Glucagon-like peptide-1 receptor signaling modifies the extent of diabetic kidney disease through dampening the receptor for advanced glycation end products–induced inflammation

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Glucagon like peptide-1 (GLP-1) is a hormone produced and released by cells of the gastrointestinal tract following meal ingestion. GLP-1 receptor agonists (GLP-1RA) exhibit kidney-protective actions through poorly understood mechanisms. Here we interrogated whether the receptor for advanced glycation end products (RAGE) plays a role in mediating the actions of GLP-1 on inflammation and diabetic kidney disease. Mice with deletion of the GLP-1 receptor displayed an abnormal kidney phenotype that was accelerated by diabetes and improved with co-deletion of RAGE *in vivo*. Activation of the GLP-1 receptor pathway with liraglutide, an anti-diabetic treatment, downregulated kidney RAGE, reduced the expansion of bone marrow myeloid progenitors, promoted M2-like macrophage polarization and lessened markers of kidney damage in diabetic mice. Single cell transcriptomics revealed that liraglutide induced distinct transcriptional changes in kidney endothelial, proximal tubular, podocyte and macrophage cells, which were dominated by pathways involved in nutrient transport and utilization, redox sensing and the resolution of inflammation. The kidney-protective action of liraglutide was corroborated in a non-diabetic model of chronic kidney disease, the subtotal nephrectomised rat. Thus, our findings identify a novel glucose-independent kidney-protective action of GLP-1-based therapies in diabetic kidney disease and provide a valuable resource for exploring the cell-specific kidney transcriptional response ensuing from pharmacological GLP-1R agonism.

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Diabetic kidney disease (DKD) occurs in up to 40% of individuals with diabetes and remains the primary cause of kidney failure worldwide,[1–3] accounting for at least a third of all cases requiring renal replacement therapy.[4] The outlook for DKD has improved over recent decades as a result of improved blood glucose control, blood pressure management with an emphasis on renin-angiotensin system blockade, and, more recently, the use of sodium-glucose cotransporter-2 inhibitors. However, a significant proportion of individuals with diabetes will still progress to kidney failure or die prematurely from a cardiovascular event.[5–7]

Glucagon-like peptide-1 (GLP-1) is a gut-derived incretin hormone, which is produced and released by the L cells of the gastrointestinal tract after meal ingestion.[8] GLP-1 potentiates glucose-dependent insulin secretion from pancreatic islets via a single canonical receptor, the GLP-1R,[8,9] but increasingly,

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Translational Statement

Considerable interest exists in identifying the actions of incretin-based therapies beyond glucose lowering, particularly in the kidney. In this study, we showed that mice with genetic disruption of *Glp1r* spontaneously develop chronic renal injury. We demonstrate that superimposition of diabetes in *Glpr1r*-deficient mice accelerated diabetic kidney disease (DKD) development and the phenotypic changes could be reversed by deletion of the proinflammatory receptor for advanced glycation end products (*Ager*). Treatment of diabetic mice with a glucagon-like peptide-1 receptor agonist (GLP-1RA), liraglutide, reduced kidney injury. Unbiased exploration using single-cell transcriptomics of kidney identified that GLP-1R agonism with liraglutide in diabetic mice remodeled a network of nutrient synthesis and transport, and promoted redox sensing signals in the proximal tubule, podocyte, and macrophage cell populations. Liraglutide treatment led to dampening of inflammatory signals in macrophages. This study demonstrates the importance of intact GLP-1R signaling in the maintenance of kidney homeostasis, provides a rationale for investigating GLP-1RA for the treatment of non-DKDs, and highlights the glucose-independent renal benefits of GLP-1RA.

extrapancreatic functions of GLP-1 are being reported. Both experimental[10,11] and more recently clinical studies such as the LEADER (Liraglutide Effect and Action in Diabetes: Evaluation of Cardiovascular Outcome Results)[12] and the REWIND (Researching Cardiovascular Events with a Weekly Incretin in Diabetes)[13] trials using GLP-1R agonists (GLP-1RA) such as liraglutide and dulaglutide, respectively, have demonstrated renoprotective actions of GLP-1 receptor agonism. However, whether these effects are glucose-independent has remained difficult to determine.

Receptor for advanced glycation end products (RAGE) is a multiligand, type 1 pattern recognition receptor and a member of the immunoglobulin superfamily that is central in mediating the proinflammatory actions of its ligands, including the danger-associated molecular patterns S100 proteins (calgranulins), AGEs, and high-mobility group box 1.[14–17] Although its basal expression is generally low, RAGE is expressed at high levels in the vascular endothelium and leukocytes and is induced in most cell types after injury, hypoxia, or inflammation, including in the kidney, in particular, the proximal tubular cells,[18] podocytes,[19] and mesangial cells.[20–22] Our group[23–25] and others[26,27] have demonstrated that the deletion of RAGE in mice confers renoprotection via dampening effects on oxidative stress and profibrotic pathways, whereas RAGE overexpression accelerates nephropathy in mouse models.[28] Results from *in vitro* studies have suggested that GLP-1 can modulate RAGE signaling and expression in multiple cell types.[29,30]

Considerable interest exists in identifying the actions of incretin-based therapies beyond glucose lowering, and a direct action of GLP-1 on the kidney has been proposed.[31,32] Although GLP-1R has been shown to be expressed within the kidney at low levels,[33–36] the role of GLP-1 signaling in the kidney is still undefined. The present study aimed to address the gap in our understanding of GLP-1 action in DKD by exploring the interaction between GLP-1 and RAGE in the kidney, in settings where glucose-lowering effects of GLP-1R agonism are absent, such as (i) loss of function studies in animal models with the absence of a functional GLP-1R, (ii) gain of function signaling through the GLP-1R pathway using a GLP-1 analog in animals with or without diabetes, and (iii) unbiased molecular network analysis using single-cell transcriptomics to assess kidney cell–specific changes in response to pharmacologic GLP-1R signaling.

METHODS

For detailed methods, refer to Supplementary Methods. The ARRIVE (Animal Research: Reporting of In Vivo Experiments) guidelines 2.0 for experimental design and reporting animal research were followed.[37]

Animal studies

Generation of RAGE x GLP-1R double knockout mice. Mice deficient in either *Ager*, *Glpr1*, or both *Ager* and *Glpr1* (*Ager-Glp1r*$^{-/-/-/-}$ double knockout [dKO]) were generated on a C57BL/6J background (wild-type) (WT; C57BL/6J). *Ager*$^{-/-}$ mice were kindly donated by Prof. Angelika Bierhaus, University of Heidelberg, Germany.[38,39] *Glp1r*$^{-/-}$ mice were kindly donated by Prof. Daniel Drucker, University of Toronto, Canada.[40] To generate the dKO mice, *Ager*$^{-/-}$ mice were crossed with *Glp1r*$^{-/-}$ KO mice. The resulting *Ager-Glp1r*$^{+-/+-}$ heterozygous mice were crossed and, after 4 generations, the *Ager-Glp1r*$^{-/-/-/-}$ mouse was generated, and a colony was established from these founders. The colony was maintained as *Ager-Glp1r*$^{+-/+-}$ heterozygous mice in order to obtain WT littermates. For details of chemical-induced diabetes and study protocol, refer to Supplementary Methods.

Ins2^{Akita} mice. Diabetic Ins2^{Akita} mice (C57BL/6-*Ins2^{Akita}*/J) and their WT counterparts were purchased from the Jackson Laboratory (Strain #003548), and at 6 weeks of age, male WT or heterozygous Ins2^{Akita} mice were randomly assigned to receive either vehicle (phosphate-buffered saline/Tween 80) or liraglutide (Lira, 50 μg/kg) by daily subcutaneous injection for 20 weeks (n = 12 mice per group), or the angiotensin-converting enzyme inhibitor (ACEi) perindopril (2 mg/kg) provided in the drinking water. A separate group of heterozygous Ins2^{Akita} mice were administered a combination therapy consisting of liraglutide and perindopril (L+A) for 20 weeks (n = 12 mice per group).

Rat subtotal nephrectomy model. Female Fischer 344 rats (Charles River) aged 8 weeks were randomized to undergo subtotal nephrectomy (SNx) or sham surgery, as previously described.[41] Briefly, animals were anesthetized with 2.5% isoflurane, the right kidney was removed via subcapsular nephrectomy, and infarction of approximately two-thirds of the left kidney was achieved via selective ligation of 2 of the 3 or 4 branches of the left renal artery. Sham surgery consisted of laparotomy and manipulation of both kidneys before wound closure. One week after surgery, rats were randomized to receive liraglutide (Novo Nordisk Canada Inc.) 0.2 mg/kg twice daily subcutaneously[42] or phosphate-buffered saline (n = 6–12 per group) for 7 weeks.

Statistical analysis

Data are expressed as means ± SD, unless otherwise stated. The analyses of experimental data were performed by either 1-way or 2-way

analysis of variance followed by *post hoc* analysis using the Tukey test or unpaired Student *t* tests. Outliers were identified and excluded using the ROUT (Robust Regression Outlier Removal) test. Data for albuminuria were not normally distributed and therefore analyzed after logarithmic transformation. The level of statistical significance was set *a priori* at $P < 0.05$. GraphPad Prism (version 9.3.1) was used for all statistical analyses.

RESULTS

Loss of GLP-1R drives a renal phenotype that is accelerated by diabetes

GLP-1-based therapies are thought to directly influence renal physiology and have indirect metabolic and hemodynamic actions that might reduce renal risk in diabetes; however, GLP-1 action on the kidney is not well understood.[31] Accordingly, to explore the physiological involvement of the GLP-1R signaling pathway in the regulation of kidney function, we studied mice with global genetic deletion of the receptor *Glp1r* (*Glpr1r*$^{-/-}$ mice) and their WT littermates (C57BL/6 background) (Figure 1a). After 24 weeks of observation, *Glp1r*$^{-/-}$ mice spontaneously developed kidney injury as shown by albuminuria (Figure 1b), a reduction in plasma cystatin C (Figure 1c), an increase in cortical fibronectin deposition (Figure 1d), and glomerulosclerosis (Figure 1e and f).

We next assessed the susceptibility of mice with GLP-1R deficiency and experimental diabetes to the development of DKD by injecting *Glpr1r*$^{-/-}$ mice with streptozotocin (STZ) to induce insulin deficiency resembling type 1–like diabetes. *Glpr1r*$^{-/-}$ mice injected with STZ exhibited worsened albuminuria compared with either *Glpr1r*$^{-/-}$ mice in the absence of STZ or WT STZ-treated mice (Figure 1b). Together, these data highlight the importance of an intact GLP-1 signaling pathway in maintaining kidney health.

Genetic deletion of RAGE in *Glp1r*$^{-/-}$ mice improves diabetes-associated kidney injury

Our group[23–25] and others[26,27] have demonstrated that the deletion of RAGE confers a protective effect on kidney function, and previous *in vitro* work has suggested that GLP-1 can interfere with RAGE signaling.[29,30] To ascertain whether the loss of RAGE attenuates renal injury in the absence of the GLP-1R, we deleted RAGE in *Glpr1r*-deficient mice by cross-breeding *Ager*$^{-/-}$ mice with *Glpr1r*$^{-/-}$ mice to produce *Ager*$^{-/-}$:*Glp1r*$^{-/-}$ dKO mice and studied these mice in the presence or absence of STZ-induced diabetes for 24 weeks (Figure 2a). The deletion of RAGE protein in the kidneys of the *Ager*$^{-/-}$ mouse and the dKO mouse was confirmed by the enzyme-linked immunosorbent assay of plasma membrane–enriched extracts of the kidney cortex (Figure 2b). The absence of GLP-1R in the *Glpr1r*$^{-/-}$ single and dKO mice was confirmed by quantitative polymerase chain reaction of the kidney cortex (Figure 2c). Primer sequences are reported in Supplementary Table S1.

After 24 weeks, STZ diabetic mice displayed reduced body weight (Supplementary Figure S1A), increased food intake (Supplementary Figure S1B), increased blood glucose (Supplementary Figure S1C), and glycated hemoglobin (Supplementary Figure S1D), which was consistent across genotypes. Water intake (Supplementary Figure S1E) and urine output (Supplementary Figure S1F) were similarly increased in all diabetic mice and were not differentially affected in the KO mice. These data indicate that the deletion of either *Ager*, *Glp1r*, or both *Ager* and *Glp1r* simultaneously does not alter glycemic control nor feeding behaviors in mice with STZ-induced diabetes, indicating that the renal phenotypes observed were independent of differences in metabolic control.

RAGE protein was upregulated in the kidneys of normoglycemic *Glpr1r*$^{-/-}$ mice (Figure 2b). In the setting of STZ diabetes, RAGE protein was further upregulated in STZ-treated WT and *Glpr1r*$^{-/-}$ mice (Figure 2b). In WT diabetic mice, *Glp1r* mRNA expression was downregulated in the kidney compared with nondiabetic controls (Figure 2c), yet a reduction of *Glp1r* mRNA expression was not observed in *Ager*$^{-/-}$ diabetic mice. Consistent with previous observations from our laboratory[23–25] and others,[26,27] the extent of albuminuria was reduced in *Ager*$^{-/-}$ mice with diabetes (Figure 2d). In contrast, *Glp1r*$^{-/-}$ mice with STZ diabetes exhibited increased albuminuria (Figure 2d). Deleting *Ager* and *Glp1r* in mice (dKO) attenuated albuminuria in the setting of diabetes (Figure 2d), indicating that RAGE is essential for increased albumin excretion rate in *Glp1r*$^{-/-}$ mice. Further investigation of the renal phenotype revealed a significant effect of diabetes status on plasma cystatin C, with a reduction in WT diabetic mice, which was not influenced by the deletion of *Ager* or *Glp1r* (Figure 2e). The glomerular sclerotic index histology score, although increased in WT diabetic mice and *Glp1r*$^{-/-}$ mice, was not increased in *Ager*$^{-/-}$ mice or dKO mice (Figure 2f and g). *Ager*$^{-/-}$ mice with diabetes had lower glomerular sclerotic index scores than WT diabetic mice, indicating a protective effect of *Ager* deletion on glomerulosclerosis (Figure 2f). The deletion of *Ager* may primarily act on structural rather than functional parameters with an effect on alleviating glomerular injury; the effect on albumin is presumed to be related to the amelioration of diabetes-associated podocyte injury.

The presence of kidney injury molecule (KIM)-1 in the urine is consistent with proximal tubular injury.[43] WT diabetic mice exhibited an increase in urinary KIM-1, and this was also seen in diabetic *Glp1r*$^{-/-}$ mice (Figure 2h). However, the deletion of *Ager* ameliorated the diabetes-induced excretion of KIM-1 (Figure 2h). Deleting both *Ager* and *Glp1r* (dKO) significantly reduced urinary KIM-1 in the context of diabetes. In addition, diabetes induced an increase in renal fibronectin expression in WT mice (Figure 2i). The deletion of *Ager* but not *Glp1r* decreased diabetes-induced fibronectin deposition. In the dKO mouse, fibronectin was reduced (Figure 2i), which is comparable to that seen in the RAGE KO mouse. Taken together, these data identify a role for GLP-1R deficiency in conferring susceptibility to kidney injury and worsening DKD outcome in an experimental model of diabetes.

GLP-1R belongs to the B family subclass of G protein–coupled receptors. GLP-1R preferentially couples to $G\alpha_S$-regulated pathways, favoring the production of cyclic adenosine

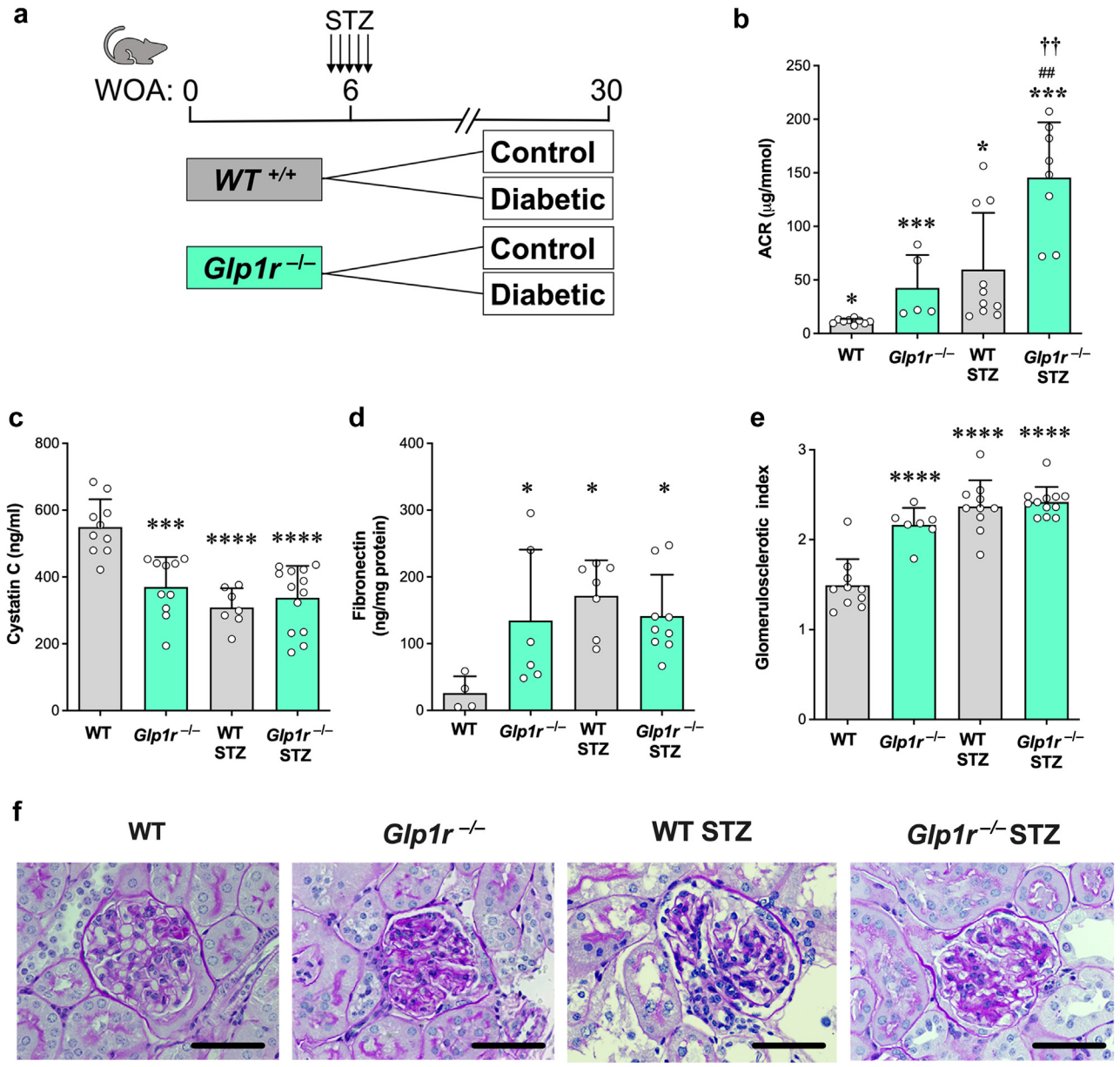

Figure 1 | Glucagon-like peptide-1 receptor (GLP-1R) deficiency is a susceptibility factor for nephropathy contributing to worsened diabetic kidney disease. (**a**) Schematic depicting the streptozotocin (STZ)-induced diabetes paradigm in wild-type (WT) and *Glp1r*$^{-/-}$ mice. After diabetes induction, mice were followed for 24 weeks. (**b**) Urinary albumin-to-creatinine (ACR) ratio and (**c**) plasma cystatin C. (**d**) Fibronectin in the kidney cortex. (**e**) Glomerular sclerotic index was quantitated using periodic acid–Schiff (PAS) staining and light microscopy under original magnification of ×400. (**f**) Representative PAS-stained kidney sections. Original magnification ×400 and Bar = 25 μm. Data are presented as mean ± SD (n = 5–10 per group). Dots represent individual mice. *P* values were determined by 2-way analysis of variance with the Tukey multiple comparison test. **P* < 0.05 versus WT, ****P* < 0.001 versus WT, *****P* < 0.0001 versus WT, ##*P* < 0.01 versus *Glp1r*$^{-/-}$, ††*P* < 0.01 versus WT STZ. *Glp1r*$^{-/-}$, *Glp1r* knockout mice; WOA, week of age. To optimize viewing of this image, please see the online version of this article at www.kidney-international.org.

monophosphate through increasing adenylate cyclase activity[44] and promoting the activation of signaling pathways coupled to protein kinase A (PKA). To establish that the decline in GLP-1R in the kidney in diabetes (WT mice) resulted in a decrease in GLP-1R canonical signaling, PKA activity was determined. Indeed, the decline in GLP-1R within the WT diabetic kidney was associated with a concomitant decrease in PKA activity compared with WT control (Supplementary Figure S2A). Moreover, RAGE-null mice with diabetes exhibited no change in kidney PKA activity compared with WT control or RAGE-null nondiabetic controls. Normoglycemic control mice with a deletion in *Glp1r* also exhibited reduced PKA activity. dKO mice did not have altered PKA activity in the diabetic setting (Supplementary Figure S2A). To assess pathophysiological

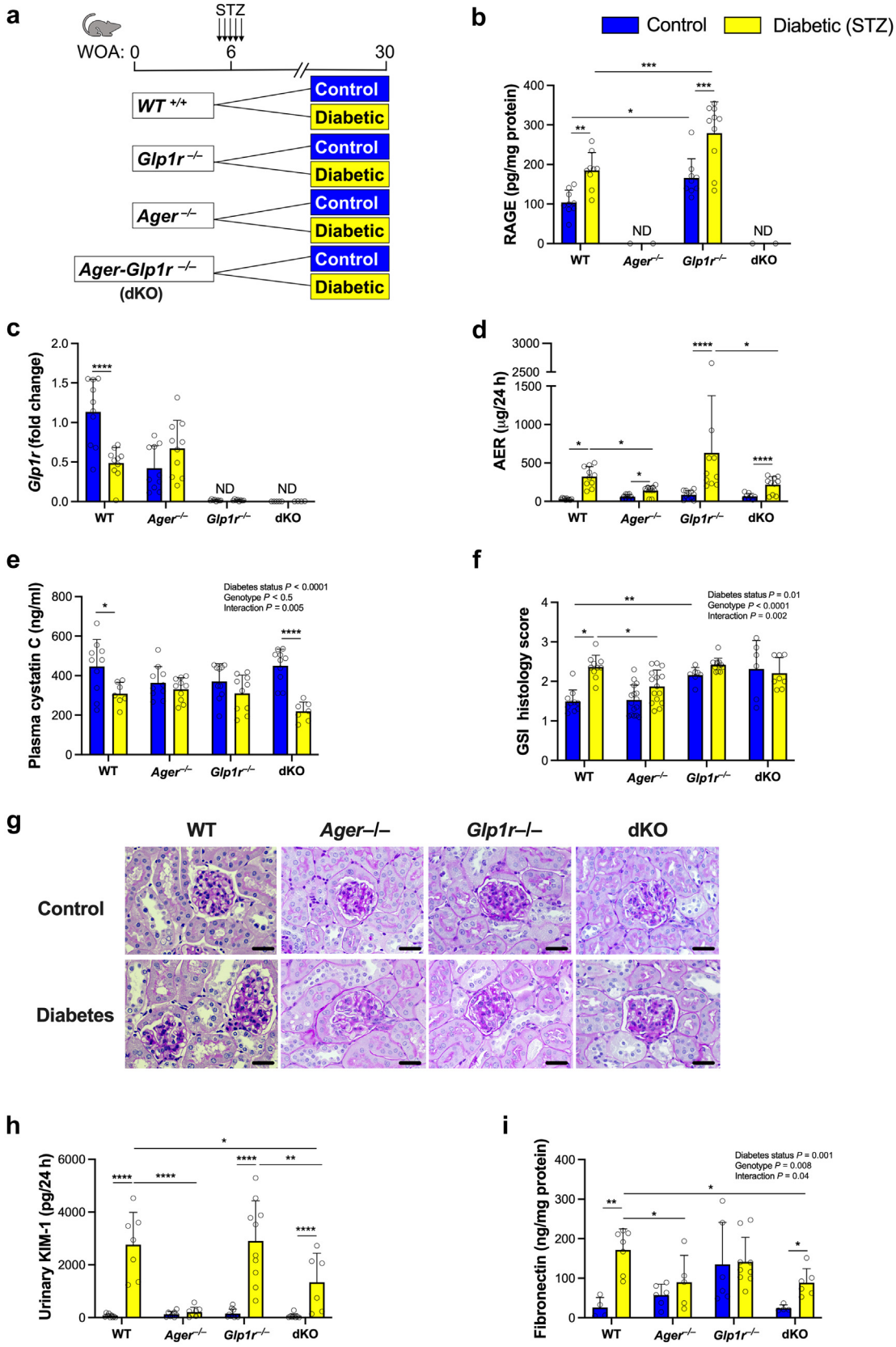

Figure 2 | Deletion of receptor for advanced glycation end products (RAGE) (*Ager*) in *Glp1r*$^{-/-}$ mice (*Ager-Glp1r*$^{-/-/-/-}$ mice) reverses key phenotypic changes in the setting of diabetic kidney disease. Wild-type (WT), *Glp1r*$^{-/-}$ mice, *Ager*$^{-/-}$ mice, and *Ager-Glp1r*$^{-/-/-/-}$ mice (dKO) with or without streptozotocin (STZ)-induced diabetes were followed for 24 weeks. (**a**) Schematic (continued)

pathways typically observed in DKD, we measured the levels of a marker of oxidative stress. Isoprostanes are prostaglandin-like compounds that are produced by free radical-mediated peroxidation of lipoproteins. Mice with diabetes had enhanced oxidative stress as reflected by an increase in urinary excretion of 15-isoprostane F_{2t}, which was dampened in response to the deletion of RAGE (Supplementary Figure S2B). Although *Glp1r*$^{-/-}$ mice did not display protection against diabetes-induced oxidative stress, urinary levels of 15-isoprostane F_{2t} were attenuated by co-deletion of *Ager* and *Glp1r* (dKO) (Supplementary Figure S2B). These data support the importance of RAGE in the development of kidney injury in *Glp1r*$^{-/-}$ mice.

The GLP-1R agonist liraglutide reverts podocyte injury and DKD

With our findings suggesting that GLP-1R deficiency exacerbates kidney injury in DKD, we next examined the actions of the clinically used GLP-1RA liraglutide in the insulin-2 *Akita* (*Ins2*^{Akita}) mouse. These animals develop pancreatic β-cell failure as a result of β-cell–selective proteotoxicity resulting from misfolding of insulin2 (*Ins2*)[45] and are a model of severe insulin deficiency suitable for the analysis of DKD.[46] Because the blockade of the renin-angiotensin system is the gold standard approach to slow the progression of DKD in subjects with diabetes, the ACEi perindopril was used alone, or in combination with liraglutide (L + A). Mice were treated from 6 weeks of age and followed for 20 weeks (Figure 3a). At the end of the study, blood glucose (Supplementary Figure S3A), glycated hemoglobin (Supplementary Figure S3B), food intake (Supplementary Figure S3C), water intake (Supplementary Figure S3D), and urinary output (Supplementary Figure S3E) were increased and body weight was reduced (Supplementary Figure S3F) in diabetic Ins2^{Akita} mice relative to WT controls. The administration of liraglutide (Lira), the ACEi perindopril, or coadministration of both liraglutide and perindopril (L + P) did not alter any of these parameters (Supplementary Figure S3A–F).

Ins2^{Akita} mice exhibited an increase in albuminuria relative to the WT littermate controls (Figure 3b). After 20 weeks of treatment, albuminuria was reduced with liraglutide or perindopril or their combination (Figure 3b). Ins2^{Akita} mice had a decrease in plasma cystatin C, and this was unaltered by liraglutide or perindopril alone or together (Figure 3c). Glomerulosclerosis was evident in Ins2^{Akita} mice and was reduced in liraglutide- but not perindopril-treated mice (Figure 3d and e). Transmission electron microscopy was performed in a subset of kidneys from control, diabetic, and diabetic mice treated with liraglutide. Image analysis of the glomerular basement membrane (GBM) demonstrated that GBM thickening was evident in the diabetic mice and that this GBM thickening was reversed with administration of liraglutide (Figure 3f and g). Liraglutide also restored the number of foot processes in podocytes per length of GBM in Ins2^{Akita} mice (Figure 3h), but it did not improve markers of tubular injury (urinary KIM-1) as was seen with the ACEi (Figure 3i). Furthermore, consistent with a liraglutide-mediated reduction in kidney fibrosis, levels of active transforming growth factor-β1 protein expression (Figure 3j), and glomerular fibronectin deposition, as measured by immunohistochemistry, were reduced in the renal cortex of liraglutide-treated mice (Figure 3k and l), emphasizing the protective role of GLP-1R agonism against podocyte injury and DKD.

Vehicle-treated Ins2^{Akita} mice showed reduced *Glp1r* expression (Supplementary Figure S4A) and PKA activity (Supplementary Figure S4B) in the kidney cortex compared with WT control mice. In Ins2^{Akita} mice, liraglutide partially restored *Glp1r* expression (Supplementary Figure S4A) and reversed the decrease in PKA activity (Supplementary Figure S4B). Vehicle-treated Ins2^{Akita} mice showed the upregulation of *Ager* expression (Supplementary Figure S4C) and RAGE protein (Supplementary Figure S4D) in the kidney cortex compared with WT control mice. In contrast, liraglutide downregulated kidney *Ager* expression (Supplementary Figure S4C) and RAGE protein (Supplementary Figure S4D) in Ins2^{Akita} mice, providing further evidence linking GLP-1R signaling to attenuation of RAGE activity.

Liraglutide attenuates diabetes-induced myelopoiesis and promotes the resolution of kidney inflammation

Because previous studies have shown that hyperglycemia associated with diabetes promotes enhanced myelopoiesis via RAGE and its ligands[47] and emerging evidence indicates that GLP-1-based therapies exhibit anti-inflammatory properties,[48,49] we explored key signals involved in "sterile" inflammation in Ins2^{Akita} mice treated with liraglutide. Canonical pathway activation of the pivotal mediator of inflammation, nuclear factor κB (p65), in the diabetic kidney was suppressed by liraglutide and perindopril (Figure 4a). Dysregulated innate immune responses mediated by macrophages are commonly observed in progressive renal injury.[50] Monocyte chemoattractant protein (MCP)-1 (also known as CC chemokine ligand 2) triggered upon exposure to inflammatory stimuli drives the chemotaxis of myeloid and lymphoid cells, regulating migration and infiltration of

Figure 2 | (continued) depicting the diabetes STZ paradigm in the various mouse genotypes. (**b**) Protein expression of RAGE was determined by the enzyme-linked immunosorbent assay (ELISA). (**c**) *Glp1r* mRNA expression was determined by quantitative polymerase chain reaction in the kidney cortex. (**d**) Urinary albumin excretion rate (AER) as determined by the ELISA in 24-hour urine collections. (**e**) Plasma cystatin C was measured by the ELISA. (**f**) Glomerular sclerotic index (GSI) histology score. GSI was quantitated using periodic acid–Schiff staining and light microscopy under original magnification of ×400. (**g**) Representative GSI images. Original magnification ×400 and Bar = 20 μm. (**h**) Urinary KIM-1 as determined by the ELISA in 24-hour urine collections. (**i**) Fibronectin in the kidney cortex. Data are presented as mean ± SD (n = 5–10 per group). Dots represent individual mice. *P* values were determined by 2-way analysis of variance with the Tukey multiple comparison test. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$. dKO, double knockout; *Glp1r*$^{-/-}$, *Glp1r* knockout mice; KIM, kidney injury molecule; ND, not detectable; WOA, week of age. To optimize viewing of this image, please see the online version of this article at www.kidney-international.org.

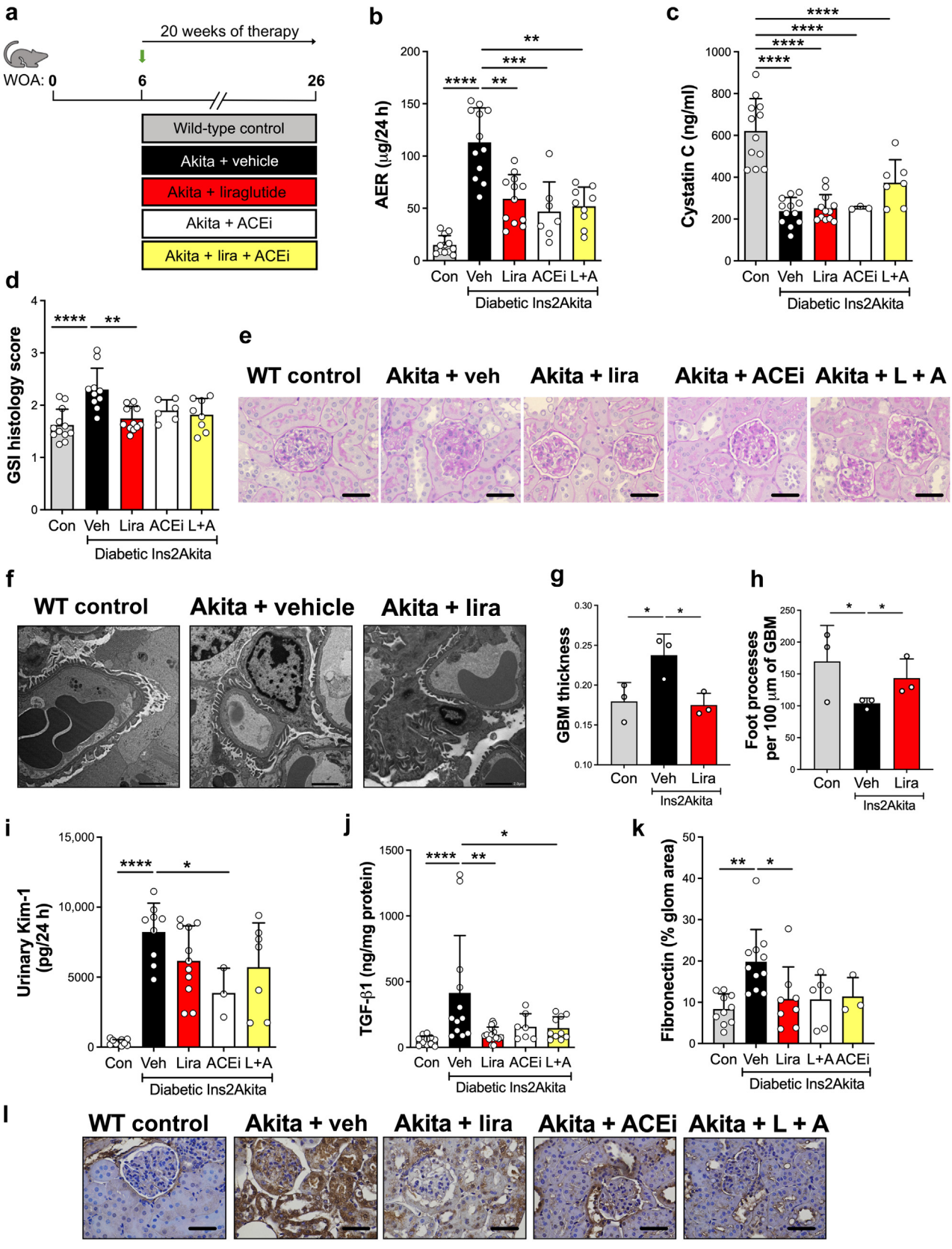

Figure 3 | Treatment with the glucagon-like peptide-1 receptor (GLP-1) receptor agonist liraglutide ameliorates podocyte injury and diabetic kidney disease. Ins2^{Akita} mice were treated with liraglutide, the angiotensin-converting enzyme inhibitor (ACEi) perindopril, or a combination of liraglutide and perindopril for 20 weeks. (**a**) Schematic depicting the treatment regimen. (**b**) Urinary albumin excretion rate (AER) as determined by the enzyme-linked immunosorbent assay (ELISA) in 24-hour urine collections. (**c**) Plasma cystatin C was (continued)

monocytes/macrophages to the sites of inflammation produced by tissue injury.[51] Indeed, MCP-1 protein expression was upregulated in the kidney cortex in vehicle-treated Ins2^{Akita} mice (Figure 4b). Both liraglutide and perindopril suppressed MCP-1 protein expression to a similar extent (Figure 4b). Interleukin-10 is a potent anti-inflammatory cytokine released from leukocytes and nonimmune cells, such as epithelial cells, and resolves inflammation and tissue injury, with monocytes and macrophages being the primary target.[52] Liraglutide-treated but not perindopril-treated Ins2^{Akita} mice displayed enhanced interleukin-10 protein expression in the kidney cortex (Figure 4c). The differentiation and polarization of macrophages into proinflammatory "M1" and anti-inflammatory "M2" states denote the 2 extreme maturation programs of macrophages during tissue injury.[53,54] Our data indicate that signaling through the GLP-1 pathway promotes an M2-like macrophage phenotype, suggestive of resolving inflammation.

Because of potential effects of liraglutide in resolving inflammation, we next investigated the possibility that this may be associated with effects on progenitor cells of the monocyte/macrophage lineage within the bone marrow. Flow cytometric analysis of bone marrow showed a diabetes-induced expansion of bone marrow–derived hematopoietic stem and multipotent progenitor cells (Figure 4d), granulocyte macrophage progenitor cells (Figure 4e), and common myeloid progenitor cells (Figure 4f), all of which were attenuated with administration of liraglutide, but not with perindopril. Together, these data demonstrate the anti-inflammatory nature of liraglutide, which extends to a reduction in the extent of enhanced myelopoiesis in the bone marrow and an inflammation-resolving macrophage phenotype in the local renal microenvironment.

We next examined the consequences of loss of GLP-1R signaling on these parameters. Kidney cortical nuclear factor κB (p65) DNA-binding activity and kidney cortical MCP-1 protein expression were increased *Glp1r*$^{-/-}$ mice compared with their WT counterparts (Supplementary Figure S5A and B). Flow cytometric analysis of bone marrow showed an expansion of bone marrow–derived hematopoietic stem and multipotent progenitor cells (Supplementary Figure S5C), granulocyte macrophage progenitor cells (Supplementary Figure S5D), and a trend toward an increase in common myeloid progenitor cells (Supplementary Figure S5E, $P = 0.08$) in the *Glp1r*$^{-/-}$ versus WT mice revealing a proinflammatory renal phenotype and dysregulated hematopoiesis in mice with loss of GLP-1R signaling.

Transcriptional profiling at the single-cell level reveals a unique expression network in the kidney in response to pharmacologic activation of GLP-1R signaling

Given the cellular heterogeneity of the kidney, single-cell sequencing was used to map the cell-specific transcriptional response to liraglutide administration in mice with DKD. We conducted an unbiased genome-wide expression profiling of individual cells freshly dissociated from the kidney of Ins2^{Akita} mice with and without 20 weeks of liraglutide therapy (Figure 5a). After initial quality filtering of demultiplexed counts, a combined 16,015 single-cell profiles across samples remained for further integration and clustering using the R package Seurat. Specific gene marker expression was examined to assign identity to clustered cell profiles, as shown in Figure 5b, including proximal tubule: (*Slc27a2*); endothelial: (*Emcn*); distal tubule: (*Slc12a3*); podocyte: (*Nphs1*); loop Henle: (*Slc12a1*); macrophage: (*Cd68*); collecting duct (intercalated): (*Atp6v0d2*); collecting duct (principal): (*Hsd11b2*); B-lymphocytes: (*Cd79a*); T-lymphocyte/N-killer: (*Cd3g*), Gzma; mesangial: (*Myl9*); neutrophil: (*S100a8*); juxtaglomerular/granular: (*Ren1*). Fifteen distinct clusters were identified and mapped according to gene expression representing the major cell types found in the kidney,[55,56] including several immune cell types (Figure 5c). Cluster distribution by the experimental group (Figure 5d) showed that each group contributed cells to every cluster, indicating the absence of dissociation bias. There was no change in the cell number in mice with diabetes after treatment with liraglutide compared with diabetic mice (Figure 5e). Overall, the heat map illustrates that liraglutide rescued gene expression changes induced by DKD across all major renal cell types (Figure 5f).

Distinct cell types responded differently to liraglutide (Figure 6a). For example, fatty acid–binding protein-4 (*Fabp4*) was upregulated in endothelial cells in DKD and downregulated by liraglutide (Figure 6a), but this change was not identified in other cell types. The top differentially expressed gene (DEG) in proximal tubular cells in DKD was DEPP1 autophagy regulator (8430408G22Rik), and this was reduced in mice treated with liraglutide (Figure 6a). In podocytes, *Ndufs5*, a subunit of mitochondrial complex I of the

Figure 3 | (continued) measured by the ELISA. (**d**) Glomerular sclerotic index (GSI) histology score. GSI was quantitated using periodic acid–Schiff (PAS) staining and light microscopy under original magnification of ×400. (**e**) Representative PAS-stained kidney sections. Original magnification ×400 and Bar = 25 μm. (**f**) Representative transmission electron micrographs (viewed at original magnification ×3000) of glomerular podocytes demonstrating thickening of the glomerular basement membrane (GBM, orange arrows) in Ins2^{Akita} mice compared with the wild-type (WT) control and prevention by liraglutide. Images also show change in podocyte foot processes (Bar = 2 μm). (**g**) GBM thickness. (**h**) Podocyte foot processes per 100 μm GBM. (**i**) Urinary kidney injury molecule (KIM)-1 as determined by the ELISA in 24-hour urine collections. (**j**) Activated transforming growth factor-β1 (TGF-β1) protein in the kidney cortex. (**k**) Fibronectin in glomeruli by immunohistochemistry. (**l**) Representative images (Bar = 25 μm). Data are presented as mean ± SD (n = 3–12 per group). Dots represent individual mice. *P* values were determined by 1-way analysis of variance with the Tukey multiple comparison test. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$. ACEi, Ins2^{Akita} perindopril; Con, WT; Lira, Ins2^{Akita} liraglutide; L + A, Ins2^{Akita} liraglutide + perindopril; Veh, Ins2^{Akita} vehicle; WOA, week of age. To optimize viewing of this image, please see the online version of this article at www.kidney-international.org.

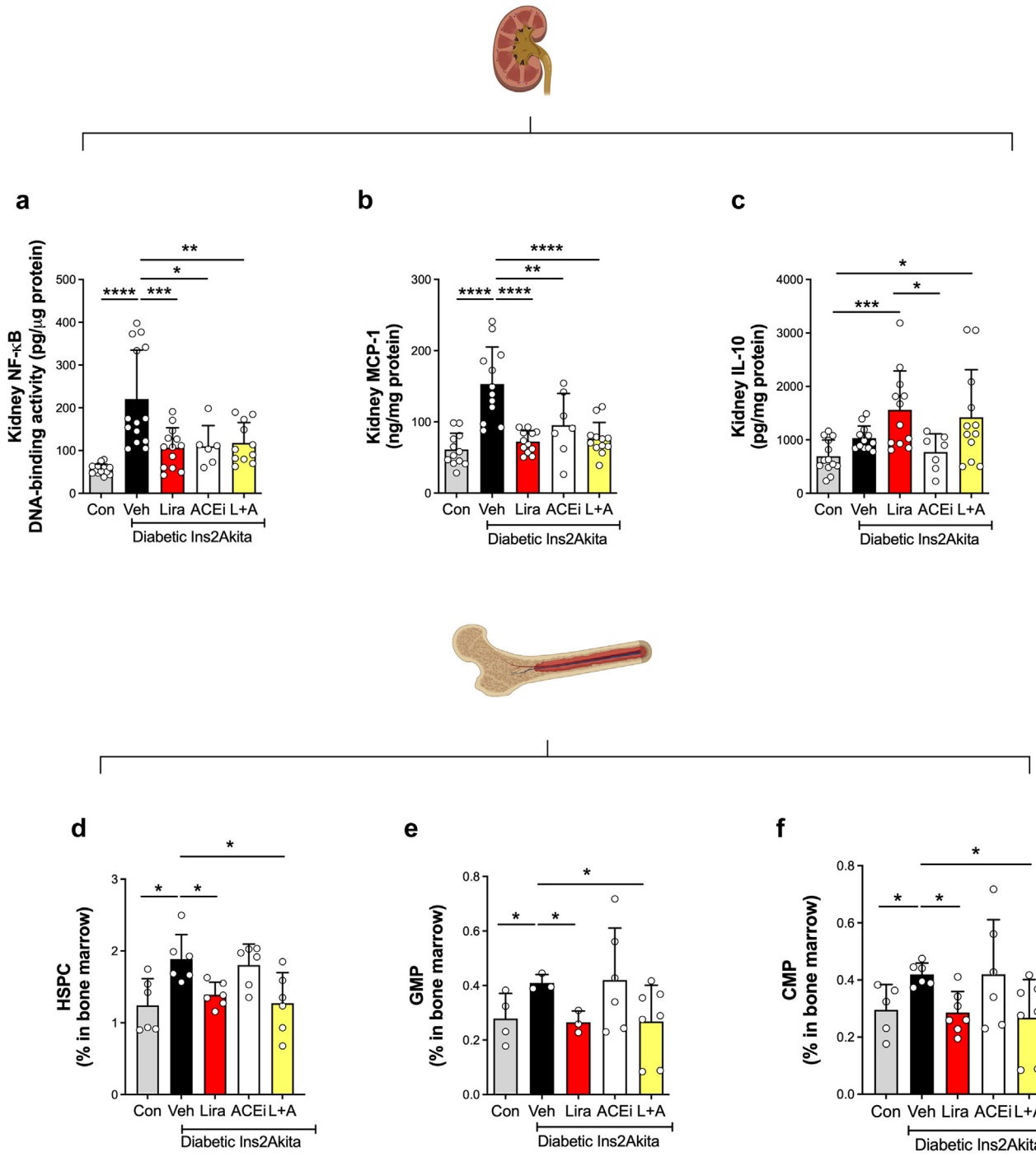

Figure 4 | Liraglutide attenuates receptor for advanced glycation end product (RAGE)-mediated myelopoiesis and influences macrophage polarization, dampening kidney inflammation. Ins2^{Akita} mice were treated with liraglutide, the angiotensin-converting enzyme inhibitor (ACEi) perindopril, or a combination of liraglutide and perindopril for 20 weeks. (**a**) Nuclear factor κB (NF-κB) DNA-binding activity in the kidney cortex. (**b**) Kidney monocyte chemoattractant protein (MCP)-1 protein. (**c**) Kidney interleukin (IL)-10 protein. Bone marrow–derived progenitor cells were measured by flow cytometry: (**d**) hematopoietic stem and multipotent progenitor cells (HSPC), (**e**) granulocyte macrophage progenitor cells (GMP), and (**f**) common myeloid progenitor cells (CMP). Data are presented as mean ± SD (n = 4–12 per group). Dots represent individual mice. *P* values were determined by 1-way analysis of variance with the Tukey multiple comparison test. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$. ACEi, Ins2^{Akita} perindopril; Con, WT; Lira, Ins2^{Akita} liraglutide; L + A, Ins2^{Akita} liraglutide + perindopril; Veh, Ins2^{Akita} vehicle.

respiratory chain, was downregulated in DKD and restored with liraglutide (Figure 6a). To gain biological insight into the gene expression data, gene set enrichment analysis was performed. This revealed that liraglutide therapy induced changes in genes participating in a number of inflammatory, metabolic, and redox sensing pathways, which were altered

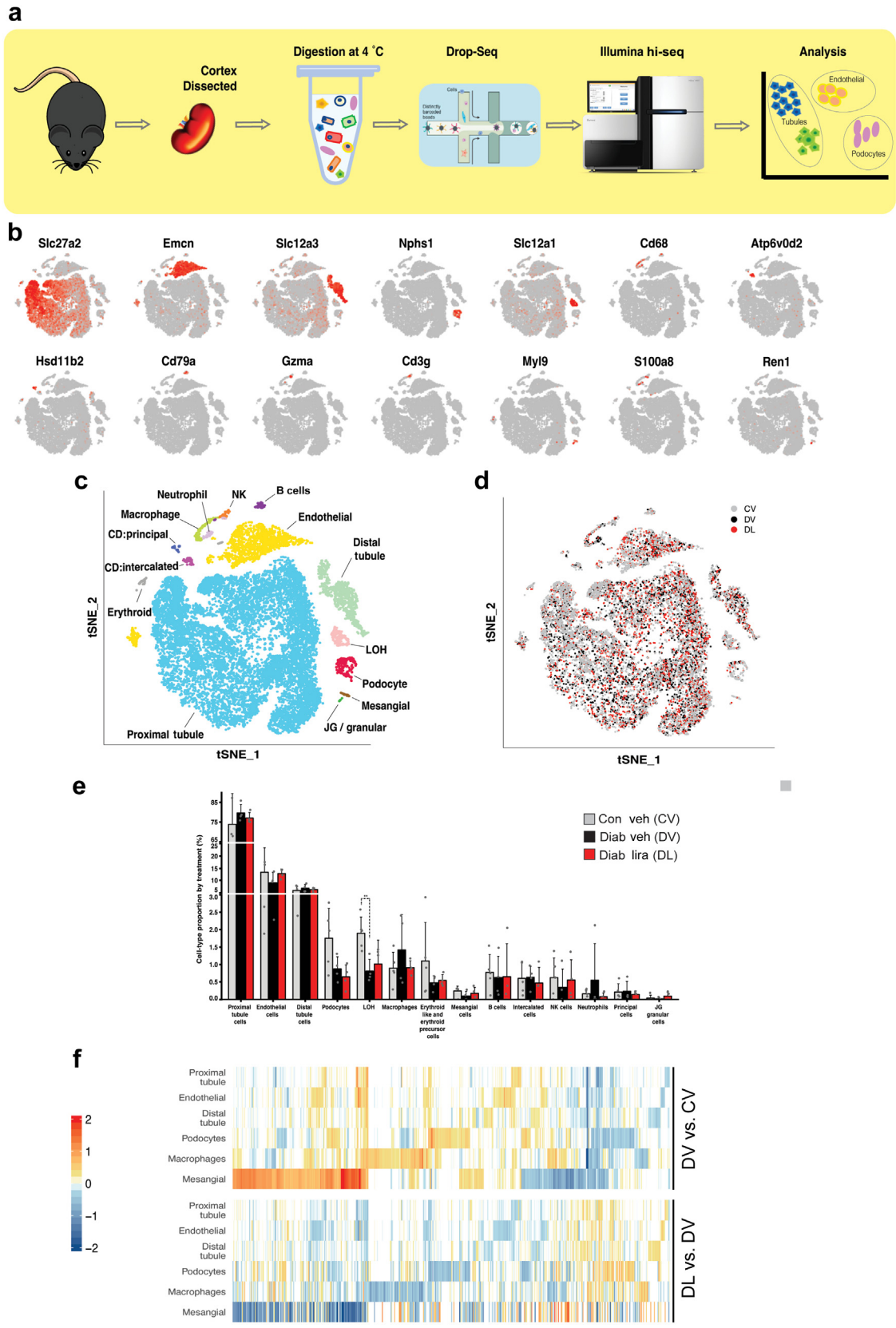

Figure 5 | Liraglutide remodels the kidney transcriptional network in diabetes. Single-cell RNA sequencing was performed to distinguish kidney cell populations in $Ins2^{Akita}$ mice treated with Lira. Cells were captured and visualized by t-distributed stochastic neighbor embedding (tSNE). (**a**) Workflow of renal single-cell RNA sequencing data creation. (**b**) Cluster identities verified by the expression of specific marker genes including proximal tubule: *Slc27a2*, endothelial: Emcn, distal tubule: Slc12a3, podocyte: Nphs1, loop of Henle (LOH): Slc12a1, (continued)

with diabetes (Figure 6b). Pathways induced by diabetes and suppressed by liraglutide included, among others, heat-shock protein signaling, mitochondrial metabolic pathways consisting of pyruvate metabolism and citric acid cycle, mitochondrial fatty acid β-oxidation, respiratory electron transport, and complex I biogenesis (Figure 6b). This mitochondrial signature was dominant in endothelial cells and proximal tubular cells. In addition, FOXO-mediated gene transcription and the solute-carrier gene superfamily pathways were dampened by liraglutide. Inflammasome pathways were also modulated by liraglutide therapy, with *Il1b* in natural killer cells decreasing, and distinct to macrophages, liraglutide rescued the diabetes-induced CLEC7A/inflammasome and NLRP3-inflammasome pathways (Figure 6b). The podocyte also displayed an inflammatory signature that was partly remodeled with liraglutide (Supplementary Figure S6). Supplementary Figure S7 shows the top 200 differentially expressed genes in the podocyte in response to liraglutide. Next, we wanted to see which cell types expressed *Ager* and *Glp1r*. *Ager* was detected in the $Ins2^{Akita}$ mouse kidney in endothelial cells, proximal tubular cells, distal tubular cells, and most predominantly, the podocytes (Supplementary Figure S8), with increased expression compared with control. Liraglutide treatment downregulated *Ager* in all of these cell types except endothelial cells. Interestingly, liraglutide led to an upregulation of *Ager* in B cells (Supplementary Figure S8). *Glpr1r* was below the limit of detection in our single-cell sequencing analysis.

We next explored some of the over-represented pathways highlighted by gene set enrichment analysis, which were downregulated in diabetes and restored in diabetic mice treated with liraglutide (Figure 6b). Gene networks relating to the resolution of inflammation predominated, including pathways such as interleukin-15 signaling and the heat shock protein 90 chaperone cycle for steroid hormone receptors, with *Hsp90ab1* expression restored by liraglutide, and ATF6 (activating transcription factor 6 alpha) activates chaperone genes, prolactin receptor signaling, activation of BAD and translocation to mitochondria, Tie2 signaling, vascular endothelial growth factor receptor-2–mediated vascular permeability, and binding and uptake of ligands by scavenger receptors (Figure 6b).

A spreadsheet summarizing the DEGs across experimental groups by cell cluster can be found in Supplementary Data File 2. Of particular interest was the identification of the following 3 genes: *Slc6a19*, *Txnip*, and *Slc11a1* (Figure 6c). The *Slc6a19* transcript level was increased in diabetes and decreased after liraglutide treatment (Figure 6c). *Slc11a1* was upregulated uniquely in the macrophage cell population in diabetes and was decreased with liraglutide treatment (Figure 6c). *Txnip* was a key signaling node reflected across proximal tubule cells, podocytes, macrophages, and endothelial cells in the $Ins2^{Akita}$ mouse and was downregulated by liraglutide (Figure 6c). This expression pattern was verified in the kidney cortex using targeted quantitative polymerase chain reaction (Figure 6d). Consistent with redox regulation, *Depp/Depp1/c10orf10* was induced in diabetes and reduced with liraglutide treatment in several cell clusters, notably proximal tubule cells (Supplementary Figure S9).

Liraglutide provides kidney protection and dampens RAGE activation in a nondiabetic rodent model of chronic kidney disease

We next aimed to corroborate these findings in a nondiabetic model of kidney injury, the subtotal nephrectomized (SNx) rat. Eight-week-old Fisher rats underwent Sham or 5/6 nephrectomy and were treated for 7 weeks after surgery with liraglutide 0.2 mg/kg twice daily subcutaneously,[42] or phosphate-buffered saline vehicle (n = 6–12 per group) (Figure 7a). A small difference in body weight was observed in the Sham + Lira, SNx, and SNx + Lira group relative to the Sham group (Figure 7b). There was no change in glucose control as evidenced by glycated hemoglobin, thus indicating that any benefits to the kidney seen in this model were independent of changes in glycemia (Figure 7c). Left kidney weight to body weight ratio was elevated in both the SNx and SNx + Lira groups relative to the Sham groups (Figure 7d). Systolic blood pressure was elevated in the nephrectomized rats and not altered with liraglutide therapy (Figure 7e). Albuminuria (Figure 7f) and urinary albumin-creatinine ratio (Figure 7g) were increased in nephrectomized rats, and urine albumin-creatinine ratio was reduced by liraglutide. Likewise, plasma creatinine was increased in the SNx model; however, there was no change with liraglutide (Figure 7h). An increase in urinary KIM-1 was evident in nephrectomized rats, and this was decreased with liraglutide therapy (Figure 7i). Glomerulosclerosis was increased in the SNx group and improved with liraglutide (Figure 7j). We next assessed whether the renal structural and functional changes seen in the SNx groups were associated with changes in local RAGE expression in the kidney. The SNx group exhibited increased kidney RAGE protein expression relative to Sham, and this was prevented by liraglutide therapy (Figure 7k and l), demonstrating RAGE suppression by liraglutide in the absence of diabetes.

Figure 5 | (continued) macrophage: *Cd68*, collecting duct (CD) (intercalated): *Atp6v0d2*, collecting duct (principal): *Hsd11b2*, B-lymphocytes: *Cd79a*, T-lymphocyte/N-killer: *Cd3g*, Gzma, mesangial: *Myl9*, neutrophil: *S100a8*, and juxtaglomerular (JG)/granular: *Ren1*. Red indicates the detection of gene expression, and gray indicates no expression/below the limit of detection. (**c**) Cluster identity mapping according to the expression of marker genes. (**d**) Cluster distribution by the experimental group (Control + Vehicle [CV], Diabetic + Vehicle [DV], and Diabetic + liraglutide [DL]). (**e**) Cell counts (percentages) in renal cell populations from single-cell transcriptomic analysis in WT and $Ins2^{Akita}$ mice with and without liraglutide treatment. Data are displayed as mean ± SD (n = 4 mice/group). *$P < 0.05$. (**f**) Heat map of gene expression changes in proximal tubule cells, endothelial cells, distal tubule cells, podocytes, macrophages, and mesangial cells in vehicle-treated mice (top) versus liraglutide-treated mice (bottom). *P* values were determined by 1-way analysis of variance with the Tukey multiple comparison test. *$P <$ 0.05. CV, WT control vehicle; DL, $Ins2^{Akita}$ liraglutide; DV, $Ins2^{Akita}$ vehicle; NK, natural killer; WT, wild type.

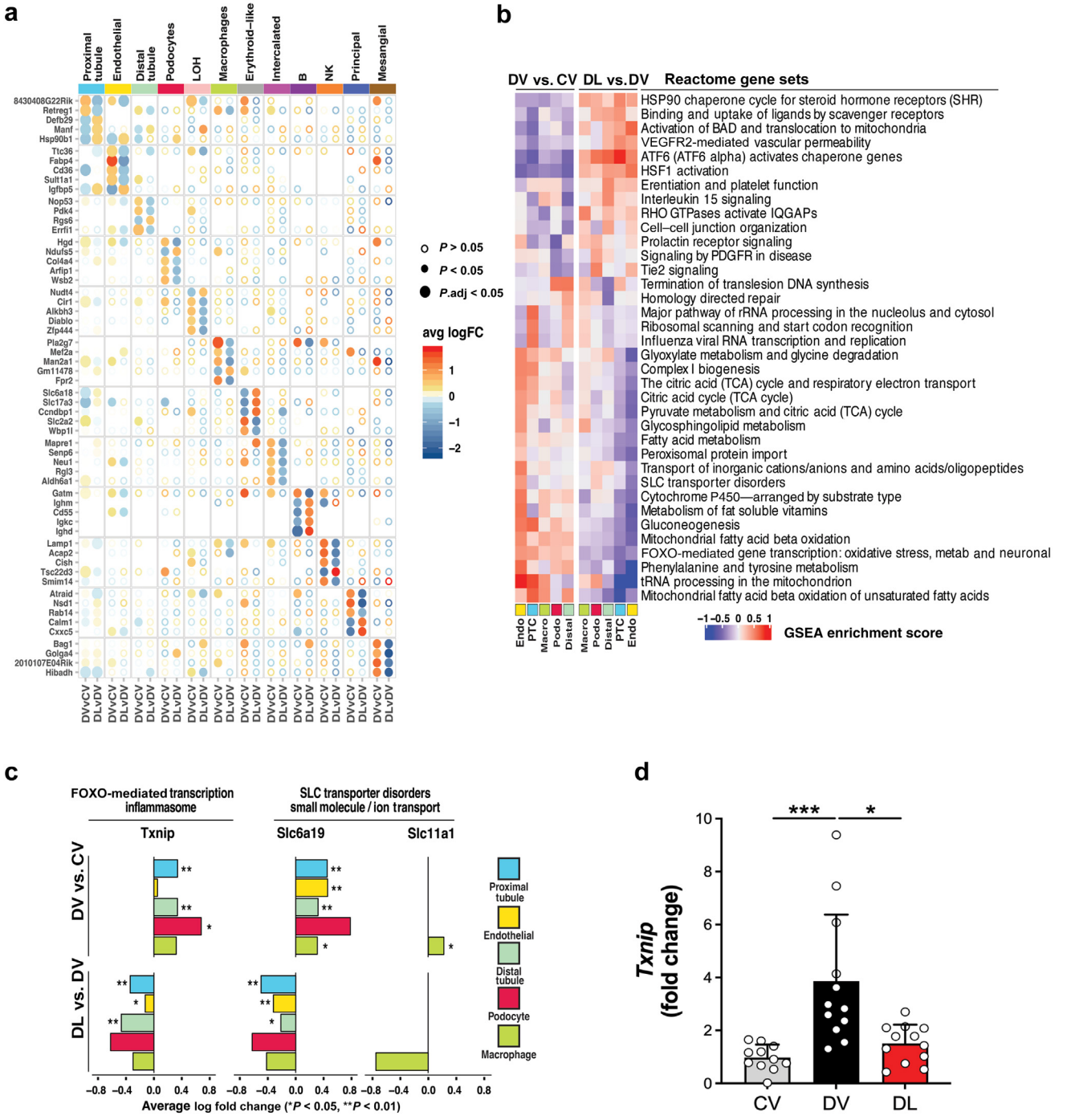

Figure 6 | Gene set enrichment analysis (GSEA) reveals novel pathways influenced by liraglutide in diabetic kidney disease (DKD). (**a**) Dot plot depicting top 5 cell-specific, upregulated, and downregulated genes during DKD ($Ins2^{Akita}$ vehicle, DV vs. control vehicle, CV) and in response to liraglutide in diabetes ($Ins2^{Akita}$ liraglutide, DL vs. DV). Color gradient indicates DEG enrichment score whereby red = higher expression and blue = lower expression of set genes. (**b**) Reactome GSEA of differential expression for the effect of diabetes "DV versus CV" and the effect of liraglutide in diabetes "DL versus DV" for clusters including proximal tubule, distal tubule, endothelial, podocyte, and macrophage. Color gradient indicates GSEA enrichment score, whereby red = higher expression and blue = lower expression of set genes. (**c**) Bar plots showing differential expression of specific genes including *Txnip*, *Slc6a19*, and *Slc11a1*. Bars indicate average log fold change (logFC) for contrasts of diabetes "DV versus CV" and the effect of liraglutide in diabetes "DL versus DV" across 5 cell types. (**d**) *Txnip* by quantitative polymerase chain reaction in the total kidney cortex. Data are presented as mean ± SD (n = 11–12 per group). Dots represent individual mice. *P* values were determined by 1-way analysis of variance with the Tukey multiple comparison test. $^{*}P < 0.05$, $^{***}P < 0.001$. ATF6, activating transcription factor 6; DEG, differentially expressed gene; GTPases, guanosine triphosphatases; HSF1, heat shock factor 1; HSP90, heat shock protein 90; LOH, loop of Henle; NK, natural killer; PDGFR, platelet-derived growth factor receptor; PTC, proximal tubular cell; SLC, solute carrier; VEGFR2, vascular endothelial growth factor receptor-2.

DISCUSSION

This study has demonstrated that GLP-1R signaling is an important pathway for controlling nonresolving inflammation in the kidney during diabetes and that loss of the GLP-1R leads to RAGE activation, inflammation, and kidney disease. Ablation of the GLP-1R caused progressive chronic kidney disease, which was accompanied by marked kidney RAGE upregulation and enhanced susceptibility to DKD. Kidney injury in *Glp1r*$^{-/-}$ mice could be rescued by RAGE deletion. GLP-1R agonism with liraglutide suppressed kidney RAGE expression and improved kidney injury in 2 distinct models of progressive chronic kidney disease, independent of glucose lowering. In mice with experimental diabetes, GLP-1R agonism reduced the expansion of bone marrow myeloid progenitor cells, promoted M2-like macrophage polarization, and suppressed inflammasome activation. GLP-1R agonism also led to signals of metabolic adaptation and tissue repair across key kidney cell populations. Collectively, these results identify novel pathways of kidney repair for GLP-1R agonism.

GLP-1RA are effective blood glucose–lowering therapies that are transforming the management of type 2 diabetes with an associated reduction in DKD and cardiovascular disease risk;[57,58] however, their mechanisms of action in reducing end-organ injury are incompletely understood. Given the impressive clinical responses to GLP-1RA therapy, understanding the central mechanisms by which incretins alter metabolism, including within the kidney, is of critical importance. The current study aimed to characterize the role of GLP-1 in kidney function, and indeed GLP-1 was found to be crucial for kidney homeostasis. We showed that mice with genetic disruption of *Glp1r* spontaneously develop renal injury exhibited by albuminuria, glomerular hyperfiltration, and fibrosis. When diabetes was chemically induced in *Glpr1r*-deficient mice, kidney injury was accelerated, beyond that observed in diabetic WT mice, as reflected by albuminuria and worsening DKD. Taken together, these findings confirm a role for the endogenous GLP-1R in conferring susceptibility to kidney injury.

We then showed that the development of STZ diabetes was associated with an increase in kidney RAGE expression and a decrease in GLP-1R expression. The deletion of RAGE (RAGE KO mice) prevented the decrease in GLP-1R, whereas *Glp1r*$^{-/-}$ mice showed RAGE upregulation, demonstrating cross-talk between these 2 pathways. To further explore regulation of these pathways *in vivo*, we generated a dKO mouse model where both RAGE and GLP-1R were deleted. The dKO mice exhibited a similar renoprotective phenotype to that of the RAGE KO mouse alone. This suggests that preventing RAGE signaling in the absence of GLP-1 is protective, emphasizing a dominant role for RAGE in DKD progression. Indeed, there is a large body of clinical and experimental evidence that the AGE-RAGE axis contributes to the onset and progression of DKD. We confirmed that genetic deletion of RAGE affords renoprotection, leading to reduced inflammation and fibrosis, as previously shown by our group and others.[23–25,59]

We next characterized changes in the kidney in response to therapy with a GLP-1RA used at a dose that did not reduce blood glucose. We identified that GLP-1R agonism with liraglutide confers renoprotection in an experimental model of insulin-deficient diabetes (Ins2^{Akita}) as well as in a nondiabetic model of renal fibrosis (SNx). This renoprotection was independent of blood glucose control and was as good as conventional treatments such as ACE inhibition, as achieved by perindopril. Our experimental findings are in agreement with the LEADER study, where liraglutide reduced the development and progression of DKD in patients with type 2 diabetes.[60] Similar findings with respect to renoprotection have been reported with other GLP-1RA, such as in the REWIND study with dulaglutide,[61] in the SUSTAIN program with semaglutide[62] and more recently the AMPLITUDE-O trial that used efpeglenatide.[57,63] With positive renal findings with GLP-1RA in numerous clinical trials albeit not performed primarily for renal endpoints, this has led to the commencement of dedicated trials enrolling people at risk for DKD such as the FLOW study (NCT03819153) to further define the role of these drugs as renoprotective therapies.

In the Ins2^{Akita} mouse model, liraglutide led to an increase in GLP-1R protein in the kidney, PKA activation, and suppressed RAGE expression. Liraglutide was efficacious against diabetes-associated albuminuria, glomerulosclerosis, renal fibrosis, and podocyte injury, including ultrastructural changes, exhibited by reducing GBM thickening. These data are consistent with previous studies by Kodera *et al.*,[10] who demonstrated renoprotective actions of the GLP-1 agonist, exendin-4, in a rat model of insulin-deficient diabetes in the absence of glucose lowering. Similarly, Fujita *et al.*[11] have shown that a 4-week treatment of liraglutide in young KK/Ta-Akita mice led to a reduction in the urinary albumin-to-creatinine ratio, although these previous experimental studies failed to elucidate an underlying mechanism at either a molecular or cellular level.

The effects of liraglutide on DKD in the Ins2^{Akita} mouse extended to a reduction in inflammation within the kidney, with suppression of nuclear factor κB DNA-binding activity and MCP-1 protein expression, whereas interleukin-10 protein was increased, indicating that signaling through the GLP-1 pathway promotes an M2-like macrophage phenotype in the kidney, suggestive of resolving inflammation. These results are supported by data from single-cell sequencing that revealed that liraglutide treatment in diabetic mice led to a dampening of inflammatory signals in macrophages. This resolving inflammation was accompanied by a reduction in myelopoiesis in the bone marrow. Together, these data confirm the anti-inflammatory nature of liraglutide systemically via direct effects on myelopoiesis and on M2-like macrophage polarization in the local renal microenvironment.

Because the kidney houses diverse cell populations,[64] we used unbiased single-cell RNA sequencing to explore the cellular landscape in response to a long-term GLP-1-incretin stimulus. Our data complement a recent and elegant study mapping the early single-cell transcriptomic response of mouse DKD to the other key therapies of DKD, including sodium-glucose

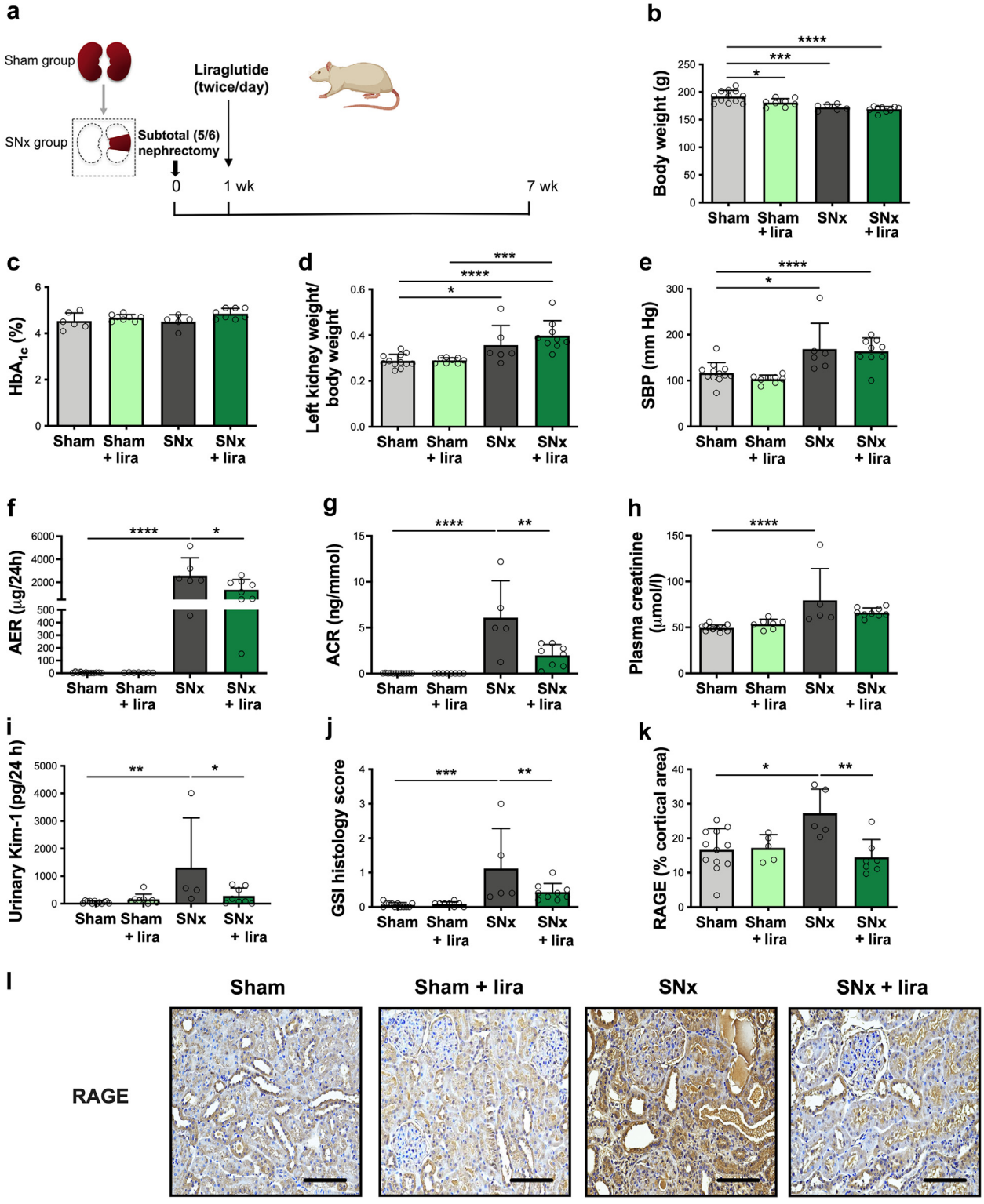

Figure 7 | The effects of liraglutide on receptor for advanced glycation end products (RAGE) and microvascular injury were corroborated in the subtotal nephrectomized (SNx) rat model. (**a**) Fisher rats were subjected to subtotal (5/6) nephrectomy and followed for 7 weeks with or without liraglutide (0.2 mg/kg twice daily subcutaneously), (**b**) body weight, (**c**) glycated hemoglobin (HbA_{1c}), (**d**) left kidney weight to body weight ratio, (**e**) systolic blood pressure (SBP), (**f**) albumin excretion rate (AER), (**g**) albumin-to-creatinine ratio (ACR), (**h**) plasma creatinine, (**i**) urinary kidney injury molecule (KIM)-1 measured, (**j**) glomerular sclerotic index (GSI) histology score, (**k**) RAGE protein in the kidney cortex by immunohistochemistry, and (**l**) representative images of RAGE staining within the kidney cortex. Original magnification ×400 and Bar = 25 µm. Data are presented as mean ± SD (n = 6–12 per group). Dots represent individual mice. *P* values were determined by 1-way analysis of variance with the Tukey multiple comparison test. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$. To optimize viewing of this image, please see the online version of this article at www.kidney-international.org.

cotransporter-2 inhibitors, rosiglitazone, and ACE inhibition.[65] In the current study, single-cell RNA sequencing identified clusters comprising endothelial, proximal tubule, podocyte, and macrophage cells influenced by pharmacologic GLP-1 signaling, associated with pathways involved in nutrient utilization, redox sensing, and resolution of inflammation. Our studies show that, independent of whole-body metabolism, GLP-1RA have effects on kidney nutrient utilization, inducing a shift toward kidney fatty acid metabolism that was particularly apparent in endothelial cells and proximal tubular cells. This was supported by a signal of increased mitochondrial complex I biogenesis and apparent flux through the mitochondrial respiratory chain in these cells, signals that were largely absent in podocytes. Fatty acids are the preferred energy source for kidney tubules, which are then metabolized by fatty acid oxidation and oxidative phosphorylation. Disturbances in kidney fatty acid metabolism have been observed in DKD.[66–68] In diabetes, the inability to make appropriate metabolic adaptations in the diabetic milieu leads to dysfunctional mitochondria and impaired renal energy generation. In DKD, there is a shift from oxidative phosphorylation[69,70] to glycolysis[71] via the Crabtree[72] or the Warburg[69] effect. An upregulation in "mitochondrial pathways" observed in the gene set enrichment analysis in our study is likely to reflect a shift in metabolism in the kidney in diabetes. However, live respiration studies would be required to determine mitochondrial function to directly assess if such changes in renal metabolism occurred in response to GLP1R agonism. This upregulation in "mitochondrial pathways" is likely a compensatory response to the increased metabolic demands of diabetes. Liraglutide reversed this mitochondrial signature in proximal tubular cells and endothelial cells. This could infer metabolic reprogramming by liraglutide and is likely due to a shift from glucose oxidation to lipid fueling, in an attempt to establish normal kidney metabolism.

GLP-1R agonism in diabetes also remodeled a network of nutrient transporters including *Slc6a19*, which encodes the sodium-dependent neutral amino acid transporter. The sodium-dependent neutral amino acid transporter is primarily expressed in early proximal tubules (S1–S2) and reabsorbs neutral amino acids.[73,74] The *Slc6a19* transcript level was increased in diabetes across most key cell populations and decreased after liraglutide treatment. A recent study found that mice with a deletion in *Slc6a19* were protected against aristolochic acid–induced nephropathy.[75] Of note, there is current interest in considering the sodium-dependent neutral amino acid transporter as a therapeutic target in diabetes.[76]

Slc11a1 is a member of the solute carrier family 11 (proton-coupled divalent metal ion transporters) and encodes a multipass membrane protein known as natural resistance-associated macrophage-1.[77,78] Natural resistance-associated macrophage-1 functions as a divalent transition metal (iron and manganese) transporter. It regulates macrophage and neutrophil activity primarily in the setting of innate immunity.[79] *Slc11a1* was upregulated uniquely in the macrophage cell population in diabetes and was decreased with liraglutide treatment, pointing toward reshaping of macrophage metabolism. *Txnip* was a key signaling node reflected across proximal tubule cells, podocytes, macrophages, and endothelial cells in the $Ins2^{Akita}$ mouse and was significantly downregulated by liraglutide. We also demonstrated that liraglutide reduced *Txnip* in the renal cortex. These data show that TXNIP is a target of the GLP-1-incretin pathway. Consistent with redox regulation, *Depp/Depp1/c10orf10*, a key hypoxia-inducible gene and transcriptional target of FOXO3, was induced in diabetes and was reduced by liraglutide treatment, predominantly in proximal tubule cells. *Depp1* localizes to peroxisomes and mitochondria and impairs cellular reactive oxygen species detoxification,[80] and it is involved in the activation of FOXO3-dependent autophagy.[81] More recently, *Depp1* was found to regulate hepatic glucose and fat metabolism partly by fibroblast growth factor 21,[82] though to our knowledge, no prior data exist regarding its specific role in the kidney.

Although the current studies have focused on direct renal mechanisms, one cannot exclude that other pathways may also be involved, including effects on weight, lipids and fatty acid metabolism, renal metabolism, kidney perfusion, and hypoxia, as has been suggested.[83] In an inflammation-driven kidney murine model, liraglutide specifically regulated genes related to the renin-angiotensin-aldosterone system, sodium glucose transporters, vasoconstriction, proliferation, angiogenesis, fibrosis, and inflammation.[84] It should be noted that this study has several limitations including the reliance on whole-body KO animals precluding precise evaluation of kidney-specific versus systemic effects.

In conclusion, these studies identify a novel glucose-independent renoprotective action of GLP-1RA in DKD, which includes the suppression of nonresolving inflammation across key kidney cell populations. This work supports the impetus to not only consider GLP-1-based therapies in DKD but also as a novel approach in nondiabetic chronic kidney diseases. In addition, these data provide a valuable resource for exploring the cell-specific kidney transcriptional response to long-term GLP-1 analog administration in diabetes.

DISCLOSURE

MEC has received honoraria from companies making GLP-1 receptor agonists and DPP-4 inhibitors including Novo Nordisk. MTC has received consultancy fees from Visterra Inc. AA has received research support from Boehringer Ingelheim. He was a recipient of a Novo Nordisk Diabetes Innovation Award and has received consultancy fees and honoraria from Novo Nordisk. LBK, DBT, and AK are the employees of Novo Nordisk. CH is past Novo Nordisk employee. LBK and DBT hold minor employee-based Novo Nordisk shares. DJD has received consulting or lecture honoraria from Altimmune, Amgen, Boehringer Ingelheim, Eli Lilly, Kallyope, Merck, Novo Nordisk, and Pfizer Inc. and Mt. Sinai Hospital has received investigator-initiated grant support from Amgen, Novo Nordisk, and Pfizer Inc. for studies in the Drucker Lab. All the other authors declared no competing interests.

DATA STATEMENT

The single-cell RNA sequencing data supporting the findings of this study are openly available in the Gene Expression Omnibus under accession GSE220045.

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AUTHOR CONTRIBUTIONS

KCS, YD, MTC, and MEC contributed equally to this work and are guarantors of this work. KCS is listed as first author because she was responsible for project management, overseeing the completion of all the experiments, running the mouse studies, analyzing the data, and completing all manuscript revisions. MTC is listed as last author as she supervised the experiments, analyzed the data and co-wrote the manuscript. KCS, YD, PK, MTC, and MEC each contributed to the conception and design of the study. KCS and YD conducted the experiments and analyzed the data. SSM and AE-O conducted the single-cell transcriptomic experiments and analysis. AJM and AA-s assisted with the bone marrow flow cytometric analysis. JMF, GR, SC, KJ-D, PK, MKM, CJR, SAP, and BEH assisted with the animal studies and drafting of the manuscript. DJD assisted with the provision of animals to establish colonies to complete the *in vivo* arm of the work. AA designed and ran the rat subtotal nephrectomy study and provided samples for further analysis. LBK, AK, DBT, and CH were involved in the study design. All of the authors have approved the final version of the manuscript.

SUPPLEMENTARY MATERIAL

Supplementary File (Word)

Supplementary Methods.

Supplementary Figure S1. No effect of genetic deletion of *Ager*, *Glp1r*, or both *Ager* and *Glp1r in vivo* on physiological or metabolic features at study endpoint. Wild-type (WT), $ager^{-/-}$, $Glp1r^{-/-}$, and *ager-glp1r* double knockout (dKO) mice were rendered diabetic with streptozotocin (STZ) and followed for 24 weeks. (**A**) Body weight, (**B**) food intake during 24-hour metabolic caging, (**C**) blood glucose, (**D**) glycated hemoglobin (HbA_{1c}), (**E**) water intake during 24-hour metabolic caging, and (**F**) urine output during 24-hour metabolic caging. Data are displayed as mean ± SD (n = 5–10 mice/group). Dots represent individual mice. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$. *P* values determined by 2-way analysis of variance with the Tukey multiple comparison test.

Supplementary Figure S2. Markers of glucagon-like peptide-1 receptor (GLP-1R) activation and oxidative stress in $Ager^{-/-}$, $Glp1r^{-/-}$, or *Ager-Glp1r* double knockout (dKO) mice. Wild-type (WT), $Ager^{-/-}$, $Glp1r^{-/-}$, and *Ager-Glp1r* dKO mice were rendered diabetic with streptozotocin (STZ) and followed for 24 weeks. (**A**) Protein kinase A (PKA) was determined in the kidney cortex by the enzyme-linked immunosorbent assay (ELISA). (**B**) 8-Isoprostanes as determined by the ELISA in 24-hour urine collections. Data are displayed as mean ± SD (n = 5–10 mice/group). Dots represent individual mice. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{****}P < 0.0001$. *P* values determined by 2-way analysis of variance with the Tukey multiple comparison test.

Supplementary Figure S3. No effect of pharmacologic treatment with liraglutide, perindopril, or combination therapy (liraglutide and perindopril) on physiological or metabolic features at the study endpoint in $Ins2^{Akita}$ mice. (**A**) Blood glucose (glucometer), (**B**) glycated hemoglobin (HbA_{1c}), (**C**) food intake during 24-hour metabolic caging, (**D**) water intake during 24-hour metabolic caging, (**E**) urine output during 24-hour metabolic caging, and (**F**) body weight. Data are displayed as mean ± SD (n = 12 mice/group). Dots represent individual mice. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$. *P* values determined by 2-way analysis of variance with the Tukey multiple comparison test.

Supplementary Figure S4. Markers of glucagon-like peptide-1 receptor (GLP-1R) activation and receptor for advanced glycation end products (RAGE) in the kidney cortex in liraglutide-treated $Ins2^{Akita}$ mice. (**A**) *Glp1r* mRNA expression in the kidney cortex. (**B**) Protein kinase A (PKA) in the kidney cortex. (**C**) *Ager* mRNA expression in the kidney cortex. (**D**) RAGE protein in the kidney cortex. Data are displayed as mean ± SD (n = 11–12 mice/group). Dots represent individual mice. $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$. *P* values determined by 1-way analysis of variance with the Tukey multiple comparison test.

Supplementary Figure S5. Markers of inflammation in $Glp1r^{-/-}$ mice. (**A**) Nuclear factor κB (NF-κB) DNA-binding activity in the kidney cortex by the enzyme-linked immunosorbent assay (ELISA). (**B**) Kidney MCP-1 protein by the ELISA. (**C–E**) Bone marrow–derived progenitor cells were measured by flow cytometry: (**C**) hematopoietic stem and multipotent progenitor cells (HSPC), (**D**) granulocyte macrophage progenitor cells (GMP), and (**E**) common myeloid progenitor cells (CMP). Data are displayed as mean ± SD (n = 4–7 per group). Dots represent individual mice. *P* values were determined by an unpaired 2-tailed *t* test. $^{*}P < 0.05$, $^{***}P < 0.001$.

Supplementary Figure S6. Podocyte inflammatory signature. Differential expression (average log fold change [logFC]) of detected inflammation set genes (Reactome) in podocytes from mice with diabetes versus control (Ins2-Akita DV vs. Control CV) and diabetes versus diabetes plus liraglutide treatment (Ins2-Akita + Lira DL vs. Ins2-Akita DV). n = 4 mice per group.

Supplementary Figure S7. Top 200 differentially expressed genes in the podocyte in response to liraglutide. Top 200 differentially expressed genes in the podocyte cell population from single-cell transcriptomic analysis in Ins2-Akita mice with or without liraglutide treatment. Right-hand column follows on from left. n = 4 mice per group. Differentially expressed genes between groups were determined using Seurat FindMarkers with Wilcoxon rank-sum testing. *P* value adjustment is based on the Bonferroni correction using all genes in the dataset. DL, diabetic liraglutide; DV, Ins2Akita vehicle.

Supplementary Figure S8. Dot plot of *Ager* gene expression in Control versus Ins2-Akita mice with and without liraglutide treatment. *Ager* expression in kidney cell populations from single-cell transcriptomic analysis in Control (CV), Ins2-Akita mice (DV), and Ins2-Akita mice treated with liraglutide (DL). Data are displayed as percentage cells expressing *Ager*. The average expression level is scaled, where 0 is not detected and 1 represents highest expression detected for *Ager*. n = 4 mice per group.

Supplementary Figure S9. Liraglutide reverses the *Depp1* gene signature in diabetes. *Depp1* expression in kidney cell populations

from single-cell transcriptomic analysis in $Ins2^{Akita}$ mice with (top) or without (bottom) liraglutide treatment. Data are displayed as log2 normalized expression per cell per group (n = 4 mice per group). Dots represent individual cells. Differentially expressed genes between groups were determined using Seurat FindMarkers with Wilcoxon rank-sum testing. *P* value adjustment is based on the Bonferroni correction using all genes in the dataset. DL, diabetic liraglutide; DV, $Ins2^{Akita}$ vehicle.

Supplementary Table S1. Primer and probe sequences for quantitative reverse transcription polymerase chain reaction**.**

Supplementary References.

Supplementary File (Excel)

Supplementary Data File S2.

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