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PEDF relieves kidney injury in type 2 diabetic nephropathy mice by reducing macrophage infiltration

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Abstract

Introduction: Pigment epithelium-derived factor (PEDF) is a multifunctional protein with anti-angiogenic, antioxidant and anti-inflammatory properties. PEDF is involved in the pathogenesis of diabetic retinopathy, but its exact role in diabetic kidneys remains unclear. P78-PEDF is an active peptide sequence consisting of 44 amino acids with biological activity similar to that of PEDF. The present study aimed to investigate whether PEDF can alleviate renal damage in type 2 diabetic nephropathy mice by inhibiting macrophage infiltration.
Material and methods: The db/db mice were randomly divided into a diabetes PEDF intervention group (DM-P78-PEDF), a diabetes empty carrier intervention group (DM-Vehicle), and a diabetes mellitus group (DM). Subsequently, they were injected subcutaneously P78-PEDF (0.3 μg/g/d) and PBS for 6 weeks. The ratio of kidney weight to body weight was observed in the mice. An automatic biochemical analyser was used to determine fasting blood glucose (GLU), blood urea nitrogen (UREA), serum creatinine (CREA), and haemoglobin (Hb) content. Histological and ultrastructural pathological changes in the kidneys were examined through H&E and PAS staining. Kidney tissue levels of interleukin-1β (IL-1β), interleukin 6 (IL-6), tumour necrosis factor alpha (TNF-α), and interferon gamma (IFN-γ) were determined by ELISA. Expression of the macrophage infiltration and typing as well as that of PEDF, NF-κB, and TLR4 was evaluated in the kidneys.
Results: PEDF was located in glomeruli, and the expression of PEDF protein and mRNA in the kidney of diabetic mice declined significantly. Compared with diabetic mice treated with vehicle, continuous infusion of P78-PEDF could reduce blood urea nitrogen, serum creatinine (CREA), renal macrophage recruitment, inflammatory cytokines, and histological changes and restore the expression of TLR4/NF-κB signalling pathway-related factors in diabetic mice.
Conclusion: These findings highlight the importance of P78-PEDF peptide as a potential treatment in the occurrence and development of diabetic renal injury. **(Endokrynol Pol 2021; 72 (6): 643–651)**

Key words: *type 2 diabetes mellitus; pigment epithelium-derived factor; macrophage infiltration; kidney injury*

Introduction

Diabetes mellitus (DM) is a metabolic disorder characterized by elevated blood glucose levels [1]. In recent years, with the improvement of people's living standards and the change of diet structure, the incidence rate of DM is increasing year by year and is affecting younger people [2]. According to incomplete statistics, DM has become the third most challenging disease threatening human health. Persistent hyperglycaemia will cause extensive vascular damage and is expected to result in various complications, including diabetic nephropathy [3]. Studies have found that intrarenal inflammation plays an important role in the development of diabetic nephropathy, and macrophages, as the representative of inflammatory cells, are abnormally activated and infiltrated in glomeruli and renal interstitium in the early stage, which is one of the key factors leading to the initiation of renal injury [4].

In a high glucose environment, glomerular endothelial cells and mesangial cells are affected by many factors, such as advanced glycation end products, oxidative stress, and polyols, which can express a large amount of monocyte chemoattractant protein 1 (MCP-1), interleukin-1, and other substances to recruit monocyte adhesion and activation, and finally to infiltrate the renal interstitium to transform into macrophages and induce irreversible glomerulosclerosis [5]. Macrophages release many lysosomal enzymes, nitric oxide, transforming growth factor-β, and cytokines such as tumour necrosis factor-α (TNF-α), interleukin-1 (IL-1), and interferon-γ (IFN-γ) in the kidney. These substances promote the occurrence and development of DM [6]. At the same time, macrophages can be polarized into two subtypes: classical activated M1 macrophages and selectively activated M2 macrophages. M1 is thought to cause inflammatory and immune injury; M2 can reduce the inflammatory response and exert the function of tissue repair. The

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proportion of M1/M2 in DM is significantly increased, resulting in renal injury [7, 8]. Therefore, inhibiting the activation and infiltration of macrophages in the kidney is important for the prevention and treatment of DM.

Although the importance of inhibiting macrophage infiltration has been recognized, current clinical interventions and efficacy of treatment are very limited [9]. Commonly used drugs for the treatment of DM, such as angiotensin-converting enzyme inhibitor/angiotensin II receptor antagonist (ACEI/ARB), can inhibit the activation of the renin-angiotensin system in the kidney, improve glomerular haemodynamics, and delay the speed of renal function injury, but they still cannot effectively alleviate the infiltration of macrophages in glomeruli and renal tubules [10]. Pigment epithelium-derived factor (PEDF) is an endogenous secretory glycoprotein first isolated from the fine products of omental pigment epithelium secreted by human embryonic visual cells in conditional culture. It belongs to the superfamily of non-inhibitory serine protease inhibitors [11]. PEDF is widely distributed in lung, kidney, prostate, vascular smooth muscle cells, and other tissues and cells [12, 13]. It has many biological functions, such as inhibiting neovascularization, inhibiting proliferation, promoting apoptosis, inhibiting inflammatory reaction, reducing peroxidation injury of nerve tissue, enhancing immunity, and so on [14, 15]. It has been confirmed that the expression of PEDF is down-regulated in the early stage of diabetic nephropathy, which is considered to be related to the increase of early permeability [16]. At the same time, PEDF plays a beneficial role in delaying the occurrence and development of diabetic nephropathy in the aspects of anti-neovascularization, anti-fibrosis and extracellular matrix accumulation, antioxidant stress, and so on [17].

The molecular weight of PEDF reaches 50 kDa, which limits its therapeutic effect, and recent studies have found that some fragments of PEDF also have similar biological activities, especially a sequence of active peptides composed of 44 amino acids (AA78-121; P78-PEDF) [18]. In this study, we used a type 2 diabetic mouse nephropathy model to observe the effect of an exogenous small fragment of PEDF on macrophage infiltration in mice, and to investigate whether small fragment PEDF could alleviate kidney injury in type 2 diabetic mice by inhibiting macrophage infiltration.

Material and methods

Experimental animals

Twenty-four five-week-old C57BL/6 male mice (db/db mice) weighing 18–25 g and 16 five-week-old C57BL/6 male mice (db/m mice) weighing 18–25 g were selected. The experimental animals were purchased from Chengdu Dashuo Experimental Animal Co., Ltd. (license no. SYXK 2019-189). The mice were kept in cages with a controlled temperature and light cycle (24°C and 12/12h light cycle) and were provided with free access to food and water. The humidity was 40%. The present study was approved by the Animal Care Unit and Use Committee of the Chongqing People's Hospital, and all efforts were made to minimize animal suffering and reduce the number of animals used.

Model preparation and animal grouping

Twenty-four five-week-old db/db mice (animal model of spontaneous type 2 diabetic nephropathy) were used as a diabetic group, and 16 five-week-old db/m mice born in the same litter were fed as a control group (Control) and normal control group (NC); all 24 mice were fed normally. At the age of 10 weeks, the tail vein blood of db/db mice was randomly taken to detect blood glucose (Omnitest plus blood glucose meter, Germany). After the blood glucose was stable for 10 days, two or more random blood glucose levels ≥ 16.7 mmol/L were considered as successful models. The db/db mice were randomly divided into a diabetes PEDF intervention group (DM-P78-PEDF), a diabetes empty carrier intervention group (DM-Vehicle), and a diabetes mellitus group (DM). Then, the kidney tissues of the control group and DM group were collected and stored at –80°C for PEDF expression and immunofluorescence assay. The DM-P78-PEDF group received a continuous subcutaneous injection of P78-PEDF (0.3 μg/g/d, GL Biochem, China) through a microperfusion pump; the DM-Vehicle group and NC group were also subcutaneously injected with the same amount of phosphate buffer (PBS) through the microperfusion pump for six weeks. After the treatment, the body weight of mice was measured and recorded, and blood samples of mice were collected through the abdominal aorta to assay the serum biochemical indexes. Then, the kidney tissue of mice in the NC group, DM-Vehicle group and DM-P78-PEDF group was collected, after weighing the kidneys of the three groups, and a portion of the kidney tissue samples were stored at -80°C for molecular index determination, while the remaining samples were fixed in 4% paraformaldehyde f*(KI) = kidney mass (mg) / mouse body weight (g).*

Assays of the serum biochemical indexes

The collected blood samples were placed at 37°C for 30 min, and then centrifuged at 3000 rpm for 10 min to collect the upper serum. The serum biochemical indexes of fasting blood GLU, UREA, CREA, and Hb were assayed using a HITACHI 7600-020 Automatic Biochemistry Analyzer (Shandong, China), and the operation was carried out in strict accordance with the manufacturer's instructions.

Haematoxylin and eosin (H&E) staining

The kidney tissues were fixed with 4% formaldehyde solution and then embedded in paraffin. Before staining, the sections were incubated at 60°C for 1 h, dewaxed with xylene, and rehydrated through a series of ethanol solutions. The embedded wax block was cut into thin slices, using a slicer, to a thickness of 3–5 μm. Finally, the sections of kidney tissues were stained with H&E and examined by light microscopy (Nikon, Japan). The degree of kidney tissue injury was obtained by the comprehensive evaluation of each section by histology. Images of each slide were captured at 400× magnification.

Periodic acid-Schiff (PAS) staining

The kidney tissues were fixed with 4% formaldehyde solution and then made into 3 μm thick paraffin sections. The sections of kidney tissues were then stained with PAS, and the glomerular segments or sclerosis were observed by light microscopy (Nikon, Japan). Glomerular sclerosis index (GSI) was used to analyse the degree of glomerular lesion: no pathological changes were found in glomeruli (0 points); lesion area < 25% (1 points); lesion area 25–50% (2 points); lesion area 50–75% (3 points); lesion area > 75% (4 points), and each section was used to analyse the 400× microscopic visual field of 30 glomeruli and 20 non-glomeruli.

Enzyme-linked immunosorbent assay (ELISA)

The kidney tissue homogenate was collected for the detection of related indexes. The expressions of IL-1β, IL-6, TNF-α, and IFN-γ in kidney tissues were detected by ELISA. The operation was carried out according to the kit instructions (Multi Sciences, China) with the double antibody sandwich ELISA. In brief, the specific anti-mice IL-1β, IL-6, TNF-α, and IFN-γ antibody were precoated on a high-affinity enzyme-labelling plate to form a solid phase antibody, and then it bound to the corresponding antigen in the tissues to form an immune complex. After washing, enzyme-labelled antibodies were added to combine with the antigen in the immune complex to form the enzyme-labelled antibody-antigen-solid antibody complex. Finally, the substrate was added for colour development, and the depth of colour was proportional to the concentration of IL-1β, IL-6, TNF-α, and IFN-γ in the sample, to determine the content in the sample.

Immunohistochemical staining

The kidney tissues were fixed, paraffin-embedded, sliced into serial sections, and dewaxed. After endogenous peroxidase was blocked using 3% H_2O_2, the sections were blotted with primary antibody anti-CD68 (1:100, Abcam, UK) at 4°C overnight, followed by incubation with the secondary antibody (1:200, Zhongshan Jinqiao, China). Finally, sections were stained with diaminobenzidine and then counterstained with haematoxylin. The expression level of CD68 was quantified using integrated optical density values generated by Image-Pro Plus 6.0 software. Positive cells in three high-power fields (HPFs) were then counted in each section at a magnification of 200× and the mean count of positive cells per HPF was reported.

Immunofluorescence staining

The kidney tissues were fixed, paraffin-embedded, sliced into serial sections (thickness 3 μm), and dewaxed. Then they were washed three times with phosphate buffer (PBS) – dripping protease K, incubated at 37°C for 40 min, and PEDF fluorescent antibody (203033, UK Abcam, 1:200) was added at 4°C overnight, they were rinsed three times with PBS, rabbit anti-mouse immunoglobulin with fluorescein was added, then they were incubated at 37°C for 40 min, rinsed three times with PBS, and sealed with glycerol. They were observed under a fluorescence microscope and photographed under an Olympus laser scanning confocal microscope. The semi-quantitative standard of immunofluorescence intensity grading refers to the current domestic and foreign, commonly used five-point (0–4+) grading.

Flow cytometry analysis

Flow-cytometric analysis was performed following the standard protocol. The kidney tissue was ground, the cell culture medium was added to make the cell suspension, and the cells were centrifuged at 300 g for 5 min at 4°C. The cells were subjected to flow cytometry of kidney macrophages with fluorochrome-conjugated antibodies for CD86, CD206, and F4/80 (1:100, Biolegend, USA). Isotype-matched controls were used as negative staining. All stained cells were analysed by flow cytometry (Cytoflex, USA) and the data were analysed using Kaluza software (Beckman Coulter).

Quantitative real-time polymerase chain reaction (qRT-PCR)

Total RNA was isolated from the kidney tissues using an RNA extraction kit (Hefei Bomei Biotechnology Co., Ltd.) according to the manufacturer's instructions. The eligible RNA was then reverse transcribed into cDNA using a cDNA kit. After cDNA synthesis (Invitrogen), mRNA expression levels of PEDF, MCP-1, CD80, CD86, IL-4, CD206, and CD163 were tested using the UltraSYBR Mixture. Relative gene expression was calculated using the $2^{-\Delta\Delta CT}$ method and normalized against GAPDH. qRT-PCR reaction conditions were as follows: initial denaturation at 95°C for 10 minutes, followed by denaturation at 95°C for 10 minutes, annealing at 60°C for 10 minutes, and extension at 72°C of 10 minutes for 45 cycles. The CT value was recorded. The used primers are listed in Table 1.

Western blot analysis

The protein expressions of PEDF, TLR4, and NF-κB p65 in kidney tissues were detected by western blotting analysis. For the determination of protein expression levels, protein samples were extracted from kidneys homogenized in RIPA buffer with the addition of Protease Inhibitor Cocktail (Solarbio, China). Then the protein concentration was quantified with the BCA protein assay (Solarbio, China). Proteins were then separated by 10% SDS-PAGE and transferred onto PVDF membranes, and anti-PEDF (sc-390172) antibody, anti-TLR4 (sc-293072) antibody, and anti-NF-κB p65 (sc-8008) antibody (Santa Cruz Biotechnology, USA) were used for immune reaction; β-actin or Lamin B was used as a loading control. The membranes were washed three times with TBST buffer for 10 min each time, followed by incubation with the secondary antibody. The polyvinylidene fluoride film was removed, and the film was obtained using the chemiluminescence method. The A value of the target band was analysed using Quantity One gel imaging software, and the ratio of the A value of the target band to the β-actin or Lamin B band was considered as the relative expression of the target protein, and the number of repetitions was three.

Statistical analysis

The data were statistically analysed using SPSS 20.0 software (IBM Corp.). All data results are tested by normal distribution first. After

Table 1. ***The primer sequences used in quantitative real time-polymerase chain reaction (qRT-PCR) for specific mRNA amplification***

Primer	Forward primer (5′-3′)	Reverse primer (5′-3′)
PEDF	GGAGCGGAGCAGCGAACAGAAT	TGCGCCACACCGAGAAGGAGA
MCP-1	GAGTCGGCTGGAGAACTACAAGA	GGTGCTGAAGTCCTTAGGGTTG
CD80	CATCACTGGAGGGTCTTCTAC	AGGATCTTGGGAAACTGTTGT
CD86	TCTGCTGCTGTAACAGGGACTA	TAGGTTCTGGGTAACCGTGTAT
IL-4	GAACACCACGGAGAACGAG	AGACCGCTGACACCTCTACA
CD206	GCTAAATGGGAAAATCTGGAATGTT	CGATGGTGTGGATACTTGTGAGG
CD163	TTTTGTCAACCAGTTCTCTTGGA	AGCCATTATTACACACGTTCC
GAPDH	AGGAGCGAGACCCCACTAACA	AGGGGGGCTAAGCAGTTGGT

verification of a normal or non-normal distribution by the Shapiro-Wilk test, two-tailed Student's *t*-test and ANOVA of Tukey's *post hoc* was performed to analyse the variables of normal distribution. When data were not normally distributed it was log-transformed. For all the analyses, $p < 0.05$ was considered to indicate a statistically significant difference.

Results

Expression of PEDF in the kidney tissue of diabetic mice

We first measured the expression of PEDF in diabetic kidney tissue. The data showed that the mRNA (Fig. 1A) and protein (Fig. 1B, C) expression of PEDF in diabetic kidneys was significantly lower than that in the control group ($p < 0.01$). Next, we used PEDF-specific antibody to identify the expression of PEDF in mouse kidneys (Fig. 1D). Immunofluorescence staining results showed that PEDF expression was mainly distributed in glomeruli, and there were obvious red target molecules in glomeruli, i.e. PEDF distribution. The above results indicated that the expression of PEDF in diabetic kidneys was markedly decreased, and the expression was distributed in glomeruli.

Protective effect of PEDF on renal function in mice with type 2 diabetic nephropathy

Due to the significant decrease of PEDF in diabetic kidneys, we questioned whether the use of P78-PEDF peptide to restore PEDF would improve diabetic renal injury. Toward this goal, we continuously injected P78-PEDF peptides or vehicle into diabetic mice. The results showed that the protein expression of PEDF in the P78-PEDF group was significantly higher than that in the vehicle group ($p < 0.05$; Fig. 2A, B). Compared with NC mice, the level of GLU, UREA, CREA, and Hb and the ratio of kidney weight/body weight were significantly increased in the vehicle-treated diabetic group ($p < 0.05$; Fig. 2C–G). Compared with vehicle-treated diabetic group, administration of P78-PEDF peptide reduced the level of GLU, UREA, CREA, and Hb and the ratio of kidney weight/body weight in diabetic mice (Fig. 2C–G). These results indicated that the administration of P78-PEDF peptide reduces the characteristics of diabetic nephropathy.

Protective effect of PEDF on pathological damage of kidney tissue in mice with type 2 diabetic nephropathy

The effect of PEDF on renal histopathology and renal inflammation in diabetic mice was observed. H&E staining of kidney sections showed that a small number of glomeruli were enlarged, and the structure of vascular glomeruli was blurred in the renal cortical area of vehicle treated mice. After administration of P78-PEDF peptide, a small number of glomerular glomeruli in the renal cortex were blurred, and there

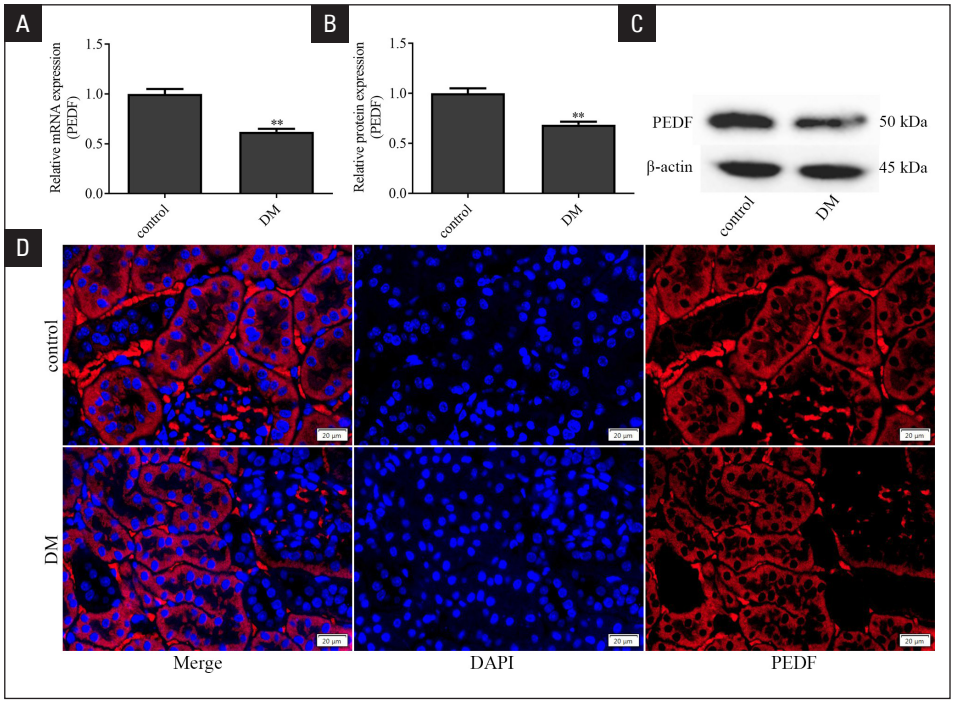

Figure 1. *Expression of pigment epithelium-derived factor (PEDF) in the kidney tissue of diabetic mice.* **A.** *Relative mRNA expression of PEDF;* **B.** *Relative protein expression of PEDF;* **C.** *PEDF protein band;* **D.** *Immunofluorescence staining for PEDF on a representative kidney section in mice. The data are expressed as the mean ± standard deviation (SD). Compared with the control group, $^{**}p < 0.01$*

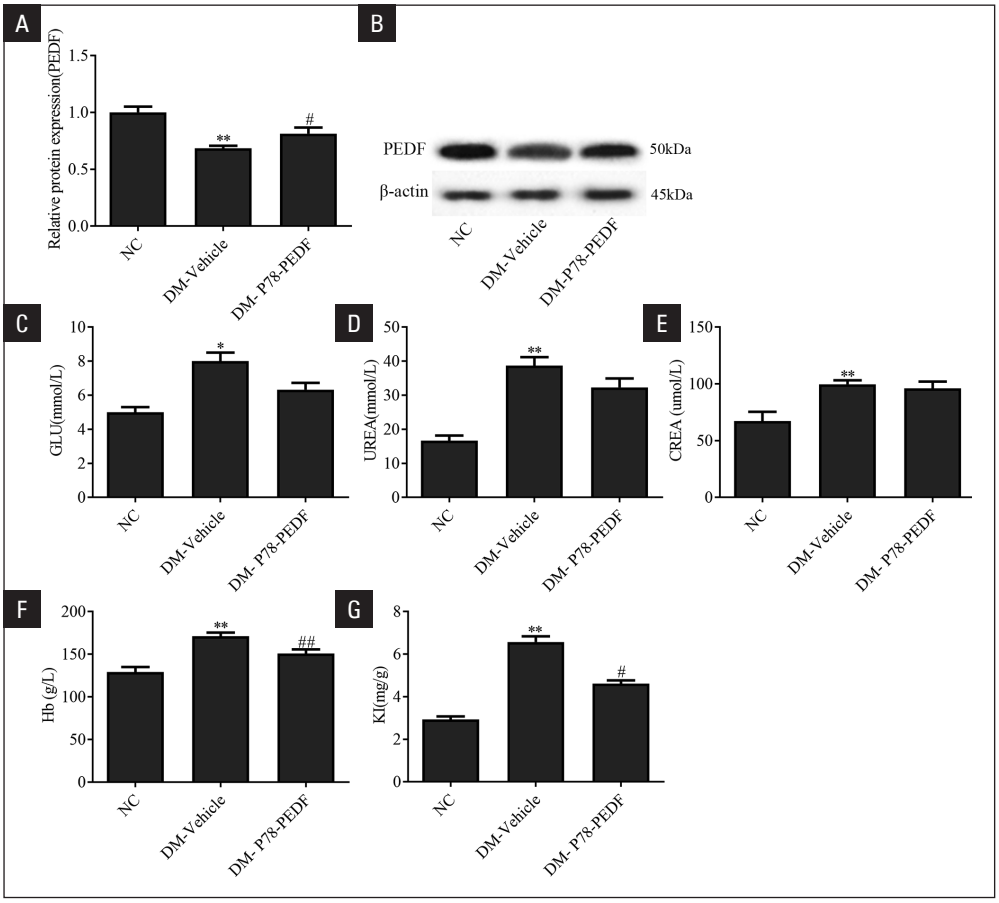

Figure 2. *Protective effect of pigment epithelium-derived factor (PEDF) on renal function in mice with type 2 diabetic nephropathy.* **A.** *Relative protein expression of PEDF;* **B.** *PEDF protein band;* **C.** *Glucose (GLU) levels in serum;* **D.** *Urea (UREA) levels in serum;* **E.** *Creatinine (CREA) levels in serum;* **F.** *Haemoglobin (Hb) levels in serum;* **G.** *Calculated kidney index in each group; the data are expressed as the mean ± standard deviation (SD). Compared with the NC group, $^{*}p < 0.05$ and $^{**}p < 0.01$; compared with the DM-Vehicle group, $^{\#}p < 0.05$ and $^{\#\#}p < 0.01$*

was no hyperaemia and inflammatory cell infiltration in the interstitium (Fig. 3A). PAS staining showed increased glomerular cells and mesangial expansion in the DM-Vehicle group. The glomerular lesions were significantly alleviated after treatment with P78-PEDF peptides (Fig. 3B). The increase of inflammatory cytokines is the main feature of DM and an important predictor of it. Therefore, we further evaluated the anti-inflammatory effect of P78-PEDF peptides in diabetic mice. Compared with NC group, the contents of IL-1β, IL-6, TNF-α, and IFN-γ were significantly increased in kidney tissue in the vehicle treated mice ($p < 0.05$, Fig. 3D-G). Compared with vehicle group, the contents of IL-6, TNF-α, and IFN-γ could be inhibited in the group that was treated with P78-PEDF peptide ($p < 0.05$, Fig 3D-G). The results showed that the administration of P78-PEDF peptide alleviated the pathological changes of the kidney and decreased the content of renal inflammatory factors in diabetic mice.

Effects of PEDF on the infiltration and typing of macrophages in the kidney tissue of type 2 diabetic nephropathy mice

To determine whether PEDF is the key to macrophage infiltration in DM, we used the immunohistochemical method to show the expression of CD68 in mouse kidneys. Compared with NC group, the expression of CD68 was significantly increased in kidney tissue of diabetic mice in the vehicle group ($p < 0.01$; Fig. 4A). In contrast, the expression of CD68 was significantly decreased in the kidney of diabetic mice treated with P78-PEDF peptide ($p < 0.05$; Fig. 4A). Flow cytometry was used to identify the expression of $F4/80^{+}CD86^{+}$ (M1) and $F4/80^{+}CD206^{+}$ (M2) in renal macrophages. Compared with the NC group, the level of $F4/80^{+}CD86^{+}$was significantly increased in renal tissue of diabetic mice in the vehicle group, and the level of $F4/80^{+}CD206^{+}$ was markedly decreased in the vehicle group ($p < 0.01$; Fig. 4B). Compared with the vehicle group, the level of $F4/80^{+}CD86^{+}$ was significantly decreased in the

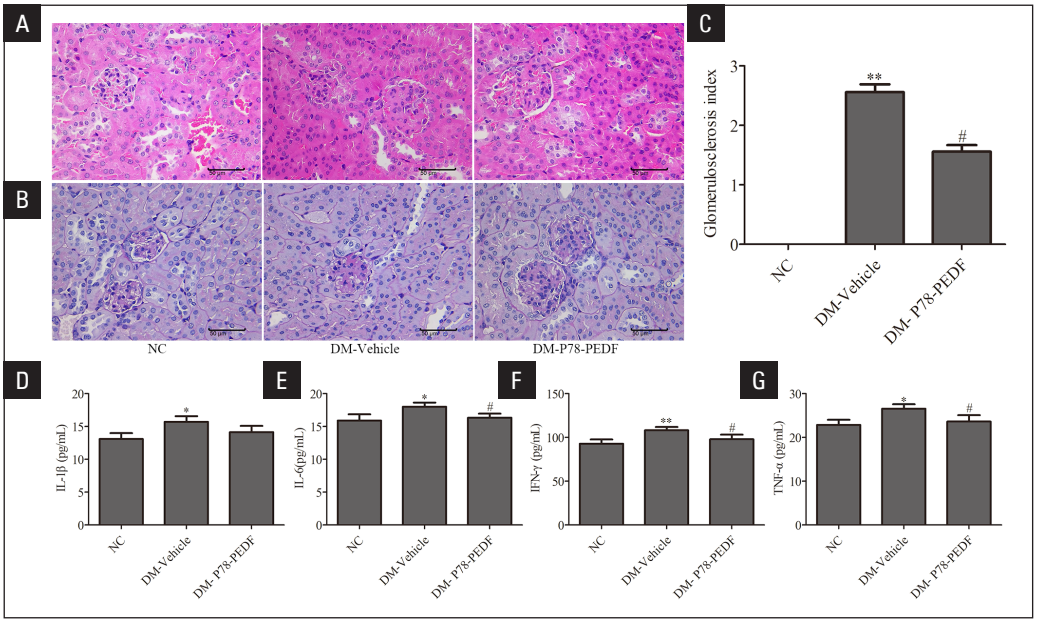

Figure 3. *Protective effect of pigment epithelium-derived factor (PEDF) on pathological damage of kidney tissue in mice with type 2 diabetic nephropathy.* **A.** *Representative micrographs of decalcified distal kidney tissue paraffin sections stained with H&E (magnification 400×);* **B.** *Representative micrographs of decalcified distal kidney tissue paraffin sections stained with PAS (magnification,400×);* **C.** *Glomerular sclerosis index in each group;* **D.** *Interleukin 1 beta (IL-1β) levels in kidney tissue;* **E.** *Interleukin 6 (IL-6) levels in kidney tissue;* **F.** *Interferon gamma (IFN-γ) levels in kidney tissue;* **G.** *Tumour necrosis factor alpha (TNF-α) levels in kidney tissue; (A and B) scale bar, 50 μm; data expressed as the mean ± standard deviation (SD). Compared with NC group, $^{*}p < 0.05$ and $^{**}p < 0.01$; compared with the DM-Vehicle group, $^{\#}p < 0.05$ and $^{\#\#}p < 0.01$*

P78-PEDF group, and the level of $F4/80^{+}CD86^{+}$ increased in the group that was treated with P78-PEDF ($p < 0.05$; Fig. 4B). The expression of MCP-1, CD80, CD86, IL-4, CD206, and CD163 mRNA in kidney tissues was detected by qRT-PCR. Compared with the NC group, the levels of MCP-1, CD80, CD86, and CD163 were significantly increased in kidney tissue of diabetic mice in the vehicle group, while the levels of IL-4 and CD206 were markedly decreased in the vehicle group ($p < 0.05$; Fig. 4C). Compared with vehicle group, the expressions of MCP-1, CD80, CD86, and CD163 were significantly reduced, and the expression of IL-4 was significantly increased, in the P78-PEDF treatment group ($p < 0.05$; Fig. 4C). These results indicate that P78-PEDF peptide administration could reduce the recruitment of macrophages in diabetic mice.

Effect of PEDF on TLR4/NF-κB signalling pathway in the kidneys of type 2 diabetic nephropathy mice

To determine changes of TLR4/NF-κB signal pathway in diabetic mice after P78-PEDF intervention, we detected the expression levels of TLR4 and NF-κB p65. Compared with the NC group, the protein expressions of TLR4 and NF-κB p65 were significantly increased in the kidney tissue of mice in the vehicle group ($p < 0.05$; Fig. 5A, B). Compared with the vehicle group, the protein expressions of TLR4 and NF-κB p65 in the P78-PEDF group were substantially decreased ($p < 0.05$; Fig. 5AB). These results show that the protein level of related factors in the TLR4/NF-κB pathway increased in diabetic mice, while P78-PEDF reduced the protein level of related factors in this pathway in diabetic mice.

Discussion

The PEDF protein is expressed in several tissues and cell types including kidney tissue [19]. In this study, a small bioactive peptide of PEDF (P78-PEDF) mediates the protective effect of renal tissue, which is characterized by the decrease of UREA, CREA, Hb, histopathological changes, renal macrophage recruitment, and the decrease of inflammatory cytokines in diabetes. In addition, P78-PEDF can inhibit the protein level of related factors in the TLR4/NF-κB pathway. These findings reveal the crucial role of P78-PEDF peptides in the pathogenesis of DM, and P78PEDF may provide a new treatment model for patients with type 2 diabetes.

In recent years, scholars have paid increasing attention to the expression and role of PEDF in the serum, urine, and renal tissue of patients with diabetes. Studies have shown that PEDF, as a protective factor of diabetic

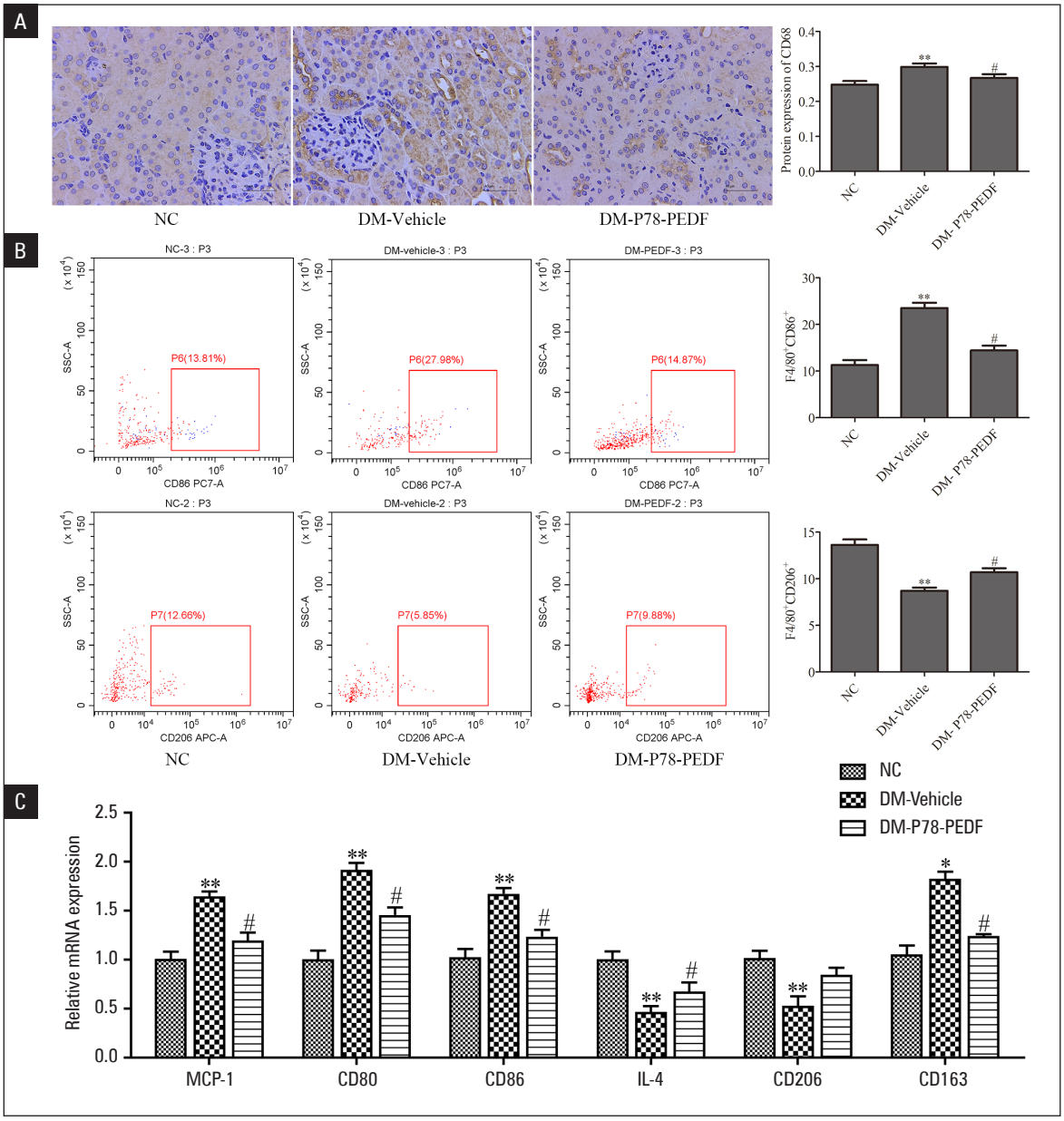

Figure 4. *Effects of pigment epithelium-derived factor (PEDF) on the infiltration and typing of macrophages in the kidney tissue of type 2 diabetic nephropathy mice.* **A.** *Immunohistochemical staining for macrophages infiltrating the kidney. Representative photomicrographs of kidney tissue sections stained for CD68. Quantification of the number of CD68-positive macrophages infiltrating the kidney;* **B.** *Flow cytometric analysis of activation levels of serum M1 (CD86) and M2 (CD206) macrophages;* **C.** *Relative mRNA expression of MCP-1, CD80, CD86, IL-4, CD206, and CD163; (A) Scale bar, 40 μm; data expressed as mean ± standard deviation (SD). Compared with NC group, $^{*}p < 0.05$ and $^{**}p < 0.01$; compared with the DM-Vehicle group, $^{\#}p < 0.05$ and $^{\#\#}p < 0.01$*

chronic microvascular complications, has the effects of anti-inflammatory reaction, antioxidant stress, and protecting vascular endothelial function [20, 21]. Awad et al. [16] showed that PEDF is expressed mainly in the kidney vasculature, interstitial spaces, glomeruli, medulla, and tubular epithelial cells, and was also able to detect PEDF expression in glomerular endothelial cells cultured in vitro in a normal glucose medium, and this effect was significantly reduced when high-glucose medium was used [17]. It is hypothesized that the reduction in PEDF may be associated with a high-glucose environment. At the same time, studies have confirmed that PEDF intervention can reduce the content of TNF-α in the urine of diabetic rats, and TNF-α is considered to be a cytokine mainly produced by monocytes and macrophages, which is an important factor leading to the increase of renal vascular endothelial permeability and urinary protein leakage in diabetic nephropathy [22]. However, there are few studies investigating whether PEDF can directly inhibit chemotaxis and activate MCP-1 and NF-κB of macrophages and inhibit CD68-positive macrophages in glomeruli, and even

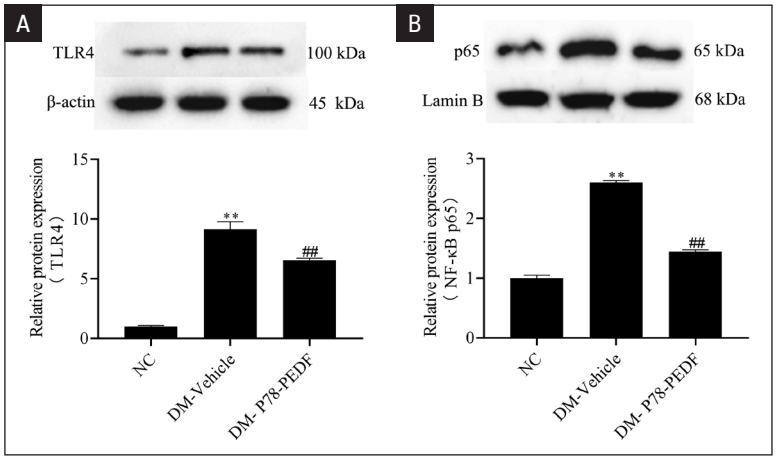

Figure 5. *Effect of pigment epithelium-derived factor (PEDF) on TLR4/NF-κB signalling pathway in the kidneys of type 2 diabetic nephropathy mice.* **A.** *Protein band and relative protein expression of TLR4;* **B.** *Protein band and relative protein expression of NF-κB p65. The data are expressed as the mean ± standard deviation (SD). Compared with NC group, $^{*}p < 0.05$ and $^{**}p < 0.01$; compared with the DM-Vehicle group, $^{\#}p < 0.05$ and $^{\#\#}p < 0.01$*

fewer assessing whether it can promote the transformation of the proportion of M1/M2 to M2, to reduce the renal injury caused by inflammation. In this experiment, we used a specific antibody to identify the expression of PEDF in the kidneys of mice. Our data show that PEDF is expressed in glomeruli. We further questioned whether PEDF has changed in diabetes. The data show that both PEDF protein and mRNA expression are significantly decreased in diabetic kidneys. These data are consistent with previous reports of reduced expression of PEDF protein and mRNA in the kidneys of diabetic rats [23].

In order to examine the role of PEDF in diabetes, we continuously injected a small bioactive peptide fragment of PEDF (P78-PEDF) into the mouse model of type 2 diabetes. P78-PEDF peptides have recently been identified and have shown excellent biological activity [24]. Our data show that the renal protective effect of P78-PEDF peptides is associated with a significant reduction of renal macrophage infiltration. The infiltration of macrophages in glomeruli and interstitium and the proliferation of glomerular extracellular matrix (ECM) are important features of diabetic nephropathy [25]. MCP-1 is mainly produced by renal tubular epithelial cells and glomerular mesangial cells, and its main function is chemotaxis and activation of mononuclear macrophages. Li et al. [26] found that MCP-1 plays an important part in the pathogenesis of type 1 diabetes by attracting monocytes/macrophages to infiltrate pancreatic islets. NF-κB is one of the important transcriptional proteins regulating the expression of MCP-1. It has been verified that the activity of NF-κBp65 protein and the expression of MCP-1mRNA in glomeruli increased significantly in STZ-induced diabetic rats, followed by macrophage infiltration, which was positively correlated with the number of CD68-positive macrophages. Therefore, MCP-1, NF-κB, and the number of CD68-positive macrophages in glomeruli can be regarded as markers of renal macrophage infiltration. Their abnormally increased expression leads directly to renal injury [27, 28]. TNF-α is mainly produced by monocytes/macrophages and is related to the increase of vascular endothelial cell permeability in diabetes mellitus [29]. This study confirmed the above results and showed that P78-PEDF peptide could dramatically increase the content of IL-1β, IL-6, TNF-α, and IFN-γ and decrease the expression of MCP-1, CD80, CD86, and CD163. Our data also showed that P78-PEDF peptide treatment decreased the expression of TLR4 in the kidney, confirming the effect of PEDF on macrophage inflammation by inhibiting NF-κB [30]. It has been suggested that the decrease of PEDF may be involved in the occurrence and/or development of diabetes, and some beneficial

Conclusions

Our study shows that PEDF fragment P78-PEDF has a renal protective effect in type 2 diabetes; in particular, P78-PEDF peptide reduces renal macrophage infiltration and improves renal histopathology. The results of this study provide support for P78-PEDF peptide as a therapeutic method for the treatment of type 2 diabetes. The exact efficacy of P78-PEDF peptide in the treatment of type 2 diabetes requires further study.

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Competing interests

The authors declare that they have no competing interests.

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