Journal Pre-proof

Glucagon Like Peptide-1 Receptor Agonism Improves Nephrotoxic Serum Nephritis by Inhibiting T Cell Proliferation

Foteini Moschovaki Filippidou, Alexander H. Kirsch, Matthias Thelen, Máté Kétszeri, Katharina Artinger, Ida Aringer, Corinna Schabhüttl, Agnes A. Mooslechner, Bianca Frauscher, Marion Pollheimer, Tobias Niedrist, Andreas Meinitzer, Daniel J. Drucker, Thomas R. Pieber, Philipp Eller, Alexander R. Rosenkranz, Akos Heinemann, Kathrin Eller

Please cite this article as: Moschovaki Filippidou F, Kirsch AH, Thelen M, Kétszeri M, Artinger K, Aringer I, Schabhüttl C, Mooslechner AA, Frauscher B, Pollheimer M, Niedrist T, Meinitzer A, Drucker DJ, Pieber TR, Eller P, Rosenkranz AR, Heinemann A, Eller K, Glucagon Like Peptide-1 Receptor Agonism Improves Nephrotoxic Serum Nephritis by Inhibiting T Cell Proliferation, *The American Journal of Pathology* (2019), doi: https://doi.org/10.1016/j.ajpath.2019.10.008.

This is a PDF file of an article that has undergone enhancements after acceptance, such as the addition of a cover page and metadata, and formatting for readability, but it is not yet the definitive version of record. This version will undergo additional copyediting, typesetting and review before it is published in its final form, but we are providing this version to give early visibility of the article. Please note that, during the production process, errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

Glucagon like peptide-1 receptor agonism improves nephrotoxic serum nephritis by inhibiting T cell proliferation

Foteini Moschovaki Filippidou[1]; Alexander H. Kirsch[1], Matthias Thelen[1], Máté Kétszeri[1], Katharina Artinger[1], Ida Aringer[1], Corinna Schabhüttl[1], Agnes A. Mooslechner[1], Bianca Frauscher[1], Marion Pollheimer[2], Tobias Niedrist[3], Andreas Meinitzer[3], Daniel J. Drucker[4], Thomas R. Pieber[5], Philipp Eller[6], Alexander R. Rosenkranz[1], Akos Heinemann[7], Kathrin Eller[1]

[1]Clinical Division of Nephrology, Department of Internal Medicine, Medical University of Graz, Graz, Austria

[2]Institute of Pathology, Medical University of Graz, Graz, Austria.

[3]Clinical Institute of Medical and Chemical Laboratory Diagnostics, Medical University of Graz, Graz, Austria

[4]Lunenfeld Tanenbaum Research Institute, Mt. Sinai Hospital, University of Toronto, Toronto, Canada

[5]Clinical Division of Endocrinology and Diabetology, Department of Internal Medicine, Medical University of Graz, Graz, Austria

[6]Intensive Care Unit, Department of Internal Medicine, Medical University of Graz, Graz, Austria

[7]Otto Loewi Research Center, Division of Pharmacology, Medical University of Graz; BioTechMed Graz, Austria

Number of text pages: 28

Number of tables 0

Number of figures: 7

Short Running Head: Glp1r agonism in glomerulonephritis.

Corresponding author: Kathrin Eller, Clinical Division of Nephrology, Department of Internal Medicine, Medical University of Graz, Auenbruggerplatz 27, A-8036 Graz, Austria. Phone: +43-316-385-80172. Fax: +43-316-385-14421. Email: kathrin.eller@medunigraz.at

Abstract

Glucagon like peptide (GLP)-1 analogs such as liraglutide improved albuminuria in patients with type 2 diabetes in large randomized controlled trials. One of the suspected mechanisms is the anti-inflammatory potential of GLP-1 receptor (Glp1r) agonism. Thus, we tested the anti-inflammatory action of Glp1r-agonism in a non-diabetic, T-cell–mediated murine model of nephrotoxic serum nephritis (NTS). The role of Glp1r in NTS was evaluated by using *Glp1r* [-/-] mice or C57BL/6 mice treated with liraglutide. *In vitro*, murine T cells were stimulated in the presence of liraglutide or vehicle. *Glp1r* [-/-] mice displayed increased renal infiltration of neutrophils and T cells after induction of NTS. Splenocyte proliferation and TH1 cytokine transcription were increased in spleen and lymph nodes of *Glp1r* [-/-]. Liraglutide treatment significantly improved the renal outcome of NTS in C57BL/6 mice by decreasing renal infiltration and proliferation of T cells, which resulted in decreased macrophage infiltration. *In vitro*, T cells stimulated in the presence of liraglutide showed decreased proliferation of TH1 and TH17 cells. Liraglutide blocked glycolysis in T cells and decreased their *Glut1* mRNA expression. Together, Glp1r agonism protects mice from a T-cell–dependent glomerulonephritis model by inhibition of T cell proliferation possibly by interacting with their metabolic program. This mechanism may explain in part the reno-protective effects of Glp1r agonism in diabetic nephropathy.

1.

Introduction

The gastrointestinal tract is the largest endocrine network in the human body. It translates environmental signals to multiple organ systems to maintain homeostasis. One of the most extensively studied gastrointestinal hormones is glucagon-like peptide-1 (GLP-1), an incretin hormone produced by the enteroendocrine L cells in the distal ileum and colon [1]. It is rapidly cleared from the circulation by the kidneys and degradation by dipeptidyl peptidase IV (DPP IV). Thus, bioactive GLP-1 in humans has a half-life of less than 2 minutes [1]. GLP-1 acts via the GLP-1 receptor (GLP1R), a G-protein–coupled receptor expressed widely in pancreas, gastrointestinal tract, kidneys, lung, and heart [2]. GLP-1 is mainly known for its ability to regulate blood glucose: it stimulates insulin secretion and contributes to glucose metabolism [3].

GLP1R agonists, such as liraglutide, are already in clinical use for patients with type 2 diabetes and obesity and have been shown to improve surrogate renal end points in large randomized controlled trials in type 2 diabetes patients [4,5] probably due to effects beyond improved glycaemic control. GLP-1R is expressed in the kidney exclusively by pre-glomerular vascular smooth muscle cells and juxtaglomerular cells [2,6]. There, GLP-1 mediates natriuresis and diuresis, alters renal haemodynamics, and decreases systemic blood pressure [7]. It has been speculated that anti-inflammatory capacity of GLP-1 analogs might further add to the improved renal outcomes since C-reactive protein levels significantly decrease by 25% to 60% in patients with type 2 diabetes treated with GLP-1 analogs independent of changes in fasting glucose, body weight, and body fat [8]. GLP1R is expressed on various immune cells and exerts anti-inflammatory effects, namely decreasing T cell proliferation and increasing the number and function of regulatory T cells in mice and humans [9–11].

So far, studies on T-cell–specific effects of GLP1R agonism in kidney disease are lacking. Nephrotoxic serum nephritis (NTS) is a murine model of immune-complex glomerulonephritis (GN) closely resembling forms of human rapid progressive GN. This rapidly progressive disease model is induced by the injection of rabbit anti-mouse glomerular basement membrane (GBM) serum and accelerated by a preceding immunization against rabbit IgG. Animals with NTS develop proteinuria within 7 days

and present proliferative and inflammatory glomerular changes, including crescent formation and kidney infiltrating leukocytes [12–14]. The pathogenesis depends on T helper (TH) cells type 1 TH17 cells, which respectively recruit macrophages or neutrophil granulocytes to the kidney [15–17]. Furthermore, regulatory T cells have been clearly shown to limit NTS [12,13,18].

We studied the anti-inflammatory capacity of Glp1r agonism in a T-cell–dependent model of rapid progressive glomerulonephritis using *Glp1r* [−/−] mice and wild-type (WT) mice that were treated with liraglutide.

Materials and Methods

Ethics statement

All animal experiments were approved by the Committee on the Ethics of Animal Experiments of the Austrian Ministry (BMWFW-66.010/0057-WF/II/3b/2014). All efforts were made to minimize animal suffering.

Mice

C57BL/6J mice were obtained from Charles River Laboratories (Sulzfeld, Germany). *Glp1r* [−/−] [19] were kindly provided by Daniel Drucker (Toronto, Canada). Genotyping was performed by isolation of genomic DNA using the DNeasy Blood and Tissue kit (QIAGEN, Hilden, Germany), followed by PCR using the following primers: primer A Glp1r 5' (5'-TAC ACA ATG GGG AGC CCC TA-3'), primer B Glp1r 3' (5'-AAG TCA TGG GAT GTG TCT GGA-3'), primer C Neo1 (5'-CTT GGG TGG AGA GGC TAT TC-3'), and primer D Neo2 (5'-AGG TGA GAT GAC AGG AGA TC-3'). For respective experiments *Glp1r* [−/−] mice and littermate WT controls were used. Mice were housed in a pathogen-free facility and maintained on a 12 h light-dark cycle, with free access to standard rodent chow and water. Eight- to 12-week–old male mice were used for all experiments. Mice weight ranged between 19 to 24g without significant differences between groups and experiments. Mice were healthy without any bacterial or viral infections as evaluated quarterly by the animal facility.

Mice were injected daily intraperitoneally with 200µg/kg bodyweight liraglutide (Victoza, NovoNordisk, Bagsvaerd, Denmark) or vehicle (saline) starting on the day of immunization.

Induction of NTS

NTS was induced as described previously [20]. Briefly, mice were pre-immunized subcutaneously with 100µL of 2 mg/mL rabbit IgG (Jackson ImmunoResearch Laboratories Inc., West Grove, PA) dissolved in incomplete Freund's adjuvant (Sigma, St. Louis, MO) and non-viable desiccated Mycobacterium tuberculosis H37a (Difco Laboratories, Detroit, MI). After 3 days, heat-inactivated rabbit anti-mouse GBM antiserum was injected via the tail vein.

Detection of urinary albumin and creatinine

Urinary albumin excretion was determined by a double-sandwich ELISA (Abcam, Cambridge, MA). Urinary creatinine was quantified using a picric acid–based method (Sigma-Aldrich) or by liquid chromatography tandem mass spectrometry (LC-MS/MS). For the later, separations were achieved on a Hypercarb column (ThermoFisher, San Jose, CA) and detection was performed by positive electrospray ionization on a SCIEX QTRAP 4500 triple quadrupole instrument (U.S.A. Framingham, MA).

Histomorphological evaluation of renal pathology

Formalin-fixed renal tissue was embedded in paraffin and cut in 4µm sections. The sections were stained with periodic acid Schiff's (PAS) and a minimum of 50 glomerular cross sections were evaluated per sample. The evaluation was performed as previously described [21]. In brief, PAS positive material was scored within glomeruli following a semi quantitative scoring system with a scale from 0 to 3. Cell proliferations in the different glomerular compartments were assessed as follows: Mesangial hypercellularity was subclassified as mild (score 1) = 4 to 5 cells/mesangial area, moderate (score 2) = 5 to 6 cells/mesangial area, and severe (score 3) = >6 cells/mesangial area. Endocapillary hypercellularity defined as hypercellularity due to increased number of cells within glomerular

6

capillary lumina was subclassified as mild (score 1) = present in single glomerula, moderate (score 2) = <50%, and severe (score 3) = >50% affected glomerula. Extracapillary hypercellularity/crescents defined as cell proliferation of more than two cell layers were subclassified as mild (score 1) = present in single glomerula, moderate (score 2) = <50%, and severe (score 3) = >50% affected glomerula. For detecting proliferating cells in kidneys, slides were stained with mouse anti-human/mouse/rat proliferation cell nuclear antigen (PCNA) antibody (clone PC10, Biolegend, San Diego, CA) using the three-layer immunoperoxidase staining. The M.O.M. Immunodetection Kit for detecting mouse primary antibodies on mouse tissue was used (Vector Laboratories, Burlingame, CA). Glomerular PCNA$^+$ cell quantitation was performed by counting the positive cells in 50 glomeruli per sample and further calculating the average number of positive cells per glomerulus. Periglomerular PCNA$^+$ cells were quantified by counting the number of positive cells located in the periglomerular region in six adjacent high-power fields (Hpf) of renal cortex. To evaluate fibrotic changes, kidney sections were stained with picrosirius red staining (Sigma-Aldrich). Samples were blinded before evaluation.

Immunofluorescence staining of kidney sections

For the detection of autologous and heterologous IgG deposition in kidneys, 4μm frozen kidney sections were stained with direct immunofluorescence staining. FITC-conjugated goat anti-mouse IgG (Jackson ImmunoResearch Laboratories) and FITC-conjugated goat anti-rabbit IgG (Jackson ImmunoResearch Laboratories) were used in a 1:800 dilution. Slides were mounted in a mounting medium for fluorescence (Vector Laboratories, Burlingame, CA). Slides were analyzed on a LSM510 META (Zeiss, Oberkochen, Germany).

Immunomorphological evaluation of infiltrating renal immune cells

The three-layer immunoperoxidase staining of frozen tissue 4-mm sections was used for the detection of macrophage, T cell, and neutrophil subpopulations in the kidney. Macrophages were stained with rat anti-mouse anti-CD68 antibody (clone FA-11, Biorad). A semi quantitative scoring

system for kidney-infiltrating macrophages was performed as follows: 0 = 0 to 4 cells stained positive; 1 = 5 to 10 cells; 2 = 10 to 50 cells; 3 = 50 to 200 cells; and 4 = >200 cells stained positive per low-power field. For the detection of T helper cells, cytotoxic T cells and neutrophils, rat anti-mouse anti-CD4 (clone YTS191.1, Biorad), rat anti-mouse anti-CD8 (clone KT15, Biorad), and rat anti-mouse anti-neutrophil (clone NIMP-R14; Abcam) antibodies were used respectively. T cell and neutrophil quantitation was performed by counting the number of positive cells in six adjacent high-power fields (Hpf) of renal cortex and medulla. For all evaluations a biotin-conjugated goat anti-rat IgG (Jackson ImmunoResearch Laboratories) was used as a secondary antibody. Samples were blinded before evaluation.

Reverse transcription (RT) real-time PCR

Total RNA was isolated from kidneys, spleens, and inguinal lymph nodes using TRI Reagent (Sigma-Aldrich). Subsequently, 2µg of total RNA was reverse transcribed using Superscript III Transcription Kit (Invitrogen, Carlsbad, CA) and random primers (Invitrogen). Real-time PCR was performed in duplicates on a CFX96 Real-Time System (BioRad, Vienna, Austria) usingTaqMan gene expression assays (Applied Biosystems, Foster City, CA) for Il-10 (Mm00439616_m1), Il-6 (Mm00446190_m1), Ifn- γ (Mm00801778_m1), Tnf- α (Mm00443258_m1), Tbet (Mm00450960_m1), Rorγt (Mm012611022_m1), *Gata3* (Mm00484683_m1), and *Foxp3* (Mm00475162_m1). *Hprt* was used as reference gene for kidney and spleen tissues, whereas for lymph node tissue *Rpl0* was used. The primers were, respectively: forward 5'-GCT TCC TCC TCA GAC CGC TTT TTG C-3'; reverse 5'-ATC GCT AAT CAC GAC GCT GGG ACT G-3'; forward 5'-TTG GCC AAT AAG GTG CCA GC-3'; and reverse 5'-CTC GGG TCC TAG ACC AGT GT-3'. Both reference genes as well as gene *Col1A1* (primers: forward 5'-CAA TGC AAT GAA GAA CTG GAC TGT-3'; reverse 5'-TCC TAC ATC TTC TGA GTT TGG TGA-3') were assessed using SYBR Green Mastermix (Invitrogen). The data were evaluated using the 2ΔΔCT method.

Flow Cytometry

Cell suspensions from lymph nodes were stained with antibodies against CD4, CD25, and FoxP3 according to the manufacturer's instructions (BioLegend, San Diego, CA). Samples were analyzed on LSRII and FACSCalibur cytometers (both BD Biosciences, San Jose, CA).

Cell culture experiments

Splenocytes were isolated from WT and *Glp1r$^{-/-}$* mice subjected to NTS for 14 days. Briefly, spleen specimens were minced and suspended after passage through a 70μm cell strainer. The cells were stimulated with 100μg/mL pre-coated rabbit IgG or 200 ng/mL lipopolysaccharides (LPS) for 24 and 16 hours, respectively. The proliferation of the cells was evaluated with the EZ4U cell proliferation assay (Biomedica, Vienna, Austria). T cells were isolated from spleens of C57BL/6J as well as *Glp1r$^{-/-}$* mice and WT littermates using the MagniSort Mouse T cell Enrichment Kit (Invitrogen). The cells were stimulated with aCD3/CD28 antibodies (eBioscience, San Diego, CA) and treated with liraglutide (60μg/mL) for 72 hours. The anti-mouse CD3e antibody (clone 145-2C11) was coated on the plates in a concentration of 5μg/mL and incubated overnight at 4 ^{o}C. Anti-mouse CD28 was added to the cells in a concentration of 2μg /mL. IFN-γ, Il-6, Il-10, Il-17, Il-4, and TNF-α levels in the supernatant of the cells were determined using commercially available ELISA kits (BD, San Jose, CA). Glycolysis in T cells was measured using the Enzychrom Glycolysis Assay kit (BioAssay Systems, Hayward, CA). For the TH1 and TH17 polarization, cells were isolated from spleens of C57BL/6J mice using the CD4^{+}CD62L^{+} T cell isolation kit (Miltenyi Biotec, Bergisch Gladbach, Germany). TH1 stimulation was achieved as following; the anti-mouse CD3e antibody (clone 145-2C11) was coated on the plates in a concentration of 2.5μg/mL and incubated overnight at 4 ^{o}C. Anti-mouse CD28 (eBioscience), anti-mouse IL4 (BioLegend, San Diego, CA), recombinant mouse IL2 (BioLegend), and recombinant mouse IL12 (BioLegend) were added to the cells in the following concentrations respectively: 3μg /mL, 10 μg /L 5ng/mL, and 10ng/mL. Cells were incubated for 5 days. For the TH17 polarization the cells were kept in culture for four days with the addition of anti-mouse CD28 (eBioscience), anti-mouse IL4 (BioLegend), anti-mouse IFN-γ (BioLegend), recombinant mouse IL23 (BioLegend), recombinant mouse IL6 (Immunotools, Friesoythe, Germany), and recombinant human TGF beta (Immunotools) in

9

the concentrations: 1 μg/mL, 10 μg/mL, 10 μg/mL, 10 ng/mL, 40 ng/mL, 5 ng/mL, respectively. The plate had been priory coated with anti-mouse CD3e antibody (clone 145-2C11) as described for the TH1 stimulation but in a concentration of 0.5 μg/mL. The proliferation of the cells was evaluated with the EZ4U cell proliferation assay (Biomedica). To verify the success of the polarization, IFN-γ and Il-17 levels in the supernatant of the cells were determined using commercially available ELISA kits (BD, San Jose, CA). Furthermore, to investigate the expression of *Glp1r* in lymphocytes, T cells were isolated from spleens and lymph nodes of C57Bl/6 and *Glp1r*^{-/-} mice using the MagniSort Mouse T cell Enrichment Kit (Invitrogen). The cells were stimulated for 72 hours with aCD3/CD28 stimulation (as described above), or used directly for RNA isolation. RNA was isolated from the cells using the RNeasy Mini Kit (QIAGEN, Hilden, Germany). Subsequently, 2 μg of total RNA was reverse transcribed using Superscript III Transcription Kit (Invitrogen, Carlsbad, CA) and random primers (Invitrogen). PCR was performed using primers for *Glp1r* and β-Actin (forward 5'-GGC CAT GTG TAC CGG TTC TG-3'; reverse 5'-GGT GCA GTG CAA GTG TCT GA-3'; forward 5'-GAA GTG TGA CGT TGA CAT CCG-3'; reverse 5'-TGC TGA TCC ACA TCT GCT GGA-3', respectively). The PCR products were loaded on a 2% agarose gel and ran at 80V for 50 min. A no template control was used as a negative control. As a positive control, cDNA synthesized from 2μg RNA isolated from mouse brain was used. Finally, the gene expression of Glut-1 has been monitored using TaqMan gene expression assays (Mm00441473_m1). *Rpl29* was used as a reference gene (primers: forward 5'-CGC GGG TTA CCG TGA GTG T-3'; reverse 5'-TGT CTG CAC CTC GCG ACC-3') and was assessed using SYBR Green Mastermix (Invitrogen).

Statistical analysis

Data are presented as mean ± SEM. The normal distribution of the data was assessed by the Kolmogorov-Smirnov test with Dallal-Wilkinson-Lilliefors correction or the Shapiro-Wilk normality test. When comparing two groups, according to the distribution, an unpaired *t*-test or a Mann-Whitney-U test was used. When comparing scores, the Chi-square test was used. A *P* < 0.05 was

considered statistically significant. All statistical analyses were performed using GraphPad Prism 7.0 (GraphPad Software, La Jolla, CA). Data are presented as mean ± SEM.

Results

Glp1r^{-/-} mice demonstrate an increased renal infiltration of immune cells after NTS induction

To evaluate the role of Glp1r in NTS, the disease was induced in *Glp1r^{-/-}* mice and wild-type (WT) mice. Mice developed NTS within the observed time period of 21 days as shown by albuminuria without a difference between the two groups (Fig. 1A, Supplemental Table S1). As expected from previous studies, mice displayed glomerular hypercellularity, endothelial cell proliferation, focal deposits, and crescent formation in about 10% to 20% of glomeruli, but differences were not observed in histologic changes between the two groups after 14 days (Fig. 1B and C, Supplemental Table S1, Supplemental Fig. S1, and Supplemental Table S2) and 21 days of NTS (Fig. 1B and Supplemental Fig. S1). Of note, relevant glomerular scaring was not observed during the observation period of 21 days (data not shown). The infiltration of CD68^{+} macrophages and CD4^{+} T cells was significantly higher in *Glp1r^{-/-}* mice after 21 days of NTS, whereas no difference was noted between the two groups on day 14 (Fig. 1D,F,H, Supplemental Table S1). No difference was observed in the infiltration of CD8^{+} T cells throughout the observation period (Fig. 1E, Supplemental Table S1). Fourteen days after induction of NTS a significant increase was observed in renal infiltration of polymorphonuclear granulocytes (PMN) in *Glp1r^{-/-}* mice compared to wild-type mice. This difference was lost by day 21 after disease induction (Fig. 1G and I, Supplemental Table S1). No difference was detected in the linear deposition of mouse and rabbit IgG on the glomerular membrane 14 days after NTS induction (Supplemental Fig. S2).

The expression of inflammatory genes is increased in lymph nodes and spleens of Glp1r^{-/-} mice in NTS

Cytokine expression profiling by qPCR in inguinal lymph nodes of wild-type and *Glp1r^{-/-}* mice after 14 days of NTS revealed increased mRNA expression of Il-10, Ifn-γ, and Tnf-α in *Glp1r^{-/-}* mice as compared to WT controls. Additionally, the expression of TH17 marker *Rorγt* and TH1 and TH2

master regulator Tbet and *Gata3* was up-regulated in the lymph nodes of *Glp1r^-/-* mice (Fig. 2A, Supplemental Table S3). The same genes were also evaluated in the spleens of those mice. In detail, the expression of Ifn-γ, Tbet, Rorγt, *Gata3,* and *Foxp3* was up-regulated in the spleens of *Glp1r^-/-* mice (Fig. 2B, Supplemental Table S3). In further experiments, splenocytes isolated from WT and *Glp1r^-/-* mice 14 days after NTS induction were cultured and stimulated with plate-coated rabbit IgG or LPS. Both stimulation methods significantly increased proliferation of splenocytes isolated from *Glp1r^-/-* as compared to WT mice (Fig. 2C, Supplemental Table S3). Of note, CD4^+CD25^+FoxP3^+ Tregs evaluated by flow cytometry in lymph nodes did not differ between the two groups either in absolute or in relative numbers (Supplemental Fig. S3A-B, Supplemental Table S4). No difference was seen in gene expression profiles of TH1, TH2, TH17, and Treg markers in the kidneys of *Glp1r^-/-* and WT mice 14 days after NTS induction (data not shown).

Interestingly, cytokine gene expression in lymph nodes differed significantly between healthy *Glp1r^-/-* mice and WT controls. *Glp1r^-/-* mice displayed a decreased cytokine mRNA expression profile of TH1, TH2, and regulatory T cell (Tregs) markers. Only the TH17 marker Rorγt did not differ between the two groups (Supplemental Fig. S4, Supplemental Table S5). No difference was detected in cytokine mRNA transcription levels in kidneys of healthy *Glp1r^-/-* and WT mice (data not shown).

Glp1r agonism by liraglutide protects mice from NTS

Since Glp1r signalling has been implicated to have immunomodulatory effects, which were also shown in *Glp1r^-/-* mice subjected to NTS, C57BL/6 mice were treated with the Glp1r agonist liraglutide starting on the day of NTS induction. On day 14 after NTS induction, albuminuria as well as urinary neutrophil gelatinase-associated lipocalin (NGAL) levels were significantly lower in mice treated with liraglutide as compared to vehicle-treated mice (Fig. 3A-B, Supplemental Table S6). In line with the difference in albuminuria, kidney histology revealed more severe renal damage in vehicle-treated mice than in liraglutide treated mice, which was quantified by scoring PAS-positive deposits and crescents in glomeruli (Fig. 3C-E, Supplemental Table S6). Further histomorphological evaluation

revealed significantly decreased glomerular and tubular changes in liraglutide-treated mice compared to vehicle controls (Supplemental Fig. S5, Supplemental Table S7). Only marginal fibrotic changes were observed in kidneys subjected to 14 days of NTS (Figure 3F, Supplemental Table S6), but mRNA expression collagen type I alpha 1 chain (*Col1A1*) as a marker for fibrosis was significantly decreased in liraglutide-treated mice as compared to vehicle-controls (Figure 3G, Supplemental Table S6). Of note, no differences were found for *Col1A1* mRNA expression in kidneys of *Glp1r$^{-/-}$* and WT mice 14 days after NTS induction (data not shown). At the same time point, a significant decrease was observed in renal infiltration of CD68$^+$ macrophages, CD8$^+$ cytotoxic T cells, and CD4$^+$ TH cells in liraglutide-treated mice as compared to vehicle-treated controls (Fig. 4B-E, Supplemental Table S8). A trend was detected towards less infiltrating PMNs in the liraglutide group (Fig. 4A, Supplemental Table S8). Consistent with these findings, the expression of several inflammatory genes related to TH1 cells and Tregs was decreased in kidneys of mice treated with liraglutide compared to vehicle controls (Fig. 4F, Supplemental Table S8). In contrast, no difference was noted in the expression of the TH17 marker Rorγt and the TH2 marker *Gata3*. Of note, proliferating cells in glomeruli as well as within the periglomerular region detected by PCNA stain were significantly decreased in kidneys of liraglutide-treated mice compared to controls (Fig. 4G-I, Supplemental Table S8). No difference was detected in the linear deposition of mouse and rabbit IgG on the glomerular membrane 14 days after NTS induction (Supplemental Fig. S2).

Liraglutide acts via the Glp1r to improve NTS

To prove whether the immunosuppressive effects of liraglutide in NTS are signalled via the Glp1r, *Glp1r$^{-/-}$* mice were subjected to NTS for 14 days and they were treated with liraglutide or vehicle starting on the day of disease induction. Neither albuminuria (Fig. 5A, Supplemental Table S9) nor renal immune cell infiltration (Fig. 5B-E, Supplemental Table S9) differed significantly between the two groups 14 days after NTS induction. Additionally, no differences were noted in the renal histology or the expression of inflammation-related genes in the kidney (data not shown).

Liraglutide inhibits the proliferation of stimulated mouse TH1 and TH17 cells

To prove whether the Glp1r agonist liraglutide decreases T cell proliferation, T cells were isolated from spleens of C57BL/6J mice, stimulated with aCD3/CD28, and treated with liraglutide. T cell proliferation was significantly inhibited by liraglutide as compared to vehicle (Fig. 6A, Supplemental Table S10), whereas liraglutide did not influence proliferation of T cells isolated from *Glp1r*^{-/-} mice as compared to WT littermate controls (Fig. 6B, Supplemental Table S10). No differences were found in the protein levels of Il-4, Ifn-γ, Il-17, Tnf-α, and Il-10 between supernatants of treated and untreated cells (Fig. 6C-D, H, Supplemental Table S10). Only the amount of Il-6 was significantly lower in the supernatant of T cells treated with liraglutide (Fig. 6G, Supplemental Table S10). Of note, T cells transcribed the *Glp1r* mRNA before and after stimulation (Supplemental Fig. S6). Furthermore, T cells from C57BL/6J mice were polarized into TH1 and TH17 cells *in vitro* (Supplemental Fig. S7B-C, Supplemental Table S11) and proliferation was evaluated in the presence or absence of liraglutide. Liraglutide significantly decreased proliferation of TH1 and TH17 polarized cells (Fig. 6I, Supplemental Table S10). *Glp1r* mRNA was detected in both TH1 and TH17 polarized cells (Supplemental Fig. S7A, Supplemental Table S11).

Liraglutide influences glucose metabolism in T cells

T cells isolated from spleens of C57BL/6J mice were stimulated with aCD3/CD28 and treated with liraglutide. L-lactate as a marker for glycolysis was measured after 72 hours with normalization to cell numbers. L-lactate was found to be significantly decreased in stimulated T cells treated with liraglutide (Fig. 7A, Supplemental Table S12). Stimulated T cells were additionally analyzed for the mRNA expression of the glucose transporter Glut-1. Liraglutide significantly reduced the mRNA expression of *Glut-1* in stimulated T cells.

Discussion

This study provides evidence that Glp1r agonism by liraglutide suppresses T cell proliferation and thereby leads to a significant improvement of a T-cell–mediated kidney disease, namely nephrotoxic

serum nephritis (NTS), which closely resembles human forms of immunocomplex-mediated rapid progressive glomerulonephritis. Our data provide additional evidence about *in vivo* effects of Glp1r agonism that go beyond blood glucose lowering effects.

These data are highly relevant from a clinical perspective since large-randomized controlled trials using Glp1r analogs namely liraglutide in the LEADER trial and semaglutide in the SUSTAIN-6 trial have shown renal-protective effects [5,22]. These effects were mainly attributed to a decreased rate of new onset of macroalbuminuria. The mechanisms responsible for this effect are unclear so far, but may involve anti-inflammatory mechanisms induced by GLP1R agonism [7]. Since the NTS model used throughout the experiments is a model with rapid development of macroalbuminuria dependent on pro-inflammatory responses in normoglycemic conditions [23], it allows us to focus on the anti-proteinuric and anti-inflammatory capacity of Glp1r agonism.

The anti-inflammatory and immunomodulatory potential of Glp1r agonism was first mentioned in 2010 when Hadjiyanni provided evidence that peripheral lymphocytes of *Glp1r^{-/-}* mice display a hyperproliferative response to mitogenic stimuli [9]. In accordance with their observations, Glp1r agonism by liraglutide also decreased the proliferation of T cells *in vitro* in our hands. This was underlined by our *in vivo* findings in a T-cell–dependent model of NTS. Liraglutide treatment resulted in less proliferating cells in glomeruli as well as within the periglomerular region, where mainly immune cells are found to infiltrate during disease [12,24]. Furthermore, antigen-specific re-stimulation with rabbit IgG of splenocytes isolated from *Glp1r^{-/-}* mice after 14 days of NTS resulted in an increased proliferative response as compared to controls. In line with these findings, TH1 cytokine transcripts were significantly increased in lymph nodes and spleens from *Glp1r^{-/-}* mice 14 days after NTS induction. Interestingly, alterations were not detected in TH17-related transcripts such as their transcription factor Rorγt, which first prompted us to hypothesize that signalling via the Glp1r might preferentially influence TH1 cells rather than TH17 cells in NTS. In the *in vitro* evaluations, only IL-6 was found to be decreased whereas other TH1 cytokines such as IFN-γ and the TH17 cytokine IL-17 remained unaltered by liraglutide treatment. The liraglutide effect on proliferation seems to be signalled via the Glp1r, since liraglutide had no effect on proliferation of T cells isolated from *Glp1r^{-/-}*

mice. When T cells were polarized to TH1 and TH17 cells, both were found to express *Glp1r* mRNA. Both polarized T cell populations showed decreased proliferation when treated with liraglutide, which provides evidence that TH1 and TH17 cells are equally inhibited by liraglutide. It has been proposed that Glp1r agonism might exert its anti-inflammatory potential by increasing the number of peripheral Tregs [9,11,25]. Since Tregs play a crucial role in limiting disease activity in NTS by acting in the lymph nodes but also locally within the kidney [12,14,18,26], the number of Tregs in *Glp1r*^{-/-} as well as liraglutide-treated mice compared to respective controls were studied after NTS induction, but no differences were observed. Interestingly, both TH1 and TH17 cells have been proven recently to change their glucose metabolism to glycolysis when clonal proliferation is initiated, which is not the case for Tregs [27]. *In vitro* stimulated T cells produced significantly less L-lactate in the presence of liraglutide, which is indicative for a change in their metabolic program resulting in less glycolysis. The blockade of glycolysis by liraglutide might be mediated by a down-regulation of the glucose transporter *Glut1*, which plays a key mechanism in glycolysis of T cells [27]. Nevertheless, additional experiments are needed to unravel the regulation of metabolic processes in T cells by liraglutide since opposing effects have been observed for Glp1r agonism in other cell types such as pancreatic beta-cells and cardiomyocytes [28,29].

A significantly aggravated NTS phenotype was not observed in *Glp1r*^{-/-} mice. This is somewhat counterintuitive, since liraglutide significantly reduced the severity of NTS. This finding might be explained by the fact that healthy *Glp1r*^{-/-} mice have an altered immune system as reflected by decreased lymph node cytokine transcripts, which could have delayed the development of NTS. Hadjiyanni and coworkers showed that though peripheral Glp1r deficient T cells are hyperproliferative, Glp1r deficient thymocytes showed severely decreased proliferation [9].

From a clinical perspective these data provide new insights not only into anti-inflammatory mechanisms of GLP-1R agonists, but also into new clinical applications. Our data support further investigation into the putative role(s) of Glp1r agonism in inflammatory, T-cell–mediated diseases. Besides our observation, Glp1r agonism has already been proven to improve T-cell–mediated murine disease models such as type 1 diabetes and experimental autoimmune encephalitis [25,30,31], but clinical

data are lacking. The anti-inflammatory effects of liraglutide described in this manuscript might also partly explain the improved surrogate renal end points observed in large randomized control trials in type 2 diabetes patients [4,5,22]. Pre-clinical studies provide evidence that immune cells including T cells play a key role in the development of insulin resistance and diabetic nephropathy [32,33]. This is supported by clinical data showing that an immune-modulatory drug, bardoxolone, improves kidney function in type 2 diabetics with advanced diabetic kidney disease. The clinical picture of diabetic nephropathy and the degree of inflammation varies significantly between each individual with type 2 diabetes and inhomogeneity of the study cohorts might thereby explain different response rates to tested drugs [34].

The renal improvement of liraglutide-treated mice subjected to NTS, might also be partly explained by the fact that Glp1r agonism suppresses activation of the renin-angiotensin-aldosterone system thereby increasing natriuresis and decreasing blood pressure [35–37]. In humans, acute administration of GLP1R agonists leads to a transient increase in blood pressure. Chronic administration of liraglutide however leads to a modest, but significant decrease in blood pressure (-3.59 mmHg) [38]. Lovshin and coworkers studied the haemodynamic effects of liraglutide in patients with hypertension and type 2 diabetes. In their hands, 3-week administration of liraglutide resulted in increased natriuresis without any effect on blood pressure [37]. Since the mice were treated only for a limited time interval with liraglutide, a blood pressure lowering effect of liraglutide may not be responsible for the renal-protective effect in NTS. Nevertheless, it cannot be excluded that the increased natriuresis and improved renal haemodynamics might add to the protective effects of liraglutide in NTS.

Together, we provided evidence that Glp1r agonism improves NTS by inhibiting the proliferation of T cells. Our findings further explain the renal-protective effects of Glp1r agonism observed in large clinical trials in patients with type 2 diabetes and might open the field for new therapeutic options in T-cell–mediated diseases.

Acknowledgements

References

1. Drucker DJ, Nauck MA: The incretin system: glucagon-like peptide-1 receptor agonists and dipeptidyl peptidase-4 inhibitors in type 2 diabetes. Lancet 2006, 368:1696–1705.

2. Pyke C, Heller SR, Kirk RK, Ørskov C, Reedtz-Runge S, Kaastrup P, Hvelplund A, Bardram L, Calatayud D, Knudsen L: GLP-1 Receptor Localization in Monkey and Human Tissue: Novel Distribution Revealed With Extensively Validated Monoclonal Antibody. Endocrinology 2014, 155:1280–1290.

3. Nauck MA, Meier JJ: Incretin hormones: Their role in health and disease. Diabetes Obes Metabolism 2018, 20:5–21.

4. Marso SP, Daniels GH, Brown-Frandsen K, Kristensen P, Mann JF, Nauck MA, Nissen SE, Pocock S, Poulter NR, Ravn LS, einberg W, Stockner M, Zinman B, Bergenstal RM, Buse JB, Committee L, Investigators L: Liraglutide and Cardiovascular Outcomes in Type 2 Diabetes. New Engl J Medicine 2016, 375:311–322.

5. Mann JF, Ørsted DD, Brown-Frandsen K, Marso SP, Poulter NR, Rasmussen S, Tornøe K, Zinman B, Buse JB, and Investigators L: Liraglutide and Renal Outcomes in Type 2 Diabetes. New Engl J Medicine 2017, 377:839–848.

6. Richards P, Parker HE, Adriaenssens AE, Hodgson JM, Cork SC, Trapp S, Gribble FM, Reimann F: Identification and Characterization of GLP-1 Receptor–Expressing Cells Using a New Transgenic Mouse Model. Diabetes 2014, 63:1224–1233.

7. Muskiet MH, Tonneijck L, Smits MM, van Baar M, Kramer MH, Hoorn EJ, Joles JA, van Raalte DH:

GLP-1 and the kidney: from physiology to pharmacology and outcomes in diabetes. Nat Rev Nephrol 2017, 13:605–628.

8. Bunck MC, Diamant M, Eliasson B, Cornér A, Shaginian RM, Heine RJ, Taskinen M-R, Yki-Järvinen H, Smith U: Exenatide Affects Circulating Cardiovascular Risk Biomarkers Independently of Changes in Body Composition. Diabetes Care 2010, 33:1734–1737.

9. Hadjiyanni I, Siminovitch K, Danska J, Drucker D: Glucagon-like peptide-1 receptor signalling selectively regulates murine lymphocyte proliferation and maintenance of peripheral regulatory T cells. Diabetologia 2010, 53:730–740.

10. Chaudhuri A, Ghanim H, Vora M, Sia C, Korzeniewski K, Dhindsa S, Makdissi A, Dandona P: Exenatide Exerts a Potent Antiinflammatory Effect. J Clin Endocrinol Metabolism 2012, 97:198–207.

11. Xue S, Wasserfall CH, Parker M, Brusko TM, McGrail S, McGrail K, Moore M, Campbell-Thompson M, Schatz DA, Atkinson MA, Haller MJ: Exendin-4 Therapy in NOD Mice with New-Onset Diabetes Increases Regulatory T Cell Frequency. Ann Ny Acad Sci 2008, 1150:152–156.

12. Eller K, Weber T, Pruenster M, Wolf AM, Mayer G, Rosenkranz AR, Rot A: CCR7 Deficiency Exacerbates Injury in Acute Nephritis Due to Aberrant Localization of Regulatory T Cells. J Am Soc Nephrol 2010, 21:42–52.

13. Wolf D, Hochegger K, Wolf AM, Rumpold HF, Gastl G, Tilg H, Mayer G, Gunsilius E, Rosenkranz AR: CD4+CD25+ Regulatory T Cells Inhibit Experimental Anti-Glomerular Basement Membrane Glomerulonephritis in Mice. J Am Soc Nephrol 2005, 16:1360–1370.

14. Hochegger K, Jansky GL, Soleiman A, Wolf AM, Tagwerker A, Seger C, Griesmacher A, Mayer G,

Rosenkranz AR: Differential Effects of Rapamycin in Anti-GBM Glomerulonephritis. J Am Soc Nephrol 2008, 19:1520–1529.

15. Hopfer H, Holzer J, Hünemörder S, Paust H-J, Sachs M, Meyer-Schwesinger C, Turner J-E, Panzer U, Mittrücker H-W: Characterization of the renal CD4+ T-cell response in experimental autoimmune glomerulonephritis. Kidney Int 2012, 82:60–71.

16. Summers SA, Steinmetz OM, Li M, Kausman JY, Semple T, Edgtton KL, Borza D-B, Braley H, Holdsworth SR, Kitching RA: Th1 and Th17 Cells Induce Proliferative Glomerulonephritis. J Am Soc Nephrol 2009, 20:2518–2524.

17. Paust H-J, Turner J-E, Steinmetz OM, Peters A, Heymann F, Hölscher C, Wolf G, Kurts C, Mittrücker H-W, Stahl R, Panzer U: The IL-23/Th17 Axis Contributes to Renal Injury in Experimental Glomerulonephritis. J Am Soc Nephrol 2009, 20:969–979.

18. Paust H-J, Ostmann A, Erhardt A, Turner J-E, Velden J, Mittrücker H-W, Sparwasser T, Panzer U, Tiegs G: Regulatory T cells control the Th1 immune response in murine crescentic glomerulonephritis. Kidney Int 2011, 80:154–164.

19. Scrocchi LA, Brown TJ, Maclusky N, Brubaker PL, Auerbach AB, Joyner AL, Drucker DJ: Glucose intolerance but normal satiety in mice with a null mutation in the glucagon–like peptide 1 receptor gene. Nat Med 1996, 2:1254–1258.

20. Rosenkranz AR, Mendrick DL, Cotran RS, Mayadas TN: P-selectin deficiency exacerbates experimental glomerulonephritis: a protective role for endothelial P-selectin in inflammation. J Clin Invest 1999, 103:649–659.

21. Kitching RA, Holdsworth SR, Ploplis VA, Plow EF, Collen D, Carmeliet P, Tipping PG: Plasminogen

and Plasminogen Activators Protect against Renal Injury in Crescentic Glomerulonephritis. J Exp

Medicine 1997, 185:963–968.

22. Pfeffer MA, Claggett B, Probstfield JL: Lixisenatide in Type 2 Diabetes and Acute Coronary

Syndrome. New Engl J Medicine 2016, 374:1094–1096.

23. Artinger K, Kirsch AH, Aringer I, Moschovaki-Filippidou F, Eller P, Rosenkranz AR, Eller K: Innate

and adaptive immunity in experimental glomerulonephritis: a pathfinder tale. Pediatr Nephrol 2017,

32:943–947.

24. Kurts C, Panzer U, Anders H-J, Rees AJ: The immune system and kidney disease: basic concepts

and clinical implications. Nat Rev Immunol 2013, 13:738–753.

25. Hadjiyanni I, Baggio LL, Poussier P, Drucker DJ: Exendin-4 Modulates Diabetes Onset in Nonobese

Diabetic Mice. Endocrinology 2008, 149:1338–1349.

26. Ostmann A, Paust H-J, Panzer U, Wegscheid C, Kapffer S, Huber S, Flavell RA, Erhardt A, Tiegs G:

Regulatory T Cell–Derived IL-10 Ameliorates Crescentic GN. J Am Soc Nephrol 2013, 24:930–942.

27. Palmer CS, Ostrowski M, Balderson B, Christian N, Crowe SM: Glucose Metabolism Regulates T

Cell Activation, Differentiation, and Functions. Front Immunol 2015, 6:1.

28. Carlessi R, Chen Y, Rowlands J, Cruzat VF, Keane KN, Egan L, Mamotte C, Stokes R, Gunton JE, de

Bittencourt P, Newsholme P: GLP-1 receptor signalling promotes β-cell glucose metabolism via

mTOR-dependent HIF-1α activation. Sci Rep-uk 2017, 7:2661.

29. Wallner M, Kolesnik E, Ablasser K, Khafaga M, Wakula P, Ljubojevic S, Thon-Gutschi E, Sourij H, Kapl M, Edmunds NJ, Kuzmiski BJ, Griffith DA, Knez I, Pieske B, von Lewinski D: Exenatide exerts a PKA-dependent positive inotropic effect in human atrial myocardium GLP-1R mediated effects in human myocardium. J Mol Cell Cardiol 2015, 89:365–375.

30. Li L, Lu J, Jia X, Hui H, Zhang J, Liu Y, Cui W, Xu Q, Zhu D: Liraglutide Enhances the Efficacy of Human Mesenchymal Stem Cells in Preserving Islet ß-cell Function in Severe Non-obese Diabetic Mice. Mol Med 2016, 22:800–808.

31. DellaValle B, Brix GS, Brock B, Gejl M, Landau AM, Møller A, Rungby J, Larsen A: Glucagon-Like Peptide-1 Analog, Liraglutide, Delays Onset of Experimental Autoimmune Encephalitis in Lewis Rats. Front Pharmacol 2016, 7:433.

32. Eller K, Kirsch A, Wolf AM, Sopper S, Tagwerker A, Stanzl U, Wolf D, Patsch W, Rosenkranz AR, Eller P: Potential Role of Regulatory T Cells in Reversing Obesity-Linked Insulin Resistance and Diabetic Nephropathy. Diabetes 2011, 60:2954–2962.

33. Feuerer M, Hill JA, Mathis D, Benoist C: Foxp3+ regulatory T cells: differentiation, specification, subphenotypes. Nat Immunol 2009, 10:689–695.

34. Heerspink HJ, Oberbauer R, Perco P, Heinzel A, Heinze G, Mayer G, Mayer B: Drugs meeting the molecular basis of diabetic kidney disease: bridging from molecular mechanism to personalized medicine. Nephrol Dial Transpl 2015, 30:iv105–iv112.

35. Rieg T, Gerasimova M, Murray F, Masuda T, Tang T, Rose M, Drucker DJ, Vallon V: Natriuretic effect by exendin-4, but not the DPP-4 inhibitor alogliptin, is mediated via the GLP-1 receptor and preserved in obese type 2 diabetic mice. Am J Physiology Ren Physiology 2012, 303:F963-71.

36. Kim M, Platt MJ, Shibasaki T, Quaggin SE, Backx PH, Seino S, Simpson JA, Drucker DJ: GLP-1

receptor activation and Epac2 link atrial natriuretic peptide secretion to control of blood pressure.

Nat Med 2013, 19:567.

37. Lovshin JA, Barnie A, DeAlmeida A, Logan A, Zinman B, Drucker DJ: Liraglutide Promotes

Natriuresis but Does Not Increase Circulating Levels of Atrial Natriuretic Peptide in Hypertensive

Subjects With Type 2 Diabetes. Diabetes Care 2015, 38:132–139.

38. Sun F, Wu S, Guo S, Yu K, Yang Z, Li L, Zhang Y, Quan X, Ji L, Zhan S: Impact of GLP-1 receptor

agonists on blood pressure, heart rate and hypertension among patients with type 2 diabetes: A

systematic review and network meta-analysis. Diabetes Res Clin Pr 2015, 110:26–37.

Figure Legends

FIGURE 1. *Glp1r$^{-/-}$ mice show increased renal infiltration of inflammatory cells after NTS induction.* WT and *Glp1r$^{-/-}$* mice were subjected to NTS for 7 days (n=13 per group), 14 days (WT n=19, *Glp1r$^{-/-}$* n=18), and 21 days (WT n=9, *Glp1r$^{-/-}$* n=6). **A:** Urinary albumin/creatinine ratios (μg/mg) were evaluated at indicated time points after NTS induction. **B:** Kidney sections were quantified for crescent formation. **C:** Representative PAS-stained kidney sections of WT and *Glp1r$^{-/-}$* mice 14 days after NTS induction. The dashed line shows cellular crescent formation, the asterisks show fibrin deposition within bowman's space, and the red arrows mark endocapillary hypercellularity with significant proliferation of endocapillary cells within the glomerular capillary loops. Magnification x600. The scale bars represent 50 μm. Kidney sections were analyzed for the infiltration of CD68$^+$ macrophages (**D**), CD8$^+$ cytotoxic (**E**) and CD4$^+$ T helper cells (**F**) as well as polymorphonuclear granulocytes (PMN) (**G**). Representative pictures from kidney sections stained for CD4+ T cells (**H**) and PMNs (**I**) after 14 and 21 days of NTS are shown. Magnification x200. The scale bars represent 100 μm. hpf= high power field. *$P < 0.05$.

FIGURE 2. *Increased systemic inflammation is observed in Glp1r$^{-/-}$ mice.* **A:** The mRNA expression of inflammatory genes was evaluated in inguinal lymph nodes of WT (n=8) and *Glp1r$^{-/-}$* mice (n=10) 14 days after NTS induction. The fold increase compared to the mean mRNA expression in healthy WT and *Glp1r$^{-/-}$* mice is provided. **B:** The mRNA expression of respective genes was evaluated in the spleens of WT (n=3) and *Glp1r$^{-/-}$* mice (n=4) after 14 days of NTS. The fold increase compared to the mean mRNA expression in diseased WTs is provided. **C:** Splenocytes were isolated from WT (n=9) and *Glp1r$^{-/-}$* mice (n=9) after 14 days of NTS. Cells were stimulated *in vitro* by plate-coated rabbit IgG and LPS and proliferation was evaluated. The stimulation index is given as the ratio between the OD values of stimulated to unstimulated cells. *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$.

FIGURE 3. *Liraglutide protects mice from NTS.* WT mice were subjected to NTS for 14 days and treated with vehicle (n=14) or liraglutide (n=13) from the day of immunization. **A:** Urinary
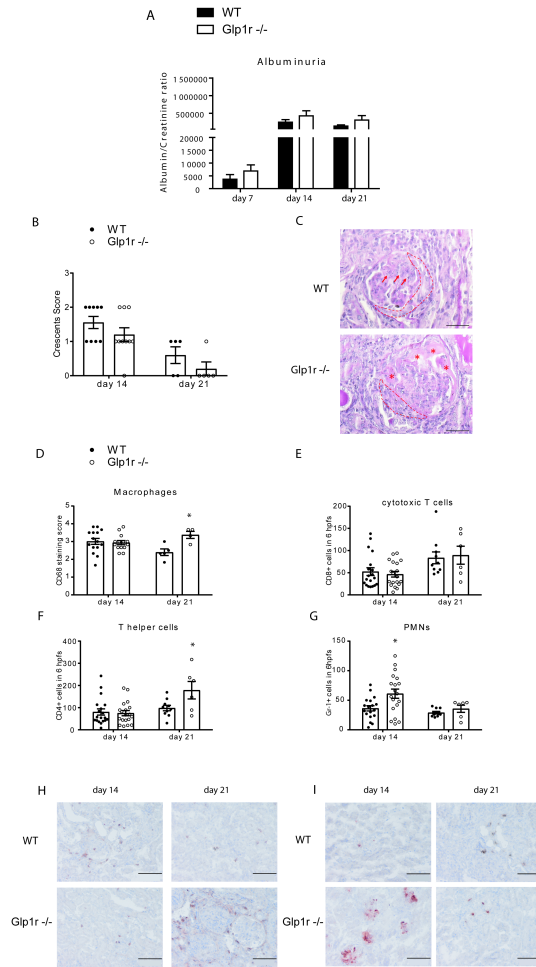
25

albumin/creatinine ratios (µg/mg) were evaluated on day 14 after NTS induction. **B:** At the same time point urinary NGAL/creatinine ratios (pg/mg; n=4 per group) were evaluated. Kidney sections were quantified for PAS positive deposits (**C**) and for crescent formation (**D**). **E:** Representative PAS-stained kidney sections of mice treated with vehicle or liraglutide. Magnification x400. The scale bars represent 50 µm. **F:** Representative pictures for Picro Sirius red stain of the kidneys. Magnification 100x. The scale bars represent 200 µm. **G:** The mRNA expression of *Col1A1* gene was evaluated in the kidneys of the mice (vehicle: n=6, liraglutide: n=8). The fold increase compared to the mean mRNA expression in vehicle-treated mice subjected to NTS is provided. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$.

FIGURE 4. *Liraglutide decreases renal inflammatory cell infiltration.* WT mice were subjected to NTS for 14 days and treated with vehicle (n=14) or liraglutide (n=13) from the day of immunization. Kidney sections were analyzed for PMNs (**A**), CD68$^+$ macrophages (**B**), CD8$^+$ cytotoxic T cells (**C**), and CD4$^+$ TH cells (**D**). **E:** Representative pictures from kidney sections from vehicle and liraglutide treated mice stained for the respective markers after 14 days of NTS. Magnification 200x. The scale bars represent 100 µm. **F:** Quantitative PCR of respective genes was performed on kidney tissues of mice subjected to NTS for 14 days treated with either vehicle (n=9) or liraglutide (n=8). The fold increase compared to the mean mRNA expression in vehicle-treated mice subjected to NTS is provided. Kidneys were stained for proliferating cells by using PCNA stain. Positive cells in glomeruli (**G**) and the periglomerular region (**H**) were counted. **I:** Representative pictures for PCNA stain in the kidneys are shown. Magnification 400x. The scale bars represent 50 µm. $*P < 0.05$, $**P <0.01$, and $***P < 0.001$.
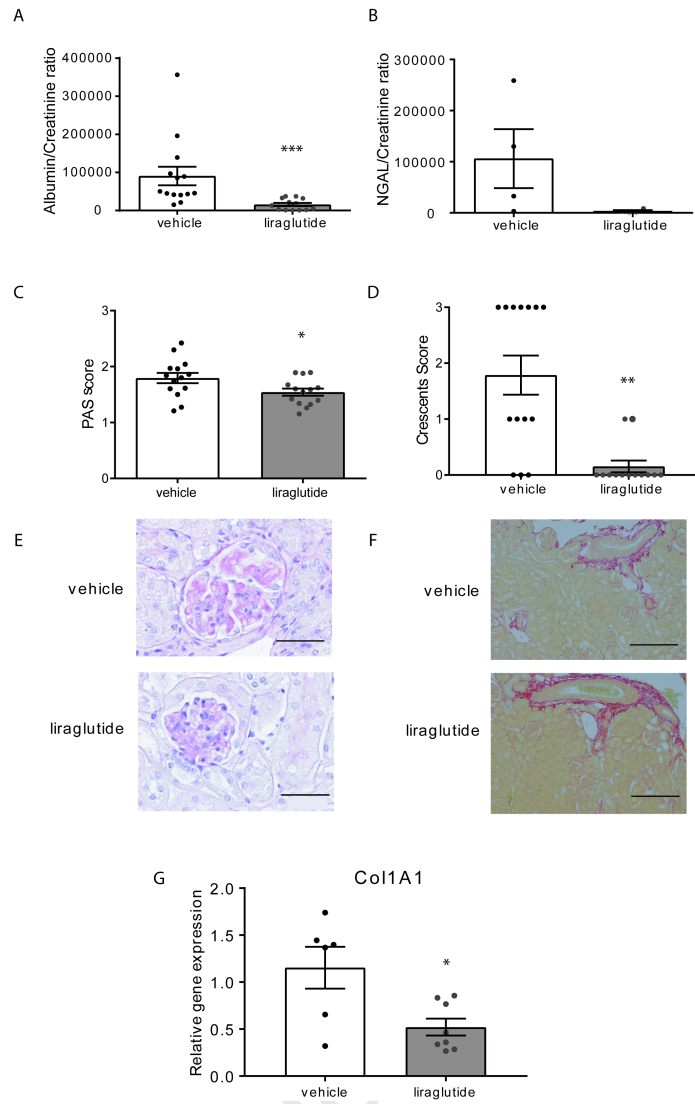
FIGURE 5. *Liraglutide treatment has no effect on Glp1r$^{-/-}$ mice subjected to NTS.* Glp1r$^{-/-}$ mice were subjected to NTS for 14 days and treated with vehicle (n=6) or liraglutide (n=8) from the day of immunization. **A:** Urinary albumin/creatinine ratios were evaluated on day 14 after NTS induction. Kidney sections were analyzed for PMNs (**B**), CD68$^+$ macrophages (**C**), CD8$^+$ cytotoxic T (**D**), and CD4$^+$ TH (**E**) cells.
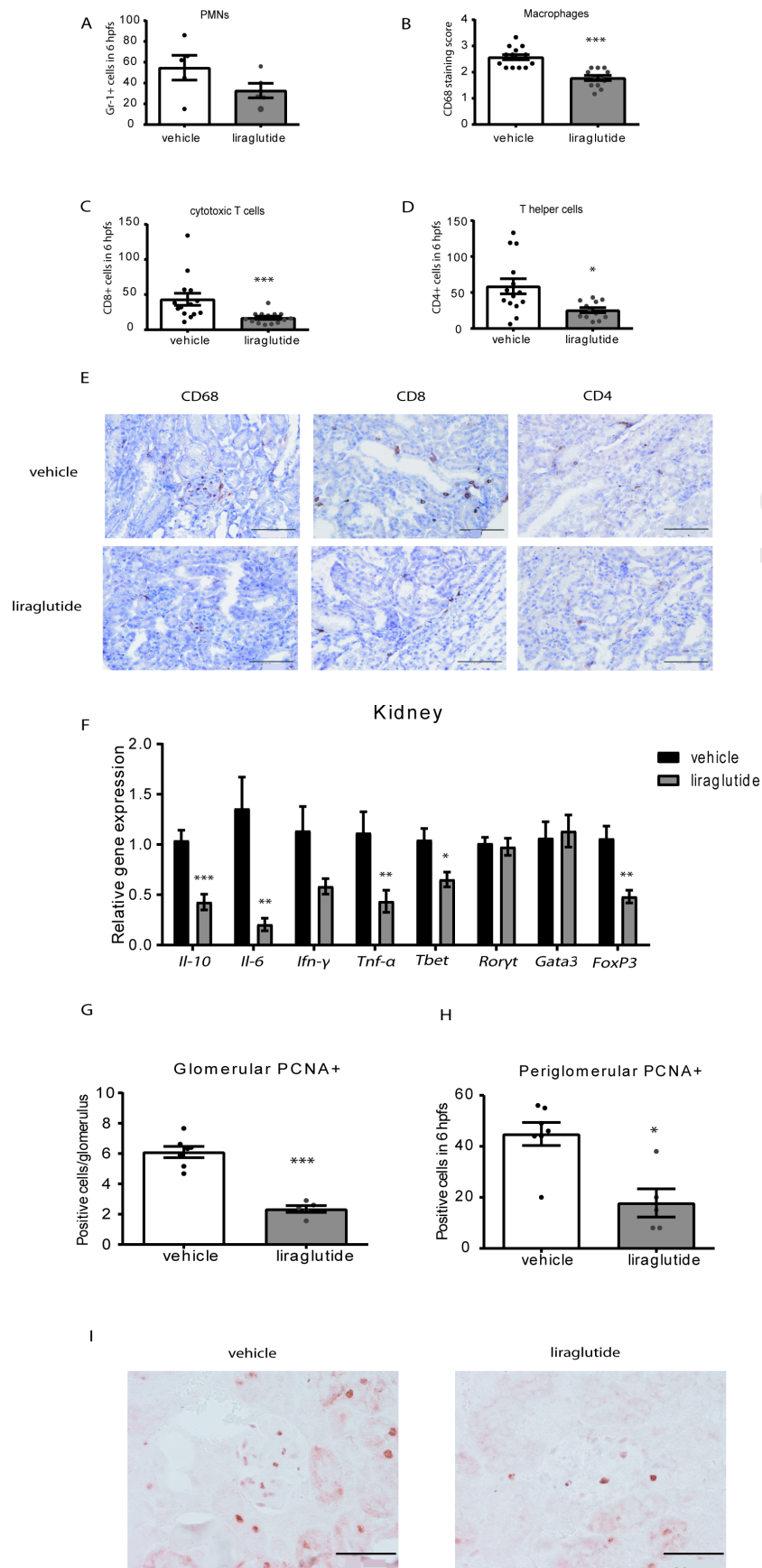
FIGURE 6. *Liraglutide inhibits the proliferation of stimulated mouse T cells and decreases the production of Il-6.* **A:** T cells were isolated from C57BL/6J mice, stimulated with aCD3/CD28 and treated with liraglutide in a concentration of 60μg/mL (n=5) or vehicle (n=5). **B:** The same procedure was followed for T cells isolated from *Glp1r*^{-/-} mice treated with vehicle (n=7) or liraglutide (n=2), which were compared to T cells isolated from WT littermates (n=5). **A** and **B:** Proliferation of cells was evaluated after 72 hours. The stimulation index is given as the ratio between the OD values of stimulated to unstimulated cells. **C-H:** The supernatant of the cells was collected after 72 hours and respective cytokine levels were measured by ELISA. **I:** TH1 and TH17 polarized cells were treated with liraglutide or vehicle (n=3 per group) and proliferation was evaluated after 4 and 5 days, respectively. *$P < 0.05$ and **$P < 0.01$.

FIGURE 7. *Liraglutide inhibits glycolysis in mouse T cells and down-regulates the expression of Glut-1.* T cells were isolated from C57BL/6J mice, stimulated with aCD3/CD28, and treated with liraglutide in a concentration of 60μg/mL (n=7) or vehicle (n=7). **A:** Glycolysis of cells was evaluated after 72 hours. **B:** The mRNA expression of Glut-1 gene was evaluated in the T cells treated with liraglutide (n=5) or vehicle (n=5). The fold increase compared to the mean mRNA expression in vehicle-treated stimulated T cells is provided. **$P < 0.01$ and ***$P < 0.001$.
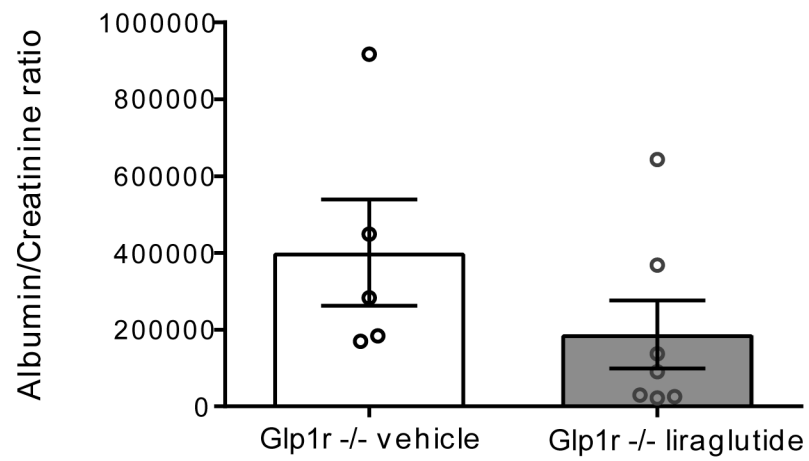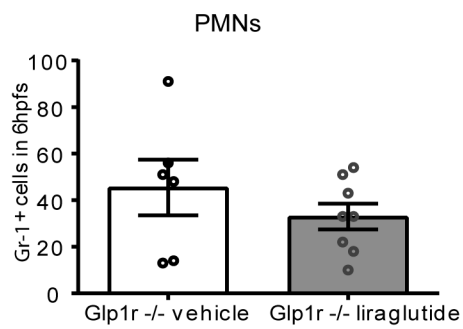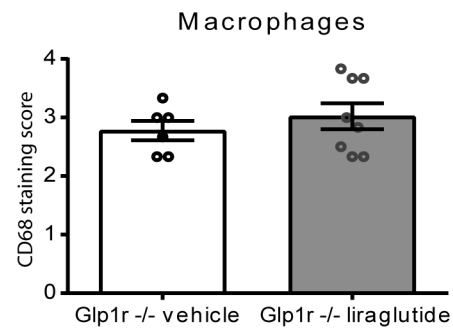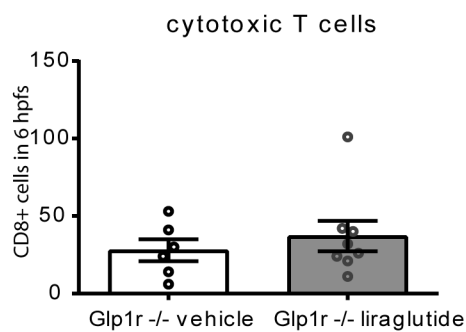
A

Lymph nodes

B

Spleen

C

Splenocyte Proliferation

A PMNs

B Macrophages

C cytotoxic T cells

D T helper cells

E

	CD68	CD8	CD4
vehicle			
liraglutide			

F

Kidney

G

Glomerular PCNA+

H

Periglomerular PCNA+

I

vehicle	liraglutide

A

B

PMNs

C

Macrophages

D

cytotoxic T cells

E

T helper cells

A   Proliferation

B   Proliferation

C   Il4

D   Ifnγ

E   Il17

F   Tnfa

G   Il6

H   Il10

I   Proliferation

A

Glycolysis

B

Glut1