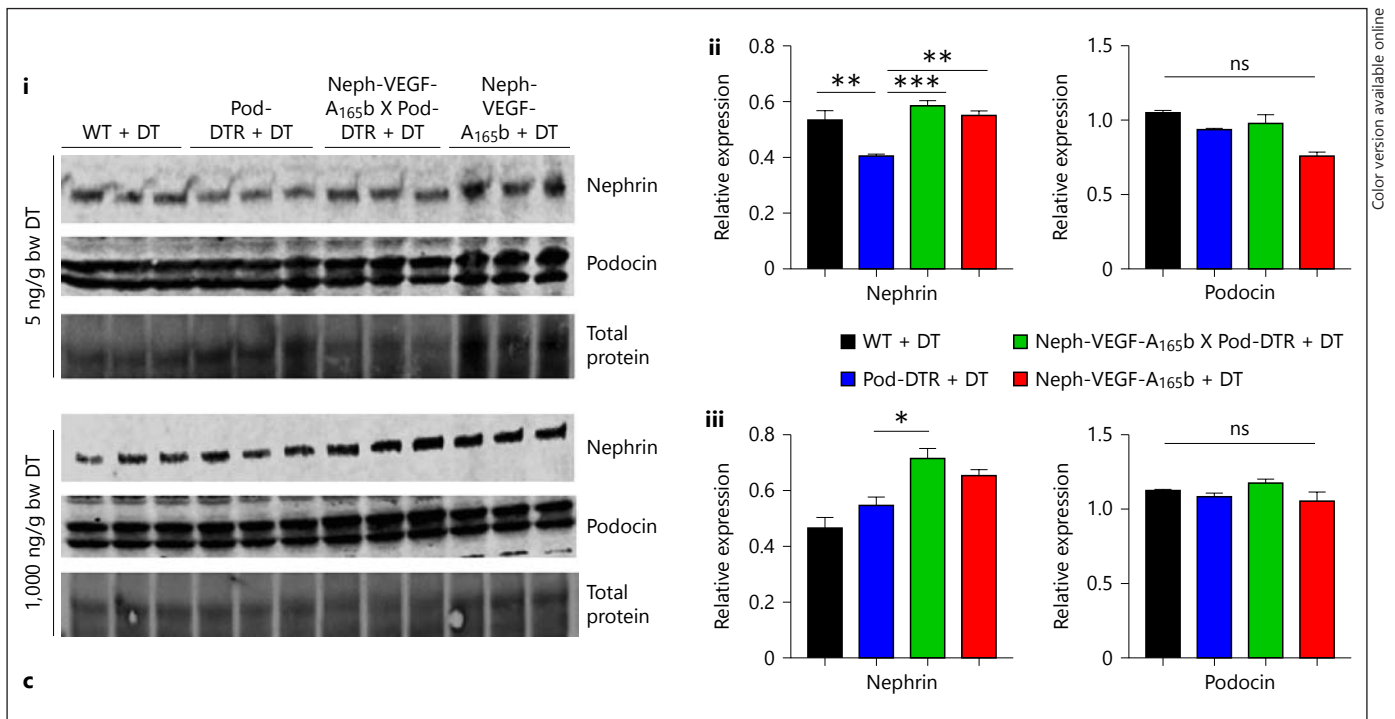


Fig. 5. Nephrin staining and expression are altered in Pod-DTR glomeruli after DT administration and this is rescued by podocyte expression of VEGF-A₁₆₅b. **a** Kidney sections from WT, Pod-DTR, Neph-VEGF-A₁₆₅b and Pod-DTR X Neph-VEGF-A₁₆₅b treated with 5 and 1,000 ng/g bw DT, and untreated Pod-DTR^{+/-} mice, were stained for nephrin as an indicator of podocyte function. Nephlin staining is expressed as a percentage of the total glomerular area (**b**). DT resulted in a decrease in glomerular nephrin staining in Pod-DTR mice (** $p < 0.01$), which is rescued at both doses by podocyte expression of VEGF-A₁₆₅b (** $p < 0.01$). DT-treated

Neph-VEGF-A₁₆₅b and untreated Pod-DTR mice showed no difference to DT-treated WT controls at either concentration of DT. Images taken at 400× magnification ($n = 3$ mice, 1 way analysis of variance [ANOVA] with Bonferroni comparison between pairs was used to analyse the data). **c** The protein expression of nephrin, but not podocin, within the glomerular cortex was decreased in Pod-DTR mice after treatment with 5 but not 1,000 ng/g bw DT, in comparison to WT, Neph-VEGF-A₁₆₅b and Pod-DTR X Neph-VEGF-A₁₆₅b mice ($n = 3$ mice, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, 1 way ANOVA with Bonferroni comparison between pairs).

Despite only observing a mild phenotype in this model, over-expression of VEGF-A₁₆₅b in the podocytes of the Pod-DTR mice did result in the prevention of the increased glomerular L_pA/V_i and proposed rearrangement of nephrin. This correlates with previous studies where VEGF-A₁₆₅b over-expression/treatment was found to reduce glomerular L_pA/V_i , both in vivo and ex vivo, in healthy murine and human glomeruli [14, 17], and in cases of murine and human glomerular disease [14, 15]. Furthermore, VEGF-A₁₆₅b exhibits cyto-protective properties when applied to podocytes in culture [24], and thus appears to be protective against detrimental alterations to nephrin induced by DT in the Pod-DTR model of glomerular disease. We speculate that the mechanism of VEGF-A₁₆₅b in reducing the glomerular L_pA/V_i may be due to its previously reported effects on reducing the glomerular endothelial fenestration density, thus reducing

permeability [17]. Furthermore, the treatment of conditionally immortalised GENCs with VEGF-A₁₆₅b has been reported to reduce the phosphorylation of VEGFR2, which in turn could decrease the permeability of the GFB [25]. On the other hand, podocyte-specific over-expression of the pro-permeability isoform, VEGF-A₁₆₄, has been reported to result in glomerular dysfunction including increased glomerular L_pA/V_i , which could be rescued by the constitutive over-expression of VEGF-A₁₆₅b [14], and a collapsing nephropathy phenotype [26]. It appears that while upregulation of the pro-permeability VEGF-A₁₆₄ isoform is detrimental to glomerular function, upregulation of the anti-permeability VEGF-A₁₆₅b isoform is beneficial to glomerular function.

DT did not induce any ultra-structural abnormalities in the Pod-DTR mice in our study. There is substantial evidence to suggest that structural defects in the GFB are

largely a result of albuminuria in specific disease conditions [27], which could explain why we do not see any structural abnormalities in our model.

The potential limitations to the present study are that we only see a mild phenotype of glomerular disease in the Pod-DTR mice. Therefore, we are not able to see the full therapeutic potential of VEGF-A_{165b} in the glomerulus, as reported in the previous studies mentioned. In addition, the number of mice per group was relatively low; however, we overcame this limitation by using multiple doses of DT, and thus had multiple groups of mice being treated with DT. Furthermore, the genetic background of the Pod-DTR mouse used in this study was not ideal to study glomerular disease.

In conclusion, the lower dose Pod-DTR model used in this study is a mild model of glomerular disease, with no measurable albuminuria apparent within the first weeks after administration of DT and no detectable structural/ultra-structural defects. However, an increased glomerular water permeability correlated to a change in nephrin within the podocytes was observed in this model. The resultant phenotype disagrees with the severe podocyte ablation reported in published models, which could be due to a number of factors including a development of insensitivity to DT, different animal

housing conditions or reduced expression of hDTR over time. Despite the drawbacks of this model, we can conclude that the mild phenotype of glomerular disease in Pod-DTR mice was successfully prevented by constitutive over-expression of cyto-protective VEGF-A_{165b} within the podocytes, underlying the importance and therapeutic potential of the VEGF-A_{165b} splice variant in glomerular disease.

Ethics Statement

This study did not require informed consent nor review/approval by the appropriate ethics committee.

Disclosure Statement

The authors have no conflicts of interest to declare.

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