



IMMUNOPATHOLOGY AND INFECTIOUS DISEASES

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



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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, the anti-inflammatory action of Glp1r agonism was tested in a nondiabetic, 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*^{-/-} mice. 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 renoprotective effects of Glp1r agonism in diabetic nephropathy. (*Am J Pathol* 2020, 190: 400–411; <https://doi.org/10.1016/j.ajpath.2019.10.008>)

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 <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

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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 endpoints in large randomized controlled trials in type 2 diabetes patients,^{4,5} probably due to effects beyond improved glycemic 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 hemodynamics, 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 because 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 closely resembling forms of human rapid progressive glomerulonephritis. 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 and 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}

The authors 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 (BMFWF-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 D.J.D. 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' (forward, 5'-TACACAATGGGGAGCCCCTA-3'), primer B *Glp1r* 3' (reverse, 5'-AAGTCATGGGATGTGTCTGGA-3'), primer C *Neo1* (forward, 5'-CTTGGGTGGAGAGGCTATTC-3'), and primer D *Neo2* (reverse, 5'-AGGTGAGATGACAGGAGATC-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-hour 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 weighed between 19 and 24 g 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 intraperitoneally with 200 µg/kg bodyweight liraglutide (once daily) (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 preimmunized subcutaneously with 100 µL of 2 mg/mL rabbit IgG (Jackson ImmunoResearch Laboratories, West Grove, PA) dissolved in incomplete Freund's adjuvant (Sigma, St. Louis, MO) and nonviable 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 enzyme-linked immunosorbent assay (ELISA) (Abcam, Cambridge, MA). Urinary creatinine was quantified using a picric acid-based method (Sigma-Aldrich, St. Louis, MO) or by liquid chromatography tandem mass spectrometry. For the later, separations were achieved on a Hypercarb column (Thermo Fisher Scientific, San Jose, CA), and detection was performed by positive electrospray ionization on a SCIEX QTRAP 4500 triple quadrupole instrument (SCIEX, Framingham, MA).

Histomorphologic Evaluation of Renal Pathology

Formalin-fixed renal tissue was embedded in paraffin and cut into 4-µm sections. The sections were stained with periodic acid-Schiff (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 semiquantitative 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 capillary lumina, was subclassified as mild (score 1), present in single glomeruli; moderate (score 2), <50%; and severe (score 3), >50% affected glomeruli. Extracapillary hypercellularity/crescents defined as cell proliferation of more than two cell layers were subclassified as mild (score 1), present in single glomeruli; moderate (score 2), <50%; and severe (score 3), >50% affected glomeruli. 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 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 of renal cortex. To evaluate fibrotic changes, kidney sections were stained with Picrosirius Red (Sigma-Aldrich). Investigators were blinded to the samples before evaluation (F.M.F., A.H.K., M.T., M.P.).

Immunofluorescence Staining of Kidney Sections

For the detection of autologous and heterologous IgG deposition in kidneys, 4- μ m frozen kidney sections were stained by direct immunofluorescence staining. Fluorescein isothiocyanate-conjugated goat anti-mouse IgG (Jackson ImmunoResearch Laboratories) and fluorescein isothiocyanate-conjugated goat anti-rabbit IgG (Jackson ImmunoResearch Laboratories) were used in 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).

Immunomorphologic Evaluation of Infiltrating Renal Immune Cells

The three-layer immunoperoxidase staining of 4-mm frozen tissue 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, Bio-Rad Laboratories, Hercules, CA). A semiquantitative 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, Bio-Rad Laboratories), rat anti-mouse anti-CD8 (clone KT15, Bio-Rad Laboratories), 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 of renal cortex and medulla. For all evaluations, a biotin-conjugated goat anti-rat IgG (Jackson ImmunoResearch Laboratories) was used as a secondary antibody. Investigators were blinded to the samples before evaluation (F.M.F., A.H.K., M.T.).

Real-Time RT-PCR

Total RNA was isolated from kidneys, spleens, and inguinal lymph nodes using TRI Reagent (Sigma-Aldrich). Subsequently, 2 μ g 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) using TaqMan gene expression assays (Applied Biosystems, Foster City, CA) for Il-10 (Mm00439616_m1), Il-6 (Mm00446190_m1), interferon- γ (Ifn- γ) (Mm00801778_m1), Tnf- α (Mm00443258_m1), Tbet (Mm00450960_m1), Ror γ t (Mm012611022_m1), *Gata3* (Mm00484683_m1), and Foxp3 (Mm00475162_m1). *Hprt* was used as the reference gene for kidney and spleen tissues, whereas for lymph node tissue, *Rpl0* was used. The primers were, respectively: forward 5'-GCTTCCTCCTCAGACCGCTTTTTGC-3'; reverse 5'-ATCGCTAATCACGACGCTGGGACTG-3'; forward 5'-TTGGCCAATAAGGTGCCAGC-3'; and reverse 5'-CTCGG GTCCTAGACCAGTGT-3'. Both reference genes, as well as the *Col1A1* gene (primers: forward 5'-CAATGCAATGAAGAAC TGGACTGT-3'; reverse 5'-TCCTACATCTTCTGAGTTTGG TGA-3'), were assessed using SYBR Green Master Mix (Invitrogen). The data were evaluated using the $2\Delta\Delta$ CT method.

Flow Cytometry

Cell suspensions from lymph nodes were stained with antibodies against CD4, CD25, and FoxP3 according to the manufacturer's instructions (BioLegend). 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 70- μ m cell strainer. The cells were stimulated with 100 μ g/mL precoated rabbit IgG or 200 ng/mL lipopolysaccharides 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 anti-CD3/CD28 antibodies (eBioscience, San Diego, CA) and treated with liraglutide (60 $\mu\text{g}/\text{mL}$) for 72 hours. The anti-mouse CD3e antibody (clone 145-2C11) was coated on the plates in a concentration of 5 $\mu\text{g}/\text{mL}$ and incubated overnight at 4°C. Anti-mouse CD28 was added to the cells in a concentration of 2 $\mu\text{g}/\text{mL}$. 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 Biosciences). 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 follows: the anti-mouse CD3e antibody (clone 145-2C11) was coated on the plates in a concentration of 2.5 $\mu\text{g}/\text{mL}$ and incubated overnight at 4°C. Anti-mouse CD28 (eBioscience), anti-mouse Il-4 (BioLegend), recombinant mouse Il-2 (BioLegend), and recombinant mouse Il-12 (BioLegend) were added to the cells in the following concentrations, respectively: 3 $\mu\text{g}/\text{mL}$, 10 $\mu\text{g}/\text{L}$, 5 ng/mL, and 10 ng/mL. Cells were incubated for 5 days. For TH17 polarization, the cells were kept in culture for 4 days with the addition of anti-mouse CD28 (eBioscience), anti-mouse Il-4 (BioLegend), anti-mouse Ifn- γ (BioLegend), recombinant mouse Il-23 (BioLegend), recombinant mouse Il-6

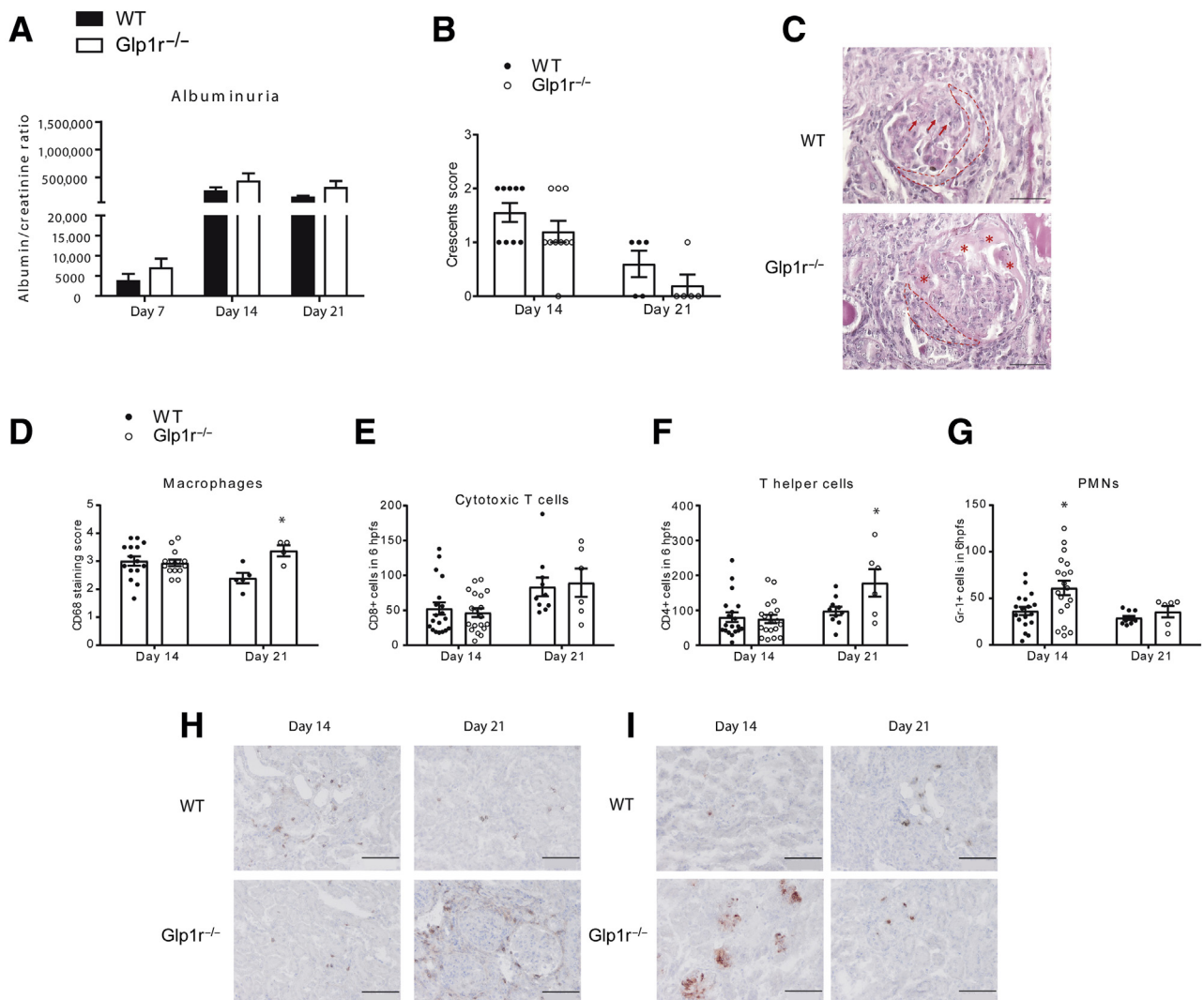


Figure 1 *Glp1r*^{-/-} mice show increased renal infiltration of inflammatory cells after NTS induction. WT and *Glp1r*^{-/-} mice were subjected to NTS for 7 days, 14 days, and 21 days. **A:** Urinary albumin/creatinine ratios ($\mu\text{g}/\text{mg}$) were evaluated at indicated time points after NTS 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. **Dashed lines** indicate cellular crescent formation; **asterisks**, fibrin deposition within Bowman space; and **arrows**, endocapillary hypercellularity with significant proliferation of endocapillary cells within the glomerular capillary loops. **D–G:** Kidney sections were analyzed for the infiltration of CD68⁺ macrophages (**D**), and CD8⁺ cytotoxic (**E**) and CD4⁺ T helper cells (**F**), as well as polymorphonuclear granulocytes (PMN) (**G**). **H** and **I:** Representative pictures from kidney sections stained for CD4⁺ T cells (**H**) and PMNs (**I**) after 14 and 21 days of NTS are shown. $n = 6$ *Glp1r*^{-/-} mice treated 21 days; $n = 9$ WT mice treated 21 days; $n = 13$ mice per group treated 7 days; $n = 18$ *Glp1r*^{-/-} mice treated 14 days; $n = 19$ WT mice treated 14 days. * $P < 0.05$ versus WT. Scale bars: 50 μm (**C**); 100 μm (**H** and **I**). Original magnification: $\times 600$ (**C**); $\times 200$ (**H** and **I**). hpf, high-power field.

(Immunotools, Friesoythe, Germany), and recombinant human TGF- β (Immunotools) in the concentrations: 1 $\mu\text{g}/\text{mL}$, 10 $\mu\text{g}/\text{mL}$, 10 $\mu\text{g}/\text{mL}$, 10 ng/mL , 40 ng/mL , and 5 ng/mL , respectively. The plate had been coated beforehand with anti-mouse CD3e antibody (clone 145-2C11) as described for the TH1 stimulation but in a concentration of 0.5 $\mu\text{g}/\text{mL}$. 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). Subsequently, 2 μg of total RNA was reverse transcribed using Superscript III Transcription Kit (Invitrogen) and random primers (Invitrogen). PCR was performed using primers for *Glp1r* and β -actin (forward 5'-GGCCATGTG-TACCGTTCTG-3'; reverse 5'-GGTGC AGTGCAAGTGTCTGA-3'; forward 5'-GAAGTGTGACG TTGACATCCG-3'; reverse 5'-TGCTGATCCACATCTGC TGGA-3', respectively). The PCR products were loaded on a 2% agarose gel and run at 80 V for 50 minutes. A no template control was used as a negative control. As a positive control, cDNA synthesized from 2 μg 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'-CGCGGGTTACCGTGAGTGT-3'; reverse 5'-TGTCTGCACCTCGGACC-3') and was assessed using SYBR Green Master Mix (Invitrogen).

Statistical Analysis

Data are presented as means \pm 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 *U*-test was used. When comparing scores, the χ^2 test was used. $P < 0.05$ was considered statistically significant. All statistical analyses were performed using GraphPad Prism software version 7.0 (GraphPad Software, La Jolla, CA).

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 WT mice. Mice developed NTS within the observed time period of 21 days as shown by albuminuria, without a difference between the two

groups (Figure 1A and Supplemental Table S1). As expected from previous studies,¹²⁻¹⁴ 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 (Figure 1, B and C, Supplemental Table S1, Supplemental Figure S1, and Supplemental Table S2) and 21 days of NTS (Figure 1B and Supplemental Figure S1). Of note, relevant glomerular scarring was not observed during the observation period of

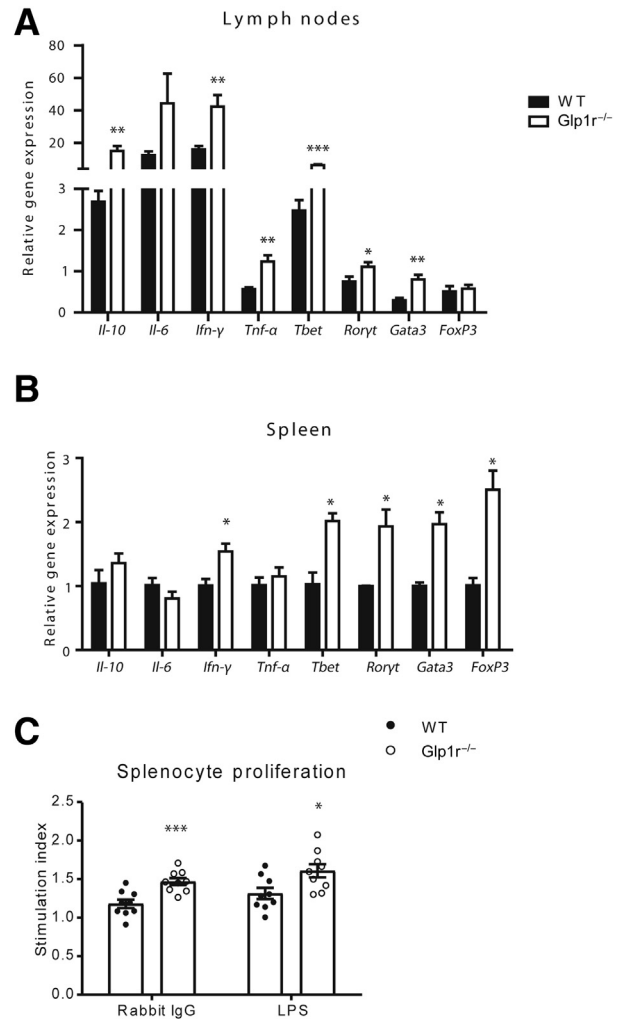


Figure 2 Increased systemic inflammation is observed in *Glp1r*^{-/-} mice. **A:** The mRNA expression of inflammatory genes was evaluated in inguinal lymph nodes of WT and *Glp1r*^{-/-} mice 14 days after NTS induction. The fold increase compared with the mean mRNA expression in healthy WT and *Glp1r*^{-/-}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

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 (Figure 1, D, F, and H, and Supplemental Table S1). No difference was observed in the infiltration of CD8⁺ T cells throughout the observation period (Figure 1E, and Supplemental Table S1). Fourteen days after induction of NTS, a significant increase was observed in renal infiltration of polymorphonuclear granulocytes in *Glp1r*^{-/-} mice compared with WT mice. This difference was lost by day 21 after disease induction (Figure 1, G and I, and 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 Figure S2).

The Expression of Inflammatory Genes Is Increased in Lymph Nodes and Spleens of *Glp1r*^{-/-} Mice in NTS

Cytokine expression profiling by real-time quantitative PCR 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 with WT controls. Additionally, the expression of TH17 marker *Ror γ t* and TH1 and TH2 master regulator *Tbet* and *Gata3* was up-regulated in the lymph nodes of *Glp1r*^{-/-} mice (Figure 2A and Supplemental Table S3). The same genes were also evaluated in the spleens of those mice. In detail, the expression of Ifn- γ , *Tbet*, *Ror γ t*, *Gata3*, and *Foxp3* was

up-regulated in the spleens of *Glp1r*^{-/-} mice (Figure 2B and Supplemental Table S3). In further experiments, splenocytes isolated from WT and *Glp1r*^{-/-} mice 14 days after NTS induction were cultured and stimulated with rabbit IgG or lipopolysaccharides coated on the plates. Both stimulation methods significantly increased proliferation of splenocytes isolated from *Glp1r*^{-/-} mice as compared with WT mice (Figure 2C and Supplemental Table S3). Of note, CD4⁺CD25⁺FoxP3⁺ regulatory T cell (Tregs) evaluated by flow cytometry in lymph nodes did not differ between the two groups either in absolute or in relative numbers (Supplemental Figure S3, A and B, and 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 Treg markers. Only the TH17 marker *Ror γ t* did not differ between the two groups (Supplemental Figure S4 and Supplemental Table S5). No difference was detected in cytokine mRNA transcription levels in the kidneys of healthy *Glp1r*^{-/-} and WT mice (data not shown).

Glp1r Agonism by Liraglutide Protects Mice from NTS

Because Glp1r signaling has been implicated in having immunomodulatory effects, which were also shown in

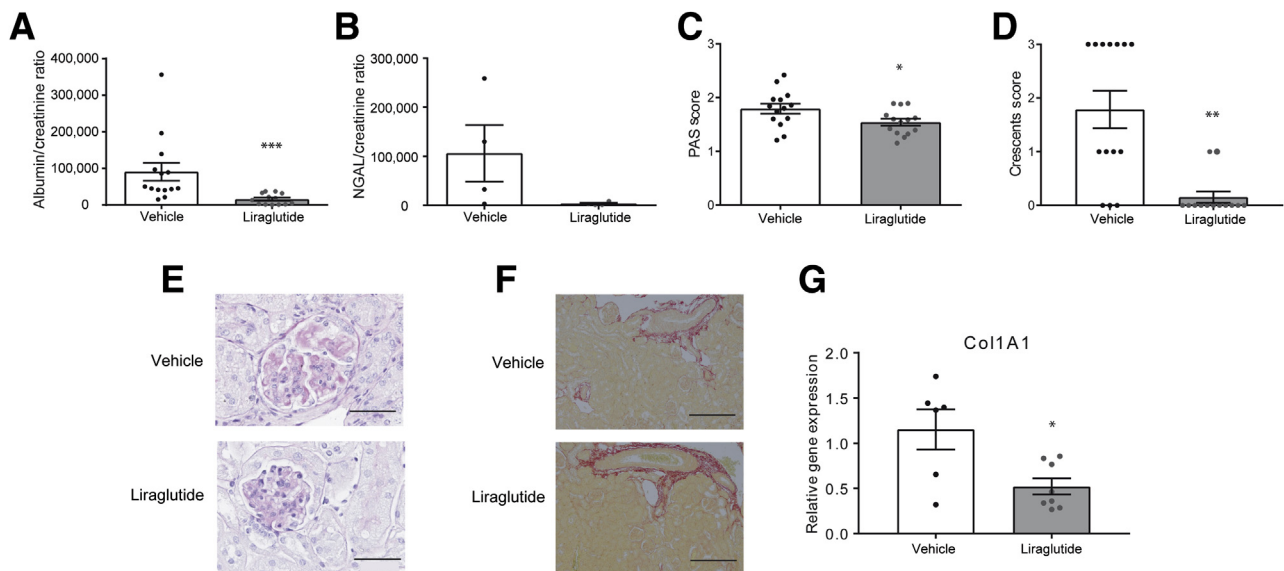


Figure 3 Liraglutide protects mice from NTS. WT mice were subjected to NTS for 14 days and treated with vehicle or liraglutide from the day of immunization. **A:** Urinary albumin/creatinine ratios ($\mu\text{g}/\text{mg}$) were evaluated on day 14 after NTS induction. **B:** At the same time point, urinary NGAL/creatinine ratios (pg/mg) were evaluated. **C** and **D:** 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. **F:** Representative pictures for Picrosirius Red stain of the kidneys. **G:** The mRNA expression of the *Col1A1* gene was evaluated in the kidneys of the mice. The fold increase compared with the mean mRNA expression in vehicle-treated mice subjected to NTS is provided. $n = 13$ liraglutide-treated mice (**A**, **C**, and **D**); $n = 14$ vehicle-treated mice (**A**, **C**, and **D**); $n = 4$ mice per group (**B**); $n = 6$ vehicle-treated mice (**G**); $n = 8$ liraglutide-treated mice (**G**). * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ versus vehicle. Scale bars: 50 μm (**E**); 200 μm (**F**). Original magnification: $\times 400$ (**E**); $\times 100$ (**F**).

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 with vehicle-treated mice (Figure 3, A and B, and 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 (Figure 3, C–E, and Supplemental Table S6). Further histomorphologic evaluation revealed significantly decreased glomerular and tubular changes in liraglutide-treated mice compared with vehicle-treated controls (Supplemental Figure S5, and Supplemental Table S7). Only marginal fibrotic changes were observed in kidneys subjected to 14 days of NTS (Figure 3F and 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 with vehicle-treated controls (Figure 3G and 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 with vehicle-treated controls (Figure 4, B–E, and Supplemental Table S8). A trend was detected toward fewer infiltrating polymorphonuclear granulocytes in the liraglutide group (Figure 4A and 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 with vehicle-treated controls (Figure 4F and Supplemental Table S8). By contrast, no difference was noted in the expression of the TH17 marker *Roryt* and the TH2 marker *Gata3*. Of note, proliferating cells in glomeruli as well as within the periglomerular region

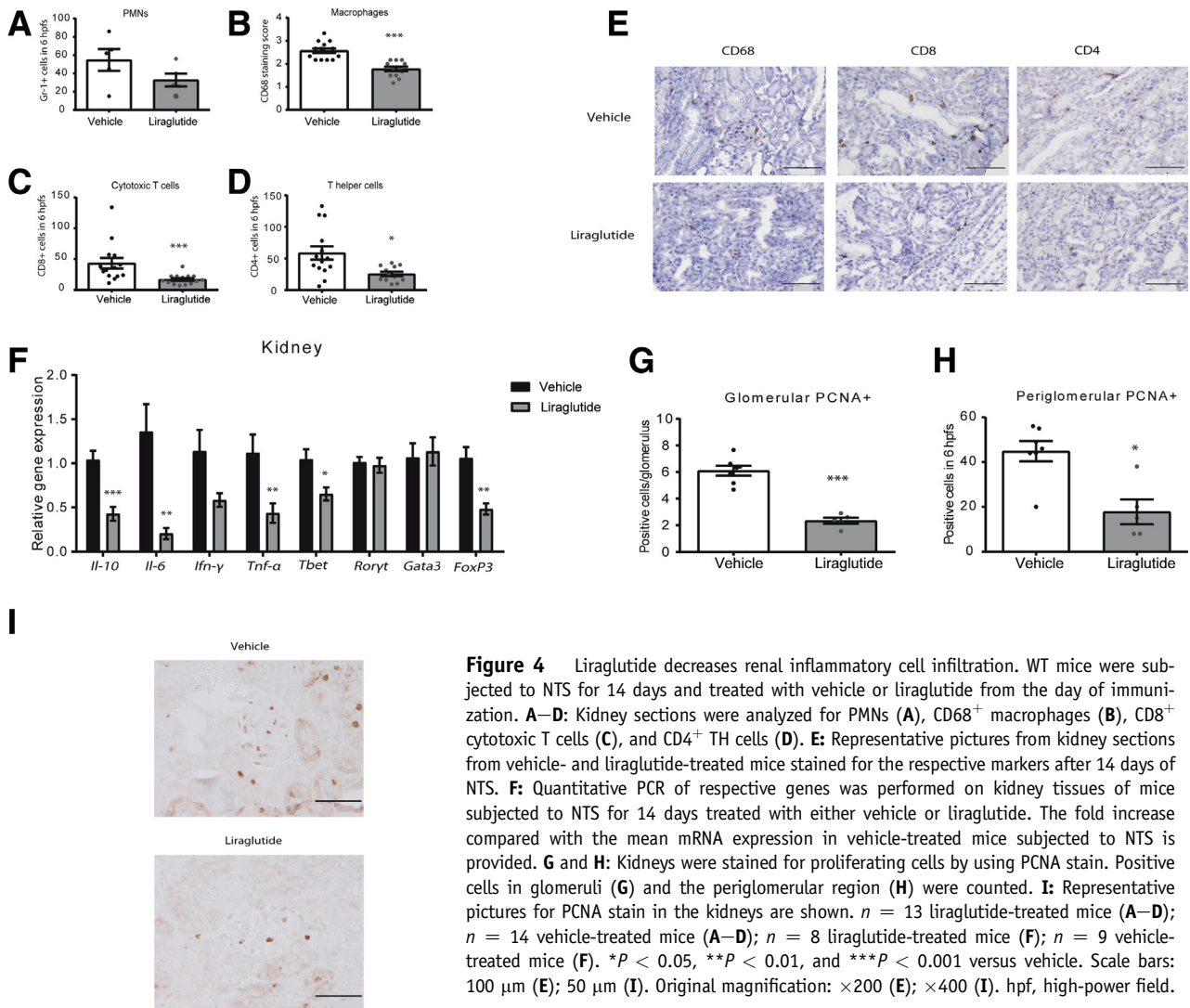


Figure 4 Liraglutide decreases renal inflammatory cell infiltration. WT mice were subjected to NTS for 14 days and treated with vehicle or liraglutide from the day of immunization. **A–D**: Kidney sections were analyzed for PMNs (**A**), CD68⁺ macrophages (**B**), CD8⁺ cytotoxic T cells (**C**), and 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. **F**: Quantitative PCR of respective genes was performed on kidney tissues of mice subjected to NTS for 14 days treated with either vehicle or liraglutide. The fold increase compared with the mean mRNA expression in vehicle-treated mice subjected to NTS is provided. **G** and **H**: 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. *n* = 13 liraglutide-treated mice (**A–D**); *n* = 14 vehicle-treated mice (**A–D**); *n* = 8 liraglutide-treated mice (**F**); *n* = 9 vehicle-treated mice (**F**). **P* < 0.05, ***P* < 0.01, and ****P* < 0.001 versus vehicle. Scale bars: 100 μm (**E**); 50 μm (**I**). Original magnification: ×200 (**E**); ×400 (**I**). hpf, high-power field.

detected by PCNA stain were significantly decreased in kidneys of liraglutide-treated mice compared with controls (Figure 4, G–I, and 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 Figure S2).

Liraglutide Acts via the Glp1r to Improve NTS

To prove whether the immunosuppressive effects of liraglutide in NTS are signaled 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 (Figure 5A and Supplemental Table S9) nor renal immune cell infiltration (Figure 5, B–E, and 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 with vehicle (Figure 6 and Supplemental Table S10), whereas liraglutide did not influence proliferation of T cells isolated from *Glp1r*^{-/-} mice as compared with WT littermate controls (Figure 6 and 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 (Figure 6 and Supplemental Table S10). Only the amount of Il-6 was significantly lower in the supernatant of T cells treated with liraglutide (Figure 6 and Supplemental Table S10). Of note, T cells transcribed the *Glp1r* mRNA before and after stimulation (Supplemental Figure S6). Furthermore, T cells from C57BL/6J mice were polarized into TH1 and TH17 cells *in vitro* (Supplemental Figure S7, B and C, and Supplemental Table S11), and proliferation was evaluated in the presence or absence of liraglutide. Liraglutide significantly decreased proliferation of TH1 and TH17 polarized cells (Figure 6 and Supplemental Table S10). *Glp1r* mRNA was detected in both TH1 and TH17 polarized cells (Supplemental Figure S7A and Supplemental Table S11).

Liraglutide Influences Glucose Metabolism in T Cells

T cells isolated from the 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

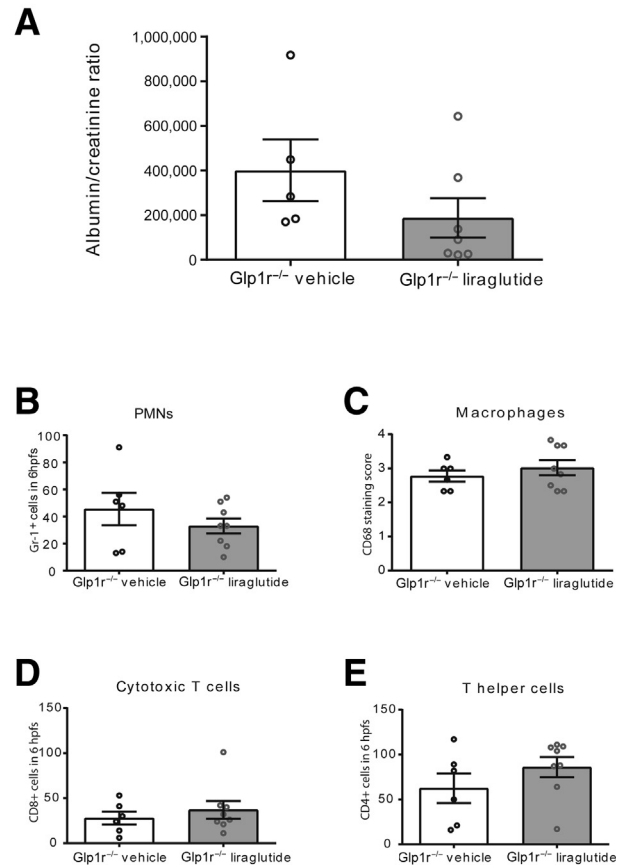


Figure 5 Liraglutide treatment has no effect on *Glp1r*^{-/-} mice subjected to NTS. *Glp1r*^{-/-} mice were subjected to NTS for 14 days and treated with vehicle or liraglutide from the day of immunization. **A:** Urinary albumin/creatinine ratios were evaluated on day 14 after NTS induction. **B–E:** Kidney sections were analyzed for PMNs (**B**), CD68⁺ macrophages (**C**), CD8⁺ cytotoxic T (**D**), and CD4⁺ TH (**E**) cells. *n* = 6 vehicle-treated mice; *n* = 8 liraglutide-treated mice. hpfs, high-power field.

treated with liraglutide (Figure 7 and 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 NTS, which closely resembles human forms of immunocomplex-mediated rapid progressive glomerulonephritis. These 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 because large randomized controlled trials using Glp1r analogs, namely liraglutide in the Liraglutide and Cardiovascular Outcomes in Type 2 Diabetes (LEADER) trial and semaglutide in the Semaglutide and Cardiovascular

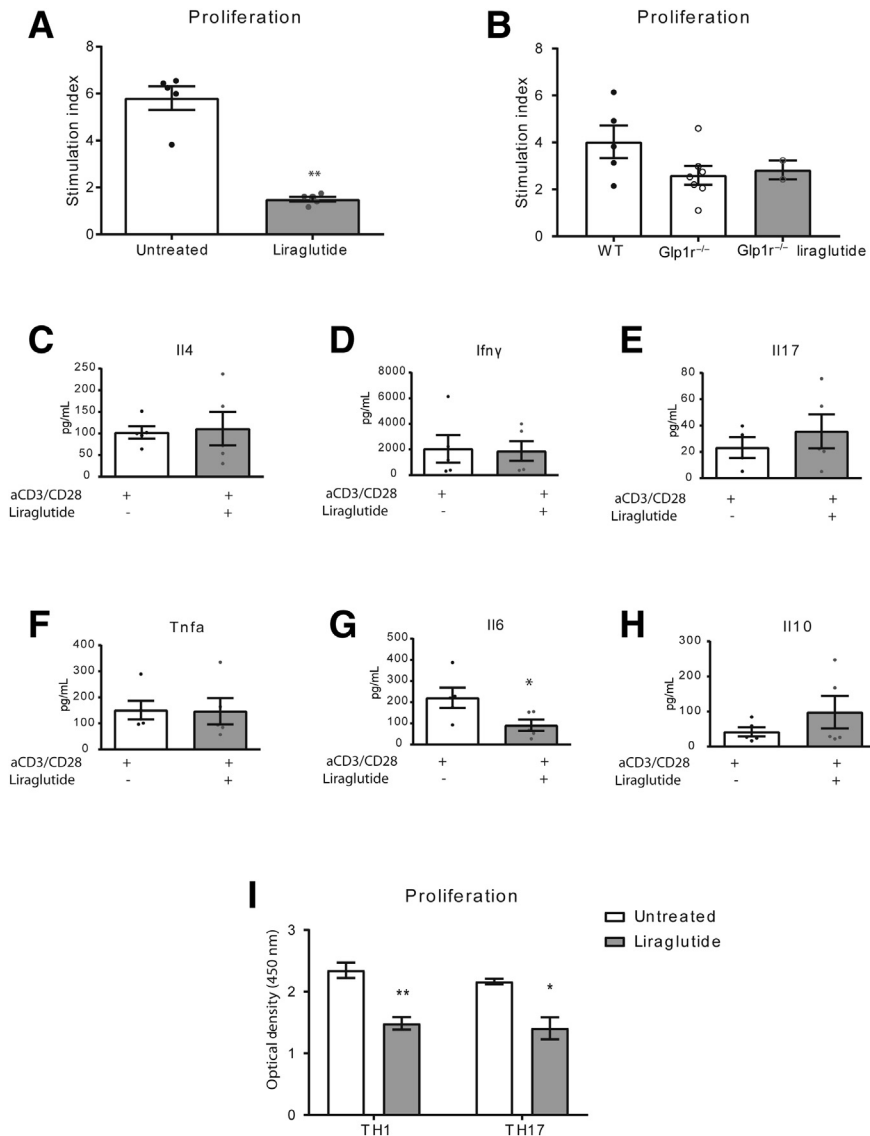


Figure 6 Liraglutide inhibits the proliferation of stimulated mouse T cells and decreases the production of IL-6. **A:** T cells were isolated from C57BL/6J mice, stimulated with aCD3/CD28, and treated with liraglutide in a concentration of 60 µg/mL or vehicle. **B:** The same procedure was followed for T cells isolated from *Glpr^{-/-}* mice treated with vehicle or liraglutide, which were compared with T cells isolated from WT littermates. **A and B:** Proliferation of cells was evaluated after 72 hours. The stimulation index is given as the ratio between the optical density 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, and proliferation was evaluated after 4 and 5 days, respectively. *n* = 5 mice per group (**A**); *n* = 2 liraglutide-treated mice (**B**); *n* = 5 littermates (**B**); *n* = 7 vehicle-treated mice (**B**); *n* = 3 mice per group (**I**). **P* < 0.05, ***P* < 0.01 versus untreated.

Outcomes in Patients with Type 2 Diabetes 3 (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 Because the NTS model used throughout the experiments is a model with rapid development of macroalbuminuria dependent on proinflammatory responses in normoglycemic conditions,²³ it allows us to focus on the antiproteinuric and anti-inflammatory capacity of Glp1r agonism.

The anti-inflammatory and immunomodulatory potential of Glp1r agonism was first mentioned in 2010 when Hadjiyanni et al⁹ provided evidence that peripheral lymphocytes of *Glpr^{-/-}* mice display a hyperproliferative response to mitogenic stimuli. In accordance with their observations, Glp1r agonism by liraglutide also decreased the proliferation of T cells *in vitro*. This was underlined by the authors' *in vivo* findings in a T-cell-dependent model of NTS.

Liraglutide treatment resulted in fewer 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 restimulation with rabbit IgG of splenocytes isolated from *Glpr^{-/-}* mice after 14 days of NTS resulted in an increased proliferative response as compared with controls. In line with these findings, TH1 cytokine transcripts were significantly increased in lymph nodes and spleens from *Glpr^{-/-}* mice 14 days after NTS induction. Interestingly, alterations were not detected in TH17-related transcripts such as their transcription factor Ror γ t, which first prompted the authors to hypothesize that signaling 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 signaled via the Glp1r, because liraglutide had no effect on

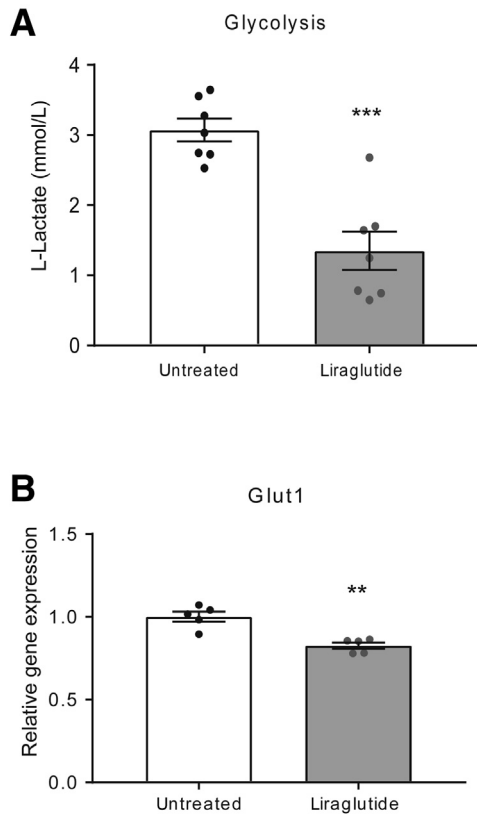


Figure 7 Liraglutide inhibits glycolysis in mouse T cells and down-regulates the expression of Glut-1. T cells were isolated from C57BL/6J mice, stimulated with aCD3/CD28, and treated with liraglutide in a concentration of 60 $\mu\text{g}/\text{mL}$ or vehicle. **A:** Glycolysis of cells was evaluated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

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} Because Tregs play a crucial role in limiting disease activity in NTS by acting, not only 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 with 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 because opposing effects have been observed for Glp1r agonism in other cell types such as pancreatic β -cells and cardiomyocytes.^{28,29}

A significantly aggravated NTS phenotype was not observed in *Glp1r*^{-/-} mice. This is somewhat counterintuitive, because 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 et al⁹ showed that although peripheral Glp1r deficient T cells are hyperproliferative, Glp1r-deficient thymocytes showed severely decreased proliferation.

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. This study's data support further investigation into the putative role(s) of Glp1r agonism in inflammatory, T-cell-mediated diseases. Besides this 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 paper might also partly explain the improved surrogate renal endpoints observed in large randomized control trials in type 2 diabetes patients.^{4,5,22} Preclinical 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 diabetic patients 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, so 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 mm Hg).³⁸ Lovshin et al³⁷ studied the hemodynamic 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. Because the mice were treated only for a limited time 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 hemodynamics might add to the protective effects of liraglutide in NTS.

Together, this study provided evidence that Glp1r agonism improves NTS by inhibiting the proliferation of T cells. The authors 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.

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Author Contributions

F.M.F. and M.T. performed experiments and analyzed data; A.H.K. and K.E. designed and performed experiments and analyzed data; M.K., K.A., I.A., C.S., A.A.M., B.F., M.P., T.N., and A.M. performed experiments; D.J.D. provided knock-out mice; T.R.P., P.E., A.R.R., and A.H. designed experiments and analyzed data; all authors wrote and approved the manuscript.

Supplemental Data

Supplemental material for this article can be found at <http://doi.org/10.1016/j.ajpath.2019.10.008>.

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