The dipeptidyl peptidase 4 inhibitor linagliptin ameliorates renal injury and accelerated resolution in a rat model of crescentic nephritis

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Background and Purpose: Dipeptidyl peptidase 4 (DPP-4) inhibitors are a class of oral glucose-lowering drugs used in the treatment of type 2 diabetes. In a pilot study using human kidney biopsies, we observed high DPP-4 expression in early crescent formation. This glomerular lesion occurs in different kidney diseases and is a hallmark in the pathogenesis of renal dysfunction. Therefore, we investigated the potential involvement of DPP-4 in the pathogenesis of nephritis induced by anti-glomerular basement membrane (GBM) antibody in rats.

Experimental Approach: Linagliptin and vehicle were used to treat anti-GBM nephritis in a 2- and 8-week regimen, that is either preventive or therapeutic (treatment started 7 days or 4 weeks after disease induction). Kidney function, morphologic changes, inflammation and fibrosis were monitored.

Key Results: In the long-term experiment, linagliptin preventive treatment in anti-GBM nephritic rats significantly reduced the number of crescents, glomerulosclerosis, tubular injury and renal fibrosis, compared with those in untreated nephritic rats. Both linagliptin regimes significantly lowered the number of Pax8+ cells on the glomerular tuft in anti-GBM nephritis, indicating accelerated resolution of the cellular crescents. The linagliptin treatment did not change the podocyte stress in both therapeutic groups. Therapeutic intervention with linagliptin resulted in weaker amelioration of renal disease on Week 8 than did preventive intervention.

Conclusion and Implications: DPP-4 inhibition with linagliptin ameliorates renal injury in a rat model of anti-GBM, indicating that linagliptin not only is a secure therapy in diabetes but also can improve resolution of glomerular injury and healing in non-diabetic renal disease.

KEYWORDS
crescent formation, DPP-4 inhibitor, fibrosis, non-diabetic renal disease

Abbreviations: DPP-4, dipeptidyl peptidase 4; GBM, glomerular basement membrane; GLP-1, glucagon-like peptide 1; Ki67, proliferation marker; MCP-1, monocyte chemotactic protein 1; Pax8, paired-box-protein 8; P-ERK1/2, phospho-ERK1/2; P-STAT3, phospho-STAT 3; SDF-1α, stromal-derived factor 1 alpha; CXCL12α; SMA, smooth muscle actin; Sox9, (sex determining region Y)-box 9.

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INTRODUCTION

Crescent formation is a histologic hallmark of severely damaged glomeruli occurring in different renal diseases but with the highest frequency in anti-glomerular basement membrane nephritis, immune-complex glomerulonephritis and pauci immune glomerulonephritis (Jindal, 1999). Podocytes, parietal epithelial cells, macrophages and T-lymphocytes are involved in the process of crescent formation (Boucher, Droz, Adafer, & Noel, 1987; Jennette & Hipp, 1986; Min, Gyorkey, Gyorky, Yium, & Eknayan, 1974; Moeller et al., 2004; Morita, Suzuki, & Churg, 1973). However, the exact mechanism of crescent formation is unclear. Interestingly, in a pilot study, we observed high expression of dipeptidyl peptidase 4 (DPP-4) in crescents in human biopsies with different forms of glomerulonephritis. DPP-4 functions as an exopeptidase to cleave or inactivate several physiological substrates and also occurs as a membranous glycoprotein along with a circulating variant in blood (Gorrell, Gysbers, & McCaughan, 2001). By inactivating the incretins, glucagon-like peptide 1 (GLP-1) and glucose dependent insulinotropic peptide (GIP), DPP-4 reduces insulin secretion and thus plays a major role in glycemic regulation (Neumiller, Wood, & Campbell, 2010). In this respect, different DPP-4 inhibitors are available for the treatment of type 2 diabetes mellitus having different binding and pharmacokinetic characteristics (Schnapp, Klein, Hoevels, Bakker, & Nar, 2016). Interestingly, DPP-4 is found in various tissues and cells with the kidney having high level of enzymatic activity (Wang et al., 2014). DPP-4 inhibition is currently restricted to the treatment of diabetes mellitus but is potentially beneficial for renal diseases. The CARMELINA clinical trial recently demonstrated reduced progression of albuminuria in diabetes patients treated with linagliptin but no improvement on hard renal endpoints (Hannsen & Jandeleit-Dahm, 2019). However, own unpublished data has shown reduced glomerular DPP-4 activity in renal biopsies from diabetic patients compared to non-diabetic healthy controls, while DPP-4 activity was up-regulated in other renal diseases. The role of renal DPP-4 in the pathogenesis of glomerular diseases and therapeutic effects of DPP-4 inhibition, beyond regulation of the glucose metabolism, is still unclear. DPP-4 cleaves a wide range of other substrates including peptide hormones such as stromal-derived factor 1 alpha (SDF-1α; CXCL12α) or monocyte chemoattractant protein 1 (MCP-1 or CCL2) (de Meester, Lambeir, Proost, & Scharpe, 2003). Furthermore, DPP-4 can serve as a surface receptor and co-stimulatory protein involved in T-cell activation mediated by interaction with caveolin-1 (Ohnuma et al., 2007; Ohnuma et al., 2015). Therefore, it is conceivable that DPP-4 is directly or indirectly involved in the pathogenesis of crescent formation. While other DPP-4 inhibitors are cleared renally, linagliptin can be given during renal impairment because its clearance primarily occurs non-renally via a hepatobiliary pathway (Fuchs, Binder, & Greischel, 2009; Fuchs, Tillement, Urien, Greischel, & Roth, 2009; Heise et al., 2009; Huttner, Graefe-Mody, Withopf, Ring, & Dugi, 2008). Thus, it can be used in patients with renal impairment and does not require dose adjustment (Fuchs et al., 2009; Gallwitz, 2013). Using a rat model for anti-glomerular basement membrane nephritis and different treatment regimens for the DPP-4 inhibitor linagliptin, we followed the hypothesis that DPP-4 is directly or indirectly involved in the pathogenesis of glomerular basement membrane nephritis, especially in crescent formation.

METHODS

2.1 Human renal tissue specimens and evaluation of glomerular DPP-4

In our study, 158 formalin-fixed paraffin-embedded specimens of archival kidney biopsies (from the Department of Nephropathology, Friedrich-Alexander University Erlangen-Nürnberg) were used to evaluate glomerular DPP-4 expression in different renal diseases using immunohistochemistry. In our pilot study, we included biopsies from hypertensive and non-hypertensive patients with clinically documented blood pressure. In addition, biopsies from transplanted kidneys without rejection (n = 16) and macroscopically normal portions of kidneys surgically excised due to the presence of a localized neoplasm (control, n = 11) were included as healthy controls. The analysis of archived renal biopsies was approved by the local ethics committee (reference number 4415).

Kidney biopsies comprised the following diseases: IgA nephropathy (n = 23), acute humoral rejection (antibody-mediated rejection; n = 10), hypertensive nephropathy (n = 12), membranous glomerulonephritis (n = 10), diabetic nephropathy (n = 16), minimal change glomerulopathy (n = 11), lupus nephritis class IV membranonephritis (n = 14), membranoproliferative glomerulonephritis (n = 12), pauci immune glomerulonephritis (n = 13) and glomerular basement membrane nephritis (n = 10).
2.2 Glomerular basement membrane nephritis model in the rat and treatment with linagliptin

In the passively induced glomerular basement membrane nephritis model, proteinuria was observed from Day 3 onwards and reaching a plateau on Day 5 (Kohda et al., 2004). Anti-glomerular basement membrane antibodies bind to glomerular basement membrane, inducing glomerular injury including severe endocapillary hypercellularity and extracapillary changes, such as capsular adhesion and crescent formation via complement or Fc-receptor-mediated processes. While glomerular CD8-positive T-cells are only barely increased after disease induction, a marked glomerular influx of macrophages can be observed early on Day 1 and further increases during the first 10 days (Kado et al., 2006). To investigate short-term versus long-term effects of linagliptin on the pathogenesis of glomerular basement membrane nephritis, rats were randomized into two different experiments: four groups in a short-term regimen for 14 days (Figure 1a) and four groups in a long-term regimen for 8 weeks (Figure 1b). The group size in the animal model was calculated in the expectation that administration of the DPP-4 inhibitor would lead to a biologically relevant reduction of crescents by at least 35%. With a determined effect size of 1.26 and a first type error of 5% and a second type error of 20%, a group size of \( n = 11 \) was determined using G-Power software (Version 3.1) (Faul, Erdfelder, Lang, & Buchner, 2007). In total, 88 male Wistar Kyoto rats (WKY/NicoCrI, Charles River, Sulzfeld, Germany) with a body weight of 200–220 g and an age of 6–7 weeks were maintained in a specific pathogen-free facility in a temperature- and light-controlled environment and had ad libitum access to chow and water.

The rats are kept on classical aspen wood bedding (sniff-Spezialdiäten GmbH, Soest, Germany) in type IV macrolon cages (Tecniplast Deutschland GmbH, Hohenpeilstein, Germany) with a maximum population of three. The experimental protocol for the animal studies was approved by the German regional committee for animal care and use, which is equivalent to the US IACUC and authorized by the governmental department (“Regierung von Unterfranken” Permit number: 55.2-2532-2-324) before the animal studies were performed in strict accordance with the German welfare act and adherence to the ARRIVE guidelines (Percie du Sert et al., 2020) and with the recommendations made by the British Journal of Pharmacology (Lilley et al., 2020). Glomerular basement membrane nephritis was induced in 66 of the 88 male Wistar Kyoto rats by a single intravenous tail vein injection of 30 μg of mouse monoclonal anti-\( \alpha 4(IV)\)NC1 of rat anti-glomerular basement membrane antibody clone a84 (Chondrex Inc., Redmond, WA, USA) solved in PBS (Sigma Aldrich, Deisenhofen, Germany) in each regimen. All rats were randomized into four different groups: non-treated anti-glomerular basement membrane nephritic rats (Figure 1a,b; anti-glomerular basement membrane) and healthy rats only receiving PBS (Figure 1a,b control) served as diseased and healthy control groups. Linagliptin-treated rats received standard chow (sniff-Spezialdiäten GmbH, Soest, Germany) supplemented with 83 mg·kg\(^{-1}\) of linagliptin (provided by Boehringer Ingelheim, Biberach, Germany) resulting in a daily linagliptin dose of approximately 5 mg·kg\(^{-1}\) of linagliptin per kg body weight.

FIGURE 1 Experimental design. Linagliptin effects on pathogenesis of anti-glomerular basement membrane (GBM) nephritis were investigated using an early (a) and a late regimen (b). In both regimens, rats were either treated preventively (starting with disease induction) or therapeutically (starting on Day 7 after disease induction in short-term regimen and on Week 4 in long-term regimen) (a, b). Blood samples and urine were collected for analysis of proteinuria, dipeptidyl peptidase 4 (DPP-4) activity and kidney function. Therefore, rats were housed in metabolic cages before start of the experiment on Days 6 and 13 or on Weeks 3, 5, and 8 to collect urine for 24 h. For investigation of disease progression, renal survival biopsies were taken on Day 7 in the short-term regimen and on Week 4 in the long-term regimen. Finally, rats were killed on Day 14 (a) or 8 weeks after induction of anti-GBM nephritis (b) (Kröller-Schön et al., 2012). In both regimens, rats were either treated preventively (starting with disease induction) or therapeutically (starting on Day 7 after disease induction in short-term regimen and on Week 4 in long-term regimen) (Figure 1a,b). Blood samples and urine were collected for analysis of proteinuria, DPP-4 activity and kidney function. Therefore, rats were housed in metabolic cages before starting the experiment, on Days 6 and 13 or on Weeks 3, 5 and 8 to collect urine for 23 h. On Days 1, 7 and 14 or on Weeks 3, 5 and 8 blood samples taken from tail vein under isoflurane anesthesia were collected in tubes containing lithium heparin and after sedimentation for 10 min at 1,300 g plasma supernatants were kept at −20°C.
until analysis of kidney function (urinary creatinine, serum creatinine) and DPP-4 activity. Proteinuria was measured using Bio-Rad Protein Assay (Bio-Rad Laboratories GmbH, Munich, Germany) (Figure 1). For investigation of disease progression, renal survival biopsies were taken on Day 7 in the short-term regimen and on Week 4 in the long-term regimen. For this purpose, the animals were administered an opioid analgesic buprenorphine (Buprenovet; Bayer, Leverkusen, Germany) in a dose of 0.05 mg·kg⁻¹ body weight s.c. before surgery. After anaesthesia induced with Isoflurane, the left flank was shaven and disinfected with KODAN tincture forte (Schülke & Mayr GmbH, Norderstedt, Germany), the flank was opened with a 1.5 cm incision and the kidney was carefully lifted out and fixed with two sterile swabs. Then the upper pole of the kidney was resected with a scalpel and the cut surface was closed with a collagen sponge (Resorba Medical GmbH, Nürnberg, Germany). Finally, the kidney was returned to its original position, the abdominal incision was closed with an absorbable suture and a continuous seam as well as the skin was closed with single button seams. Postoperatively, activity, fluid intake, weight, coat texture and behaviour of the animals were assessed but showed no signs of post-operative pain. However, analgesia given over at least 3 days post-operatively. Finally, the rats were killed under isoflurane anaesthesia on Day 14 by bleeding (Figure 1a) or 8 weeks after induction of glomerular basement membrane nephritis (Figure 1b). For perfusion of the anesthetized rats, the aorta was punctured with an indwelling venous catheter and perfused with 10% dextran 40 (Pharmacy of the University Hospital Erlangen, Erlangen, Germany) supplemented with 0.2% procaine (Steigerwald Arzneimittel GmbH, Darmstadt, Germany) followed by 0.9% NaCl and the vena cava was opened at the same time.

2.3 | Isolation of glomeruli for quantitative RNA analysis

Glomeruli were isolated from rat kidneys using the graded sieving method as described previously (Wittmann et al., 2008). Total glomerular RNA was isolated using RNeasy Mini columns (Qiagen, Hilden, Germany). Reverse transcription reactions and Real-time PCR were performed using Power SYBR Green on a 7500 Fast Real time PCR system (both Applied Biosystems, Weiterstadt, Germany) according to the manufacturer’s instructions. Real-time PCR data were analysed using the SDS v1.3 software (Applied Biosystems) and relative expression of target gene mRNA levels was calculated using the comparative delta Ct method (Dimmeler et al., 2003). Normalization was conducted against the endogenous 18S rRNA levels applied to the resulting relative fold changes. The list of used primers is provided in Table S1.

2.4 | Immunohistochemical staining

For all immune staining, kidneys were fixed in formalin (R. Langenbrinck, Emmendingen, Germany) or zinc fixative, embedded in paraffin and cut into sections of 2 μm. After deparaffinization, endogenous peroxidase was blocked using 3% H₂O₂ (Merck KGaA, Darmstadt, Germany) for immunohistochemistry. Antigen retrieval was done using target retrieval solution (Dako Deutschland GmbH, Hamburg, Germany) and boiling in a pressure cooker for 2.5 min (this step could be omitted when using zinc fixative was used). After blocking for 10 min with 1% BSA (Merck KGaA) in 50 mM of Tris (Roth GmbH, Karlsruhe, Germany) pH 7.4, sections were incubated overnight at 4°C for immunohistochemical staining for using the antibodies (Table S2) diluted in 1% BSA (Merck KGaA) in 50 mM of Tris pH 7.4. Primary antibodies were detected using secondary antibodies as shown in Table S3; ABC-kit and ImmPACT-DAB were used as substrate (all from Vector Laboratories, Burlingame, CA, USA). The experimental detail provided for immunohistochemistry conforms with BJP guidelines. All immuno-related procedures involved comply with the editorial on immunoblotting and immunohistochemistry (Alexander et al., 2018).

2.5 | Immunofluorescence multiple staining for co-localization studies

The kidney biopsies were pretreated for immunofluorescence multiple staining in the same way as for immunohistochemistry, omitting the blocking of endogenous peroxidase. Combinations of primary antibodies (Pax8/DPP-4/synaptopodin; Pax8/podocalyxin; synaptopodin/SDF-1α; PCNA/Pax8/ED1) were incubated simultaneously overnight at 4°C (Table S2). Immunofluorescence multiple staining was performed using fluorescence-labelled secondary antibodies as listed in Table S3 and nuclei were stained with DAPI (Merck KGaA). Sections were covered with Mowiol mounting medium (Calbiochem, La Jolla, USA) and examined by a confocal laser scanning microscope (LSM Zeiss 710 and Zen software, Zeiss GmbH, Jena, Germany) and Image-J software (Abramoff, Magalhaes, & Ram, 2004). In negative controls, the primary antibody was omitted and replaced with blocking solution.

2.6 | Quantitative evaluation of renal histopathologic changes and immunohistology

In at least 30 glomeruli (at 400x magnification) and 15 fields of vision (at 200x magnification) per cross section from each kidney with the glomerular cross sections with crescents in %, the degree of glomerulosclerosis (glomerularsclerosis index) and tubular injury (tubular injury index) were evaluated using semi-quantitative scores as described previously (Schlote et al., 2013). Fifty randomly selected glomeruli per kidney section were examined for each rat at 40x magnification and the mean cell numbers per glomerular cross section that stained positive for Pax8, Sox9, Ki67, WT1, SDF-1α, ED1 and CD3 were evaluated. Glomerular staining for the myofibroblast marker a-smooth muscle actin (SMA), fibronectin, sirius red and DPP-4 in glomeruli of human biopsies were analysed using a semi-quantitative
score ranging from 0 to 4 (0, no staining; 1, marginal staining; 2, obvious staining in ≤25%; 3, >25%; 4, >50% of the glomerular section area). CD163-positive cells and CD3-positive cells were counted in 25 randomly selected cortical fields at 20x magnification and demonstrated as cell number per mm².

2.7 | Detection of DPP-4 activity in tissue and plasma

Levels of DPP-4 activity in plasma were detected using an assay with Gly-Pro 4-methoxy-β-naphthylamide (Sigma Aldrich) as substrate as described previously in detail (Wang et al., 2014). In situ renal DPP-4 activity was detected using air-dried cryosections from rat kidneys as described previously (Luippold, Mark, Klein, Amann, & Daniel, 2018). Sections were incubated with Gly-Pro 4-methoxy-β-naphthylamide (1.5 mM) and Fast blue BB salt (2.4 mM) (both provided by Sigma: G9137, F3378; Munich, Germany) in assay buffer (Tris 50 mM, NaCl 150 mM, Tween 20 0.05% at pH 7.6) for 15 min at room temperature. Samples were subsequently washed with assay buffer, underwent nuclear staining using hemalaun and mounted with Aquatech (Merck, Darmstadt, Germany). Kidney sections from non-treated rats pretreated with linagliptin 1 μM served as negative controls.

2.8 | ELISA for detection of MCP-1 and SDF-1α plasma levels

MCP-1 plasma levels were quantified using a sandwich ELISA kit according to the protocol provided by the manufacturer (R&D, MJE00, Minneapolis, USA). SDF-1α/CXCL12 plasma levels were quantified by Natural and Medical Science Institute at University of Tübingen (Reutlingen, Germany) using a sandwich ELISA.

2.9 | Data and analysis

All data were analysed in a blinded fashion and presented as scatter plots showing each single data point representing the number of independent values and means ± SEM using bars and whiskers. Group sizes in the animal experiment were n = 11 and in human biopsy study at least n = 10. Only for the complex co-localization studies in Figure 8h,i was the number of samples per group limited to 5. Outliers were excluded according to the 2-σ method, excluding all values exceeding ±2-fold SD. The number of included data per group was mentioned in the figure legends. After testing values for normal distribution using Kolmogorov-Smirnov test, data were analysed using a one-way or two-way ANOVA followed by Bonferroni post hoc tests only if F achieves the necessary level of statistical significance of P < 0.05 and there is no significant variance inhomogeneity. Non-parametric data were analysed by Kruskal-Wallis test followed by Dunn’s multiple comparison test. Hereby, P < 0.05 was accepted as statistically significant and marked by asterisks: *P-values < 0.05. Compared groups are indicated in the graphs by connecting lines. Statistical analysis was performed using GraphPad Prism 8 for Windows software (Version 8.0.0, GraphPad software Inc., San Diego, CA, USA). The manuscript complies with BJP’s recommendations and requirements on experimental design and analysis (Curtis et al., 2018).

2.10 | Nomenclature of targets and ligands

Key protein targets and ligands in this article are hyperlinked to corresponding entries in the IUPHAR/BPS Guide to PHARMA-
COLOGY http://www.guidetopharmacology.org and are permanently archived in the Concise Guide to PHARMAKOLOGY 2019/20 (Alexander et al., 2019).

3 | RESULTS

3.1 | DPP-4 was strongly expressed in nephritic glomeruli in cellular crescents

>DPP-4 was detected in renal biopsies with different renal disease entities using immunohistochemistry. DPP-4 was highly expressed in renal cortex within proximal tubular cells (Figure S1A) in healthy and diseased kidneys (Figure S1B–D). However, in kidney biopsies with progressed chronic kidney disease, DPP-4 is reduced in areas with extended fibrosis (Figure S1E, F). Glomerular DPP-4 staining was variable in all investigated diseased and healthy kidneys ranging from low expression to marked predominantly podocytic expression (Figure 2a,b,e). Glomerular DPP-4 expression was significantly increased in pauci immune glomerulonephritis, glomerular basement membrane nephritis and minimal change glomerulonephritis compared to transplants without rejection, antibody-mediated rejection, IgA nephropathy and diabetic nephropathy (Figure 2e). Interestingly, in nephritic glomeruli from patients with lupus nephritis (Figure 2c), pauci immune glomerulo-
nephritis (Figure 2d) and glomerular basement membrane nephritis (Figure 2f), DPP-4 was strongly expressed in cellular crescents (Figure 2c,d,f). Co-localization studies in anti-glomerular basement membrane nephritic glomeruli indicated strong DPP-4 expression of Pax8-positive epithelial cells within the crescent (Figure 2f). Therefore, we investigated the pathogenetic role of DPP-4 in crescent formation in a rat model of glomerular basement membrane nephritis. An in situ DPP-4 activity assay could confirm glomerular DPP-4 activity in healthy rats (Figure 3a). This activity was increased in nephritic glomeruli (Figure 3b), especially within the area of adherence to the Bowman capsule (Figure 3b, red arrowheads) and crescents (Figure 3c, red arrowheads). The DPP-4 inhibitor linagliptin was used to block DPP-4 activity, resulting in almost complete inhibition of detectable renal DPP-4 activity 7 or 14 days after start of the treatment (Figure 3d,e), without any effect on DPP-4 expression (Figure S2). In addition, plasma DPP-4 activity was strongly inhibited in linagliptin-treated groups (Figure 3f).
3.2 | Linagliptin treatment ameliorated renal function and morphology in experimental glomerular basement membrane nephritis

In order to investigate the role of DPP-4 in the pathogenesis of crescentic glomerulonephritis, we treated anti-glomerular basement membrane nephritic rats with the DPP-4 inhibitor linagliptin either in a preventive regimen starting at the day of disease induction or in a therapeutic regimen with treatment starting 7 days or 4 weeks later (Figure 1). While therapeutic DPP-4 inhibition starting after 1 week could only slightly but significantly reduce proteinuria and serum urea levels on Day 14 (Figure 4a,c), initial preventive treatment did not significantly lower proteinuria in the short-term experiment (Figure 4a). In the long-term preventive linagliptin treatment group, proteinuria was lowered by more than 25% on Weeks 3–5 but did not reach the level of significance at endpoint on Week 8 (Figure 4b). Serum creatinine was increased by less than 20% in this model (Figure 4e,f) with a tendency to reduce levels in the preventive long-term DPP-4 inhibitor group (Figure 4f). Rats injected with the anti-glomerular basement membrane antibody showed cellular crescents in more than 50% of glomerular cross sections (Figure 5a–c). In the short-term experiment, glomerulosclerosis and tubular injury were significantly lower on Day 7 (Figure 5d,f) after preventive DPP-4 inhibition with no effect on crescent formation (Figure 5b). In contrast, long-term linagliptin treatment significantly reduced crescent formation in both preventive and therapeutic treatment (Figure 5c), indicating that most likely crescent formation and resolution were affected. However, glomerulosclerosis and tubular injury were significantly ameliorated by 20–25% only after preventive DPP-4 inhibitor treatment (Figure 5e,g).

3.3 | Linagliptin modulated inflammatory response in glomerular basement membrane nephritis

Inflammatory cells are important pathophysiologic mediators in crescent formation. Therefore, we analysed how linagliptin treatment influenced renal inflammation in experimental glomerular basement membrane nephritis. Linagliptin time dependently
changed glomerular influx of ED1-positive macrophages. In short-term glomerular basement membrane nephritis on Day 14, glomerular ED1-positive macrophages were significantly increased in the therapeutically treated group compared to the non-treated nephritic group (Figure 6a,g). In contrast, at Week 8 after nephritis induction, macrophage numbers tended to be lower in the preventive treatment group compared to untreated and therapeutically treated rats (Figure 6b). No clear linagliptin effect on macrophage differentiation was observed. The number of CD163-positive M2-like macrophages was very low, with only single detectable cells in the glomeruli (data not shown) and there was no significant change due to treatment with the DPP-4 inhibitor (Figure 6c,d). Since the macrophage attracting chemokine MCP-1 is a substrate for DPP-4, we measured MCP-1 serum levels after short- and long-term treatment. Induction of glomerular basement membrane nephritis significantly increased serum MCP-1 on early and late time points, without clear effect of linagliptin treatment as assessed by ELISA (Figure 6e,f). The glomerular infiltration with CD3-positive T-cells was relatively low after induction of glomerular basement membrane nephritis (Figure 7a–c). In early glomerular basement membrane nephritis, glomerular CD3-positive T-cells were transiently reduced on Day 7 in rats preventively treated with linagliptin (Figure 7a). In late glomerular basement membrane nephritis, the T-cell reduction was less pronounced, reaching the significance level only on Week 4 in preventive linagliptin-treated rats (Figure 7b). However, the number of CD3-positive cells in the tubulointerstitial compartment was higher and increased with time in nephritic rats (Figure 7d,e). Only long-term linagliptin treatment in the preventive but not in the therapeutic regimen reduced CD3-positive T-cells by about 50% (Figure 7e).
3.4 | DPP-4 inhibition reduced migration and proliferation of parietal epithelial cells on glomerular tuft

Migration and proliferation of parietal epithelial cells on the glomerular tuft are crucial in crescent formation and are accompanied by the loss of podocytes (Figure 8c,d). Healthy control glomeruli had no PAX8-positive cells on their tufts (Figure 8a–c). In both the short-term (Figure 8a) and long-term experiments (Figure 8b), the number of Pax8-positive parietal epithelial cells on the glomerular tuft was lowered by about 50% by both the preventive as well as therapeutic linagliptin treatment on Day 14 and Week 8 (Figure 8a,b). However, co-localization studies using the proliferation marker PCNA together with Pax8 and ED1 demonstrated that linagliptin treatment reduced the number of Pax8-positive parietal epithelial cells (Figure 8g,h) but increased proliferation of ED1-positive macrophages (Figure 8g,i).

3.5 | DPP-4 inhibition reduced podocyte stress and renal fibrosis

Induction of glomerular basement membrane nephritis resulted in a marked loss of podocytes, which was hardly ameliorated by linagliptin treatment. Interestingly, linagliptin increased glomerular proliferation significantly on Day 14 (Figure 8e) and showed anti-proliferative properties on Week 8 in the preventive linagliptin treatment group (Figure 8f). Therapeutic groups were less effective in changing proliferative activity (Figure 8e,f).
Linagliptin preventive treatment in the short-term experiment, as assessed by detection of WT1-positive cells (Figure 9a,c). In the long-term experiment, both linagliptin therapy regimens resulted in slightly higher numbers of WT1-positive podocytes at endpoint of the experiment (Figure 9b). Quantitative mRNA expression analysis confirmed this weak non-significant effect for the podocyte slit diaphragm molecule nephrin (Figure 9d) but not for podocin (Figure S3C). However, podocyte stress, as assessed by glomerular desmin staining, was significantly lower in linagliptin-treated rats with the lowest values in preventive linagliptin treatment groups (Figure 9e,f).

Both preventive and therapeutic linagliptin treatment significantly reduced glomerular (Figure 10a) as well as cortical fibrosis (Figure 10b) in the long-term experiment, as assessed by sirius red stain. The anti-fibrotic effect of linagliptin was confirmed by glomerular analysis of the matrix component fibronectin (Figure 10c) and myofibroblast marker α-SMA (Figure 10d). In healthy glomeruli, SMA was absent (Figure 10g, arrowhead marks SMA-positive arteriole) but increased early after disease induction on Days 7 and 14 (Figure 10e) showing SMA-positive cells within the crescents, on the glomerular tuft and the Bowman capsule (Figure 9g, red arrowheads); but the number was not changed by linagliptin treatment in the short-term experiment (Figure 10e). In the long-term experiment, glomerular SMA myofibroblasts were significantly reduced in both linagliptin-treated groups with more pronounced reduction in the preventive group (Figure 10f).
Linagliptin transiently increased DPP-4 substrate SDF-1α (CXCL12 α) with minor changes in downstream pathways

As a potential mediator of linagliptin effects, we investigated the DPP-4 substrate in our nephritis model. Total serum SDF-1α was significantly increased in both linagliptin-treated groups (Figure 11a), while mRNA expression of SDF-1α was similarly increased in all nephritic groups in the short-term experiment and not altered by linagliptin treatment (Figure 11b), indicating reduced degradation of this chemokine. SDF-1α was expressed by podocytes and was absent in glomerular lesions, as shown by double immunofluorescence staining with synaptopodin (Figure 11g,h). However, not uncommon for a secreted protein, we could not detect significant differences in glomerular SDF-1α expression, evaluated by immunohistochemistry, between treated and non-treated anti-glomerular basement membrane nephritic groups (data not shown). Furthermore, we investigated glomerular SDF-1α signalling via ERK and STAT pathways. Both pathways were markedly increased in anti-glomerular basement membrane nephritic rats on Day 14 as well as Week 8 after disease induction, as shown by P-ERK1/2- and P-STAT3-positive cells (Figure 11c,d). In short-term glomerular basement membrane nephritis on Week 2 preventive linagliptin treatment increased ERK1/2 and STAT3 phosphorylation of glomerular cells in number compared to untreated nephritic control but failed to reach the level of significance due to high variability (Figure 11c,d). In contrast, in the long-term experiment, P-ERK1/2-positive glomerular cells were significantly reduced in therapeutically linagliptin-treated rats, showing the same...
tendency for the group treated with the preventive regimen (Figure 11c). On Week 8, the activation of STAT3 was not influenced by linagliptin treatment (Figure 11d). Since we could show in a previous study that transcription factor Sox9 was highly up-regulated in parietal epithelial cells and Sox9-positive cells could be also detected on the glomerular tuft in anti-glomerular basement membrane nephritic rats, we expected that Sox9 is an important regulator in the pathophysiology of crescent formation. Therefore, we investigated linagliptin-induced changes in Sox9 expression in our experimental setting. Linagliptin treatment significantly increased the number of Sox9-positive cells on glomerular tuft in the preventive group on Days 7 and 14, while therapeutic treatment showed only a tendency to increased Sox9-positive cells on Day 14 on the glomerular tuft compared to untreated nephritic control (Figure 11e). In contrast, in the long-term experiment on Week 4, Sox9-positive cells on the glomerular tuft were significantly reduced in the preventive group compared to untreated and therapeutically treated nephritic rats (Figure 11f). On Week 8, both linagliptin treatment groups showed reduced numbers of Sox9-positive cells on glomerular tuft compared to untreated nephritic controls (Figure 11f). Interestingly, the number of Sox9-positive cells on the tuft paralleled with linagliptin-induced changes observed for P-ERK1/2-positive cells, indicating a potential link (Figure 11c,e,f).

4 | DISCUSSION

DPP-4 inhibitor are widely used in patients with diabetes mellitus to improve glycaemic control by inhibition of incretin breakdown (Mulvihill, 2018). Due to its anti-inflammatory and anti-fibrotic effects, DPP-4 inhibitors are recently suggested for treatment of chronic kidney disease (Kanasaki, 2018). There are several preclinical studies using animal models for acute injury (Chen et al., 2017; Glorie et al., 2012; Reichetzeder et al., 2017) and chronic kidney disease (Hasan et al., 2019; Tsuprykov et al., 2016) confirming the renoprotective effects of DPP-4 inhibition. However, the role of DPP-4 in pathogenesis and progression of glomerulonephritis was not investigated in detail (Higashijima, Tanaka, Yamaguchi, Tanaka, & Nangaku, 2015). Since we had observed markedly increased DPP-4 expression in cellular crescents found in human pauci
immune glomerulonephritis, anti-glomerular basement membrane and lupus nephritis patients we investigated if the exopeptidase DPP-4 is directly or indirectly involved in the pathogenesis of crescent formation or resolution. In our experimental glomerular basement membrane nephritis, we observed reduced numbers of crescents in a preventive as well as therapeutic treatment regimen with the DPP-4 inhibitor linagliptin 8 weeks after disease induction. In contrast, in short-term glomerular basement membrane nephritis, DPP-4 inhibition did not significantly change the number nor the morphology of crescentic glomeruli, indicating that DPP-4 inhibition rather accelerated the resolution of the existing lesions than inhibited their formation. Similar to our observations in human biopsies, DPP-4 was also prominently expressed in crescents and activated parietal epithelial cells in our anti-glomerular basement membrane model. Therefore, this model was ideal to investigate DPP-4-mediated interactions in pathogenesis and resolution of crescent formation. Pax8-positive parietal epithelial cells are the dominant cells in cellular crescents, forming the crescent by migration and proliferation (Shankland, Smeets, Pippin, & Moeller, 2014).
Pax8-positive parietal epithelial cells on the glomerular tuft were reduced in the preventive and therapeutic treatment with linagliptin 14 days after disease induction and in our long-term study. Since linagliptin also reduced Pax8-positive cells on glomerular tuft when therapy started on Week 5 after disease induction, we suggest that DPP-4 inhibition rather accelerates resolution of crescentic lesions than preventing their formation. Cellular crescents can be resolved but upon gradual encasement of parietal epithelial cells with extracellular matrix a fibrous crescent develops, a lesion that is considered as irreversible (Anguiano, Kain, & Anders, 2020).

In our study, we analysed SDF-1α as a potential player of linagliptin-mediated reduction of Pax8-positive parietal epithelial cells on glomerular tuft. The chemokine SDF-1α is degraded by DPP-4 and expressed by podocytes (Miglio, Vitarelli, Klein & Benetti, 2017; Romoli et al., 2018) and thus a potential mediator of linagliptin effects. We could confirm the increase of circulating SDF-1α in nephritic rats after linagliptin in short-term treatment. SDF-1α can interact with two different receptors CXCR4 and CXCR7 that exert numerous functions (Miglio et al., 2017) and are both expressed in parietal epithelial cells and podocytes (Romoli et al., 2018). SDF-1α was described as an intrinsic podocyte progenitor feedback mechanism: podocytes produce SDF-1α to keep podocyte progenitor cells quiescent. Loss of podocytes was associated with reduced SDF-1α secretion and hereby activation of podocyte progenitors from Bowman’s capsule (Romoli et al., 2018; Sayyed et al., 2009). In vitro experiments demonstrated that SDF-1α strongly induced notch2 in podocytes without induction.
of notch2 signalling (Romoli et al., 2018). In our study, glomerular notch2 expression was significantly increased but not changed by linagliptin treatment (Figure S3E). However, it might be that locally increased SDF-1α concentrations are responsible for lesser mobilization of Pax8-positive cells from Bowman’s capsule. SDF-1α signalling via ERK1/2 and STAT-phosphorylation was not significantly altered by linagliptin and therefore probably not responsible for the observed reduced mobilization of parietal epithelial cells. Since we showed earlier that most Sox9-positive cells on glomerular tuft are also positive for Pax8 and that Sox9 is probably involved in parietal epithelial cell differentiation and podocyte regeneration (Prochnicki et al., 2018), we investigated the effect of linagliptin treatment on Sox9 expressing cells. In our model, Sox9 might be regulated by ERK1/2 phosphorylation, since Sox9 expression and ERK1/2 phosphorylation were parallel. In the short-term experiment, the number of Sox9-positive cells on glomerular tuft increased in contrast to Pax8-positive cells, whereas in the long-term experiment, Sox9-positive and Pax8-positive cells decreased in a similar manner. Therefore, reduction of Pax8-positive cells on the glomerular tuft seems at least not directly mediated by linagliptin-induced changes in Sox9 expression.

Macrophages are another important cell type in crescent formation that proliferate and undergo apoptosis during pathogenesis of crescentic glomerulonephritis (Lan et al., 1997). On Day 14, we could observe increased glomerular macrophage infiltration and proliferation in linagliptin-treated groups, which might be responsible for the lacking improvement of the disease at this time point. Accelerated macrophage acquisition in the short-term experiment could be influenced by the chemokine MCP-1, which is also a
substrate of DPP-4. Using ELISA, we could not confirm increased MCP-1 serum levels after linagliptin treatment, but the ELISA did not differentiate between active and cleaved MCP-1. Therefore, differences in MCP-1 activity by linagliptin treatment cannot be excluded. Macrophage subpopulations might be involved in glomerular repair. While other groups observed increased macrophage conversion to anti-inflammatory CD163-positive M2-like macrophages by DPP-4 inhibitor treatment (Cappetta et al., 2019; Iwakura, 2019) that reduces infiltration with, for example, T-cells, in our experiment, linagliptin treatment did not change macrophage differentiation to CD163-positive subsets. Nevertheless, preventive linagliptin treatment reduced other immune cells like CD3-positive T-cells especially early in the glomerular and late in the tubulointerstitial compartment. Immunoregulatory action of DPP-4 is mediated by a variety of mechanisms including acting as a costimulatory molecule and changes in cytokine expression (Wagner, Klemann, Stephan & von Horsten, 2016). While glomerular crescents are the hallmark of severe glomerular damage and important for disease progression, podocytes are essential for renal filtration (Pavenstadt, Kriz, & Kretzler, 2003). The number of podocytes, as assessed by WT1-positive cells, was not different in linagliptin-treated groups, which might explain why proteinuria was not significantly ameliorated. However, there was a tendency...
to more WT1 positive cells, higher expression of nephrin and significantly less podocyte stress in preventive and therapeutic linagliptin-treated groups in the long-term experiment.

We also observed anti-fibrotic effects by linagliptin therapy, as assessed by sirius red stain for collagens, fibronectin and the myofibroblast marker α-SMA, which might be responsible for the beneficial effects on kidney function and regeneration. Anti-fibrotic action of DPP-4 inhibition therapy was shown in many different preclinical studies using models for diabetic nephropathy (Kanasaki et al., 2014; Shi, Koya, & Kanasaki, 2016) or chronic kidney disease (Tsuprykov et al., 2016). Linagliptin reduced TGF-β signalling in several models by different molecular pathways (Gupta & Sen, 2019). Furthermore, DPP-4 was shown to interact with cation-independent mannose 6-phosphate receptor (CIM6PR), thus being involved in TGF-β activation (Gangadharan Komala, Gross, Zaky, Pollock, & Panchapakesan, 2015). This interaction can be inhibited by linagliptin treatment (Gangadharan Komala et al., 2015). It remains unclear if this mechanism leads to the observed anti-fibrotic effects in our glomerulonephritis model and must be evaluated in later studies. However, the anti-fibrotic action of linagliptin might be prevent the development of irreversible fibrous crescent formation and hereby allows its resolution. This theory could explain that in the long-term experiment, therapeutic administration of linagliptin is not able to dissolve already irreversibly remodelled crescent lesions, so that only a partial improvement could be achieved.

Clearly, our study is limited by focusing on selected DPP-4 substrates and we cannot exclude that additional DPP-4 substrates or interaction partners are involved. One key substrate of DPP-4 is, for example, GLP-1, but linagliptin effects on crescent formation mediated by the incretin GLP-1 are unlikely since in humans neither podocytes nor parietal epithelial cells express the GLP-1 receptor on their surface (Pyke et al., 2014).

In conclusion, our study showed renoprotective effects of DPP-4 inhibition. In the short-term treatment, DPP-4 inhibition increased glomerular macrophage proliferation and numbers while simultaneously Pax8-positive parietal epithelial cell proliferation was reduced leading to lower Pax8-positive cells on glomerular tuft. These early DPP-4 inhibitor effects might contribute to reduced crescent formation and improved crescent resolution, fibrosis and podocyte stress observed in the long-term experiment when rats were treated preventively and to some extent also therapeutically with linagliptin in a model of crescentic glomerulonephritis. The DPP-4 substrate SDF-1 might be an important mediator of these effects, but we assume a complex effect of DPP-4 inhibition with currently unknown additional players. The observed effects, however, are not yet sufficient to suggest DPP-4 inhibitor treatment as a single therapy and linagliptin effects on crescent formation fit partly to current concepts of crescentic lesion pathogenesis, indicating complex interactions that need further investigation. Nevertheless, DPP-4 inhibitor treatment by linagliptin not only is a secure therapy option for diabetic patients with kidney injury but also can improve progression of renal disease.

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AUTHOR CONTRIBUTIONS
A.-LM conducted the experiments, analysed the data and, wrote the manuscript. N.E. and I.S. carried out immunohistological staining and evaluation. T.K. supplied the linagliptin and proofread the paper. K.A. wrote the paper. C.D. conceived and designed the study, analysed the data and wrote the manuscript.

CONFLICT OF INTEREST
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DECLARATION OF TRANSPARENCY AND SCIENTIFIC RIGOUR
This Declaration acknowledges that this paper adheres to the principles for transparent reporting and scientific rigour of preclinical research as stated in the BIP guidelines for Natural Products Research, Design and Analysis, Immunoblotting and Immunohistochemistry and Animal Experimentation, and as recommended by funding agencies, publishers and other organizations engaged with supporting research.

DATA AVAILABILITY STATEMENT
The data that support the findings of this study are available from the corresponding author upon reasonable request. Some data may not be made available because of privacy or ethical restrictions.

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