Dissertation

The Effect of Glucagon Like Peptide-1 Receptor Agonism in T Cell Mediated Diseases: A Thorough Study of the Case of Nephrotoxic Serum Nephritis

submitted by

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Statutory Declaration

I hereby declare that this thesis is my own original work and that I have fully acknowledged by name all of those individuals and organisations that have contributed to the research for this thesis. Due acknowledgement has been made in the text to all other material used. Throughout this thesis and in all related publications I followed the "Standards of Good Scientific Practice and Ombuds Committee at the Medical University of Graz".

Graz, November 2019

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Disclosures

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Abbreviations and Definitions

AhR - Aryl hydrocarbon receptor

AKD - Acute kidney disease

AKI - Acute kidney injury

ANCA-mediated GN - Antineutrophil cytoplasmic antibody-mediated glomerulonephritis

ANG2 - Angiotensin II

ANP - Atrial natriuretic peptide

Anti-GBM - Anti glomerular basement membrane

APCs - Antigen presenting cells

CKD - Chronic kidney disease

CLR - C-type lectin-like receptors

CVD - Cardiovascular Disease

DC - Dendritic cells

dH2O - Distilled H2O

DPP IV - Dipeptidyl peptidase IV

ECC - enteroendocrine cells

ESKD - End stage kidney disease

FSGS - Focal segmental glomerulosclerosis

GALT - Gut-associated lymphoid tissue

GC - Germinal centre

GFR - Glomerular filtration rate

GI tract - Gastrointestinal tract

GIP - Glucose-dependant insulinotropic polypeptide

GLP - Glucagon like peptide

GLP-1 - Glucagon like peptide-1

GLP-1R - Glucagon like peptide-1 receptor

GN - Glomerulonephritis

Hpf - High-power fields

Ig - Immunoglobulin

LC-MS/MS - Liquid chromatography tandem mass spectometry

LPS - lipopolysaccharides

MHC - Major histocompatibility complex

MNC - Mononuclear cells

NC1 - Non collagenous 1

NETs - Neutrophil extracellular traps

NFκB - Nuclear factor kappa-light-chain-enhancer of activated B cells

NGAL - Neutrophil gelatinase-associated lipocalin

NK - Natural killer cells

NLR - Nod-like receptors

NTS - Nephrotoxic serum nephritis

OD - Optical density

PAMPs - Pathogen-associated molecular patterns

PAS - Periodic-Acid-Schiff

PC1 - Prohormone convertase 1

PCNA - Proliferation cell nuclear antigen

PMNs - Polymorphonuclear granulocytes

PRRs - Pattern recognition receptors

RAS - Renin angiotensin system

RBC - Red blood cells

RLR - RIG-like receptors

RPGN - Rapid progressive glomerulonephritis

TCR - T cell receptor

TH - T helper cells

TLRs - Toll-like receptors

TMB - 3,3′,5,5′-Tetramethylbenzidine

Treg - Regulatory T cell

WT - Wild type

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Abstract in German

Hintergrund: Aktuelle randomisierte kontrollierte Studien zeigten, dass Glucagon-like Peptid 1 (GLP-1) Analoga, wie beispielsweise Liraglutid, die Nierenfunktion von Patienten mit Diabetes mellitus Typ 2 verbessern können. Der zugrunde liegende Mechanismus dieser Wirkungsweise ist noch nicht vollständig geklärt. Eine mögliche Erklärung beruht auf der antiinflammatorischen Wirkung des Agonismus des GLP-1- Rezeptors (Glp1r). Aus diesem Grund untersuchten wir die antiinflammatorische Kapazität des Glp1r in einem nicht diabetischen, T-Zellen abhängigen Mausmodell der nephrotoxischen Serumnephritis (NTS).

Methoden: NTS wurde in Glp1r Knockout Mäusen sowie in Littermate-Kontrollen induziert. Zusätzlich wurde die Behandlung mit Liraglutid in C57BL/6J Mäusen mit NTS evaluiert. Für *in vitro* Experimente wurden Maus T-Zellen in der Anwesenheit von Liraglutid oder Vehicle stimuliert.

Ergebnisse: Glp1r Knockout Mäuse zeigten nach Induktion der NTS eine Zunahme der Neutrophilen Granulozyten und T-Zellen in der Niere im Vergleich zu der Kontrollgruppe. Gleichzeitig war die Proliferation der Splenozyten, sowie die Transkription von TH1 Cytokinen in der Milz als auch in den Lymphknoten von Glp1r Knockout Mäusen mit NTS erhöht. Jedoch zeigten histologische Untersuchungen der Niere sowie Messungen der Albuminurie keine Unterschiede zwischen den beiden Gruppen. Im Gegensatz dazu, resultierte die Liraglutid Behandlung von C57BL/6J Mäusen mit NTS in einer signifikanten Verbesserung des renalen Outcomes. Neben einem Rückgang der Albuminurie, konnte zusätzlich eine Reduktion von infiltrierenden T-Zellen und Makrophagen in der Niere sowie eine Reduktion der Transkription von renalen TH1 Cytokinen beobachtet werden. Da Glp1r Knockout Mäuse durch die Behandlung mit Liraglutid nicht vor NTS geschützt werden konnten, sind die positiven Effekte von Liraglutid auf die Niere Glp1r abhängig. *In vitro* Experimente zeigten, dass stimulierte T-Zellen in der Anwesenheit von Liraglutid eine verminderte Proliferation und Il-6 Produktion aufweisen. Zusätzlich blockierte Liraglutid die Glykolyse und führt zu einer reduzierten Expression von *Glut1* mRNA in T-Zellen.

Schlussfolgerung: Unsere Daten unterstützen die Hypothese, dass der Agonismus von Glp1r durch seine antiinflammatorischen Eigenschaften, die Mäuse vor einer T-Zell abhängigen Glomerulonephritis schützt. Ein möglicher Mechanismus wäre, dass der Glp1r Agonismus die T-Zell Proliferation inhibiert.

Aus diesem Grund, kann Liraglutid, ein Glp1r Agonist, als neuer therapeutischer Ansatz in der Behandlung von T-Zell abhängigen Erkrankungen bedacht werden.

Abstract in English

Background: Recent randomized controlled trials have shown that glucagon like peptide (GLP)-1 analogues like liraglutide can improve kidney function in patients with type 2 diabetes mellitus. Though the mechanism of action of this effect is not yet clear, a possibility is the anti-inflammatory potential of GLP-1 receptor (Glp1r) agonism. Thus, we aimed to test the anti-inflammatory capacity of Glp1r agonism in a non-diabetic, T cell mediated murine model of nephrotoxic serum nephritis (NTS).

Methods: NTS was induced in Glp1r-/- mice and littermate controls. Furthermore, liraglutide treatment in NTS was tested in C57BL/6J mice. *In vitro*, murine T cells were stimulated in the presence of liraglutide or vehicle.

Results: Glp1r-/- mice displayed increased renal infiltration of neutrophils and T cells after induction of NTS as compared to littermate controls. In parallel, splenocyte proliferation and Th1 cytokine transcription were increased in spleen and lymph nodes of Glp1r-/- mice after NTS induction. Nevertheless, no difference in renal outcomes such as albuminuria and histological changes was detected between the two groups. In contrast, liraglutide treatment significantly improved the renal outcome of NTS in C57BL/6 mice as reflected by decreased albuminuria and histological changes. This was accompanied by a significant decrease in the renal infiltration of T cells and macrophages and a decrease in renal Th1 cytokine transcription. Renal beneficial effects of liraglutide were mediated via the Glp1r since liraglutide failed to protect Glp1r-/- mice from NTS. *In vitro*, T cells stimulated in the presence of liraglutide showed decreased proliferation and IL-6 production as compared to vehicle. In addition, liraglutide blocked glycolysis and decreased the expression of *Glut1* mRNA in T cells.

Conclusion: Our data support the hypothesis that Glp1r agonism has anti-inflammatory potential thereby protecting mice from a T cell dependent glomerulonephritis model, possibly by inhibiting T cell proliferation and interacting with their metabolic program. Thus, Glp1r

agonism by liraglutide might also be an attractive new therapeutic tool in the treatment of other T cell mediated diseases.

Introduction

The immune system

Immunity is the condition of protection against infectious disease. The word is derived from the Latin term ‘immunis’, which can be translated to ‘exempt’. The first recorded mention of immunity has been attributed to the Greek historian Thucydides, back in the fifth century BC. During the outbreak of a deadly infectious disease, at the time mentioned as the plague, in Athens, Thucydides wrote that only survivors of the disease are to treat patients, as they would not get sick again (2, 3). Nevertheless, it took more than 2000 years until this theory was brought into practice by medicine.

The organization of cells and molecules responsible for immunity comprise the immune system (4, 5). Main function of the immune system is to defend against infectious microbes, while even noninfectious foreign molecules may trigger an immune response. The immune system can be categorized into two functionally different compartments; the innate immune system and the adaptive immune system. Innate immunity consists the first line of defense against infections and can be found in all species, from simple multicellular organisms till complex vertebrates like ourselves (4). Innate immunity is the result of mechanisms encoded in our genome, inherited from many evolutionary generations back. It consists of cellular and biochemical defense mechanisms which are present even before infection and they repeat on the exact same manner regardless of how many times the same pathogen has already been encountered. These mechanisms react fast and mostly effectively, therefore they have been maintained through many years of evolution (6). Nevertheless, innate immunity lacks specificity and memory. To cover these gaps, adaptive immunity initiated to evolve, but not until the development of the first jawed vertebrate (fish), approximately 450 million years ago (6). Adaptive immune responses are characterized by specificity, diversity, memory and self-recognition (3). It has been named adaptive immunity as it develops as a response to infections by adapting to them. The innate and the adaptive immune system communicate and act jointly to accomplish the maintenance of the integrity of the organism.

Innate immunity comprises defense mechanisms that include the following: (i) physical and chemical barriers, (ii) cellular components, more specific phagocytic cells (neutrophils,

macrophages), dendritic cells (DC) and natural killer (NK) cells, (iii) soluble components (the complement system and other mediators of inflammation and (iv) various cytokines (2).

The main components of adaptive immunity are the lymphocytes (B and T cells), while specialized molecules required for their function play an important role as well (2). All the above will be thoroughly discussed in the following chapters.

Innate immunity

Surface cellular and chemical barriers

Surface barriers consist the front line of host defence. These physical barriers are formed by intact epithelial surfaces, which separate the internal tissues and organs from the external environment and its potential insults They can be mainly summarized to the skin and the various mucosal surfaces found in the gastrointestinal, respiratory and genitourinary tracks (2, 7). Besides its role as a mechanical barrier, surface epithelia also act by the production of chemical substances that have antimicrobial properties. Fatty acids produced by the skin, enzymes like lysozyme found in saliva, tears and sweat, antibacterial peptides and the production of mucous contribute in the host's protection (8). Two district peptide families worth of mentioning are the defensins and the cathelicins, produced mainly by epithelial cells of mucosal surfaces, neutrophils and various barrier epithelia. Both peptides act by direct toxicity to microbes and by the activation of cells involved in inflammatory responses (8). Finally, microbiological barriers can also be included in the list of surface barriers. A normal flora of non-pathogenic bacteria competes with potential pathogenic intruders over nutrients and attachment sites, while some have also been found to produce antibacterial substances (8).

Cell-associated pattern recognition receptors

As earlier mentioned, the innate immune system is lacking the specificity demonstrated by the adaptive immune system. Nevertheless, it does have the ability to distinguish self from nonself, in a different manner. That is achieved by the recognition and binding of unique structural features of microbes, named pathogen associated molecular patterns (PAMPs), by host germline-encoded pattern recognition receptors (PRRs) (9, 10). PRRs are expressed mainly by phagocytes, dendritic cells and endothelial cells. They are located on membranes or in the cytoplasm of the cells.

Once activated, PRRs initiate intracellular signal transduction pathways which lead to various cellular responses promoting inflammation and other mechanism to eliminate pathogens (2). Often PRRs can translocate by membrane trafficking to different compartments and set off the innate immunity there (11).

Several classes of PRRs have been named. The most common ones include the toll-like receptors (TLRs), the nod-like receptors (NLRs), the RIG-like receptors (RLRs) and the C-type lectin-like receptors (CLRs) (12). TLRs are located on membranes and recognize various microbial molecules, such as lipoproteins, glycolipids and nucleic acids. They play an important role in innate and adaptive immunity by resulting in the activation of several signal pathways and transcription factors. NLRs are expressed in the cells' cytoplasm and identify bacterial components. After binding them, they recruit other proteins to promote inflammation. One subfamily of the NLRs responds by activating the inflammasome, which produces the inflammatory cytokine IL-1. RLRs are cytosolic receptors that sense viral RNA by detecting double-stranded RNA, which is generated during viral replication. RLRs induce the production of anti-viral type I interferons (2, 12, 13).

Soluble components

A key component of the innate immunity is the complement system. It consists of serum and plasma proteins that interact with one another and with other molecules of the immune system and induce a series of inflammatory responses, opsonize microbes, and promote phagocytosis. The activation of the complement system can be achieved by three distinct pathways; the classical pathway, the alternative pathway and the lectin pathway. They have different ways of initiation and depend on different molecules for it but eventually result to generate the same effector molecules. All three activation methods conclude in recruitment and assembly of component proteins into protease complexes. In more detail, they lead to the production of C3b, which subsequently binds to microbes, promotes their phagocytosis and initiates the late step of complement activation. C3b forms the C5 convertase that cleaves C5 and produces the C5b fragment, that initiates the formation of the membrane attack complex, which causes lysis of the cells that activated the complement.

Other proteins that can be categorized to the soluble components of innate immunity are pentraxins, collectins and ficolins. They are characterized by the ability to recognise microbial structures and lay a role in their opsonization (2, 3, 8).

Cytokines, a group of soluble mediators of immunity, produced by many different cells of the immune system can be categorized both in the innate and the adaptive immunity. Being a quite broad and varied group of molecules, they act on different target cells and often have the role of messengers both within the immune system and between the immune system and other systems of the body (4). Cells sense the presence of cytokines by specific cytokine receptors. Cytokines may have autocrine, paracrine or endocrine actions.

Within the broad group of cytokines, an important group of chemotactic cytokines called chemokines can be distinguished. Chemokines consist a large family of structurally homologous secreted proteins whose low molecular weight ranges between 8 and 10 kD. Their main function is to stimulate leukocyte movement and regulate their transit from the blood to different tissues (2, 4). Leukocytes carry specific chemokine receptors on their cell surface that guide them to the chemokines in the tissue. Chemokines are classified into four subfamilies (C, CC, CXC and CX3C) based on their structural motifs near the N terminus (2, 14, 15).

Cellular components

Explicitly listing all the cell types involved in the innate immune system is not an easy task, as many cells are involved in both adaptive and innate immunity, participate in one and stimulate the other or are simply equipped with PRRs. For the shake of this general overview about the immune system, the main cell types that will be shortly described in this part are phagocytes, dendritic cells, natural killer cells and mast cells.

Phagocytes can be defined as a group of distinct cells with a main role in immunity to identify, ingest and destroy pathogens. They can internalize and kill microbes or respond to them by producing various cytokines. Phagocytes are also responsible for removing the body's own dead and dying cell (16). Macrophages and neutrophils are the most abundant cell populations within phagocytes. Macrophages are derived from blood-borne monocytes. They are equipped with receptors that recognize carbohydrates exposed only on cells of non-vertebrates, and their main function can be summed up in ingesting and killing microbes. Additionally, they secrete cytokines that can bind to signalling receptors of other cells (2, 4, 17). Neutrophils are considered to mediate the earliest phase of inflammatory reactions. They are recruited very fast to the site of infection but have a short life span. They are certainly the most potent microbicidal phagocyte (17).

Dendritic cells (DCs) consist the major type of antigen presenting cells (APCs) of the immune system. Their main task is the initiation of T cell responses and by that they link innate and adaptive immune responses. They achieve their task by behaving as APCs, when the PRRs on their surface get attached to PAMPs on the surface of microbes (4). They can also be activated by endogenous danger signals or by the increase of heat-shock proteins (4). When inactivated they quietly endocytose extracellular antigens. More recently, a striking ability of DCs to secrete factors that play a role in T cell polarization has been discovered (18).

Natural killer (NK) cells are lymphocytes able to directly induce death of malignant and virus-infected cells. Additionally, they are major producers of IFN-γ and other cytokines and chemokines. They recognize their target by Fc receptors that bind IgG or by killer-activating receptors and killer-inhibiting receptors, a system uniquely used by these cells (4, 19). Recently, NK cells have been proven to demonstrate characteristics of the adaptive immune system (19-21), such as the ability to 'educate' to accomplish functional competence while maturation. Additionally, they have demonstrated immunological memory, a characteristic typical for adaptive immunity. To add to this, they are known to have developed from a

common lymphoid progenitor cell like B and T lymphocytes and require γ-chain-dependant cytokines during development and homeostasis (21).

Finally, mast cells, found on the skin and the mucosal epithelium, have the ability to secrete pro-inflammatory cytokines and lipid mediators. They perform those actions with the use of high-affinity receptors for IgE. Together with basophils and eosinophils, they are supplied with cytoplasmic granules filled with inflammatory and anti-microbial mediator (2, 4).

Adaptive immunity

Humoral adaptive immunity

B cells are the mediators of humoral immune responses, as the only cell type capable of producing antibody molecules. They can be defined as a cell population expressing clonally diverse cell surface immunoglobulin (Ig) receptors that act to recognize specific antigen epitopes. B cells develop in the bone marrow. When mature, B cells leave the bone marrow, each expressing a surface Ig receptor. These mature naïve B cells can be found mainly in the secondary lymphoid organs and in the circulation. When they come in contact with a matching antigen for the first time, the activation phase is initiated. In this phase the antigen and the surface Ig receptor of the naïve B cell get attached and together with other stimuli (T helper cells), the extended proliferation of the naïve B cell is stimulated. This clonal expansion is followed by the differentiation into plasma cells or memory B cells. Plasma cells are the effector B cells which no longer acquire a surface Ig receptor but secrete the antibodies. They have a short life but can produce huge amounts of antibodies during this time. Memory B cells have a longer life span and still express the surface Ig receptors.

Humoral immunity can be divided into primary and secondary humoral immune responses. A primary immune response is initiated when an antigen is encountered for the first time by naïve B cells which get activated and eventually start producing specific for the antigen antibodies. During a secondary humoral response, memory B cells encounter an antigen that has already been encountered and they start to proliferate and prodice higher amounts of specific antibodies in a shorter time period.

The function of antibodies, the actual components of the humoral immune response, can be summarized into neutralization of antigens, opsonization and phagocytosis, antibody-dependent cellular cytotoxicity and activation of the complement system (22-24).

Cell-mediated adaptive immunity

Cellular-mediated adaptive immunity is mediated by T lymphocytes or T cells. T cells, like B cells, originate from hematopoietic stem cells located in the bone marrow. In the case of T cells, their precursors leave the bone marrow and circulate to the thymus, where they complete their maturation.

During their maturation, T cells start to express a T cell receptor (TCR) on their surface. TCRs are complexes of polypeptides including CD3, a molecule present in all mature T cells and therefore a useful marker for this cell population. The TCR has similar properties with the surface Ig receptor of B cells, with the important distinguishing characteristic that a TCR is not able to recognize an antigen alone but only when it is bound to a Major Histocompatibility Complex (MHC) (3). Beside the expression of the TCR, at the first step of maturation precursors of T cells also start to express CD4 and CD8 molecules on their surface. These maturation step takes place in the thymic cortex, and these cells are characterised as double positive thymocytes. In the next step, double positive cells undergo further selection processes and they mature into CD4+ or CD8+ T cells, called single positive thymocytes. The mature single positive thymocytes move to the thymic medulla and can then abandon the thymus to populate the peripheral lymphoid tissues (2).

Based on whether they express CD4 or CD8 molecules on their surface, T cells can be divided into two basic populations, T helper cells and cytotoxic T cells, respectively. The main function of cytotoxic T cells is to kill infected cells and eliminate the reservoirs of infection. They can recognise antigens bond to MHC class II and they do not produce many cytokines but have cytotoxic abilities. T helper cells recognise and interact with MHC class II. Their main function is to excrete cytokines which are crucial for the activation of B cells, cytotoxic T cells macrophages and other cell types. The most important cytokine expressed by T helper cells is IL-2, a growth, survival and differentiation factor for T lymphocytes (3).

The activation of naïve T lymphocytes takes place in secondary lymphoid organs and resembles in many ways the activation of B cells described earlier. For the initiation of the activation process, the recognition of an antigen presented by a dendritic cell is required, together with other activating stimuli, as costimulators provided by molecules on the APCs. These result in the secretion of cytokines from the T cells, proliferation of the clone specific for this antigen and differentiation of the naïve cells into effector or memory cells. Effector T cells are responsible for actions that eventually eliminate the microbes and for inflammation and tissue

damage. Memory T cells are long-lived cells with an enhanced ability to react against the antigen (2).

T cell subpopulations

An important and much discussed chapter in T cell biology is the classification of CD4+ T helper cells into district subsets. The first time the idea of distinguishing Th clones based on cytokine profiles was proposed was over 30 years ago (25), when Th1 and Th2 cells were identified. Since then, many new T cell subsets have been added to the list. Nevertheless, there is still an ongoing debate regarding the distinct subsets. That is partly due to the discovered plasticity of the T cell subsets, but also as the subset-signature cytokines are not as stable and exclusive as initially considered (26). For example, it has been shown that subsets Th2, Th17 and Tregs can gain the ability to produce IFN-γ, a characteristic Th1 property. New ways of identifying Th subset are being discussed, as the use of chemokine receptor profiles (27), which are also critical for the T helper cells to execute their effector functions. In the next part of this small overview of the immune system, T cell subsets Th1, Th2, Th17, Th22, Th9, Tregs and Tfh will be briefly described.

Th1 cells, one of the two first subsets to be described, are responsible for enhancing the cell-mediated immunity and critical for the protection of the host against intracellular pathogens. Additionally, they are involved in the clearance of cancer cells. They function by secreting IFN-γ, which is the characteristic cytokine of this subset, as well as TNFα and IL-2. IL-12 induces the expression of T-bet and the differentiation into the Th1 subset. The chemokine receptor found on these cells is CXCR3 (27, 28).

Th2 cells' main function is the defence of the host against multicellular parasites (such as helminths), while they are involved in allergies and atopic illnesses. They act in epithelial tissues as the intestinal track and the lungs. Characteristic cytokines produced by these cells are IL-4, IL-5, IL-13, IL-9 and IL-10. The signature cytokine, IL-4, is also produced by mast cells, basophils, eosinophils and γδ T cells. Committed Th2 effectors express the transcription factor Gata3, while the factor inducing this lineage is IL-4. The major chemokine receptor expressed by Th2 is CCR4.

Th17 cells were first described in 2005. They act by secreting IL17A and IL17F, together with a list of other cytokines. Th17 cells defend the host against bacterial and fungal pathogens and can recruit neutrophils to the site of infection. Their differentiation is a result of RORγt expression induced by IL-6, TGF-β and IL-23. CCR6 is considered the characteristic chemokine receptor found on these cells (27, 29, 30).

The next T helper cell subset to be described, was discovered on the skin about 10 year ago (31-33). There is still an ongoing debate over whether Th22 cells can be considered a district T cell subset. The reason for that is that their signature cytokine, IL-22, is also secreted by Th17 cells and their master transcription factors, Tbet and Aryl hydrocarbon receptor (AhR) are not confined to Th22 lineage. The differentiation of these cells is induced by IL-6 and TNFα and they express the chemokine receptors CCR10, CCR4 and CCR6. Th22 cells act in mucosal immunity and they prevent microbial translocation across epithelial surfaces. Finally, they promote the repair of damaged epithelial barriers and enhance immune responses against pathogens (27, 28).

Tregs acting for the negative regulation of immune responses and work on maintaining tolerance against self-antigens and limiting pathogen-induced inflammation to protect extensive damage to the tissue of the host. They secrete anti-inflammatory cytokines, such as IL-10. The differentiation of Tregs is induced by high TGF-β and defined by the expression of the transcription facto FoxP3.

More recently described T helper cell subsets are Th9 and Tfh cells. The characteristic feature of Th9 is the secretion of IL-9 in absence of IL-4 production. No master transcription factor is yet shown to define Th9 and their functions are closely related to type 2 immune responses, as allergic inflammation and protection against helminths (27). Tfh cells provide help to B cells. These cells are required for high-affinity antibodies in the germinal center and for long term memory responses. Tfh cells excrete IL-21 and IL-4 and chemokine receptor CXCR5 is critical to their function and allows T cells to migrate to the follicles (27, 28).

Renal Anatomy and Physiology

The kidneys are highly complex organs which serve the body by removing the toxic waste products of metabolism from the blood. They regulate the fluid, electrolyte and acid-base balances in the body and produce several hormones necessary for a normal body function, as vitamin D, erythropoietin and rennin (34-36).

Each kidney contains over one million nephrons, namely, the basic, functionally and anatomically independent, unit of the organ. A nephron is composed of one glomerulus and one double hairpin-shaped tubule. The tubule of the nephron consists of the proximal tubule, a continuation of Bowman's capsule, the Loop of Henle, the distal tubule and finally the collecting duct. The loop of Henle and the collecting duct are mainly located in the medulla while the rest of the tubule in the kidney cortex (37).

The glomerulus consists the filtering unit of the kidney and it functions as a size-selective filter. The glomerulus is practically a bundle of capillaries that are located between two resistance vessels (38). All glomeruli are located in the kidney cortex and they are surrounded by the Bowman's capsule. The filtration barriers of the glomeruli are constructed by three district layers. The proximal component layer consists of endothelial cells and is a highly specialized fenestrated endothelium. The individual fenestrae can reach up to 70 to 100 nm in diameter. Second comes the glomerular basement membrane (GBM), a specialized extracellular matrix that is composed of four major macromolecules: type IV collagen, laminins, nidogen and proteoglycans. Additionally, the GBM contains specific basement membrane protein isoforms, some of which are crucial for the glomerular development, morphology and function. It has been widely supported that the GBM plays the most defining role in the permselectivity and the establishment and maintenance of the glomerular filtration barrier (39, 40). The third layer of the glomerular filter is composed by visceral epithelial cells, the podocytes. Characterized by their interdigitated foot processes with filtration slits in between, they are bridged by the slit diaphragm, which establishes the selective permeability of the glomerular filtration barrier and helps sustain the integrity of the free-standing capillary loops (41-43). Of note, different glomeruli situated in different regions of the kidney may significantly vary in their size and filtration rate (38).

Renal Disease

Renal disease is a general term that can include a number of variable conditions that affect the kidney structure and function. Based mainly on the duration of the condition, kidney diseases can be characterized as acute or chronic (44, 45).

Acute kidney injury (AKI) can be defined as an acute reduction in glomerular filtration rate (GFR) occurring over hours or days, with a subsequent rise in serum creatinine concentrations (46). AKI includes acute renal failure but it is not limited to it, and it consists one of many acute kidney diseases and disorders (AKD) in which damage in the kidney tissue occurs within one week (45). Over the last years, there have been various studies and literature developments regarding the link between chronic kidney disease (CKD) and AKI. CKD is a long-known risk factor for AKI, while there is growing evidence that AKI is often followed by the development of CKD (45-47). In Table 1 the definitions of AKI, CKD and AKD are compared (44, 48).

	Duration	**Functional criteria**	**Structural criteria**
AKI	Within 2-7 days	Increase in serum creatinine by 50% within 7 days OR GFR<60 ml/min per 1.73m^2 OR Oliguria	None required
CKD	≥3 months	GFR<60 ml/min per 1.73m^2	Marker of kidney damage (albuminuria)
AKD	≤ 3 months	AKI OR GFR<60 ml/min per 1.73m^2 OR Decrease in GFR by >35% OR Increase in serum creatinine by >50%	Marker of kidney damage (albuminuria, hematuria, pyuria)

Table 1: Definitions of AKI, CKD and AKD.

In the next chapters a more detailed study of CKD and glomerular diseases is given.

Chronic Kidney Disease

Chronic kidney disease (CKD) is a worldwide public health problem with significant impact on patient's quality of life and life expectancy but also on the health care system (49). CKD is defined as a condition in which GFR is lower than 60 mL/min per 1.73 m^2 or one of the markers of kidney damage are present, or both, for at least 3 months. The markers of kidney damage consist of albuminuria (albumin: creatinine ratio ≥ 30 mg/g), urinary sediment abnormality, electrolyte or other abnormality due to tubular disorder, histological abnormalities, structural abnormalities and history of kidney transplantation (50).

CKD is classified into five stages based on GFR. In stage 1 GFR exceeds 90 mL/min per 1.73 m^2. In stage 2 GFR is between 60-89 mL/min per 1.73 m^2, in stage 3 30-59 mL/min per 1.73 m^2, in stage 4 15-29 mL/min per 1.73 m^2 and in stage 5 lower than 15 mL/min per 1.73 m^2 (50, 51). At stage 5 a patient has reached an end stage kidney disease (ESKD). From this point on kidney function is no longer able to sustain life over the long term (50).

Diabetes Mellitus and hypertension consist the leading risk factors for CKD (52). 30-50% of all CKD cases consist of diabetes patients, while more than a quarter was estimated to have been diagnosed with hypertension in 2000 (50). Other risk factors are obesity, smoking and the consumption of nephrotoxins as recreational drugs and analgesic drugs (53-55). Low birth weight has been also associated with development of CKD during childhood but also during adult life (56). As renal function decreases with age, the elderly population is more prone to develop CKD. Additionally, race, ethnicity and gender are also to be taken into consideration when studying the risk factors for CKD (57).

The prevalence of CKD varies globally, but also across Europe and even within a country (58). These differences may partly be explained by the prevalence of the risk factors for CKD, as well as by the variation in health policies addressing disease prevention and treatment (59). A systematic review published in 2016 reported that a global CKD prevalence of 5 stages was 13.4% and stages 3-5 was 10.6% (60). In this review, CKD was proven to be more prevalent in women than in men. Additionally, developed areas as Europe, USA and Canada had higher rates of CKD prevalence compared to areas with growing economy as sub Saharan Africa or India.

Glomerulonephritis

Glomerulonephritis (GN) is a subclass of glomerular diseases characterized by increased glomerular cellularity. It may be caused by the proliferation of indigenous cells or by leukocyte infiltration (61). There is a variety of patterns that reflect the increased glomerular cellularity, as mesangial proliferative GN, diffuse endocapillary GN, membranoproliferative GN, crescentic GN or necrotizing and they all may lead to sclerosing GN. Based on those patterns the severity of the disease can also be evaluated (62).

The standardized classification of GN is based on aetiology. Since 2015 GN is classified into five basic groups (61): immune complex-mediated GN, antineutrophil cytoplasmic antibody (ANCA)-mediated GN, anti-glomerular basement membrane (aGBM) GN, monoclonal immunoglobulin-mediated GN and C3 glomerulopathy. Within each of these five groups there are specific distinct diseases (62). ANCA-mediated GN is typically negative for Ig or complement and it is characterized by the finding of ANCA against myeloperoxidase or proteinase 3 in the serum of the patients (63, 64). According to the International Chapel Hill Consensus from 2012, ANCA-mediated GN can be classified as microscopic polyangiitis, granulomatosis with polyangiitis or eosinophilic granulomatosis with polyangiitis, based on clinicopathologic findings (65). Monoclonal Ig-mediated GN is characterized by the finding of monoclonal Ig deposits in the glomeruli and/or along tubular basement membranes inspected by IF microscopy. In most patients the disease is associated with an underlying paraproteinemia (66). C3 glomerulopathy is defined by bright C3 staining and minimal or absent Ig staining on IF microscopy (62). Anti-GBM GN and immune complex-mediated GN will be discussed more thoroughly in the next chapters.

Anti-Glomerular Basement Membrane Disease

Anti-GBM disease, also known as Goodpasture disease, is a small vessel vasculitis that may affect the glomerular and pulmonary capillaries. It is a rare and life-threatening auto-immune disease (67). Anti-GBM disease is a common cause for rapid progressive glomerulonephritis (RPGN), consisting 10 to 15 % of cases of RPGN (68). The autoantibodies responsible for the disease are directed against the non-collagenous 1 (NC1) domain of the alpha 3 chain of type IV collagen in the basement membrane (37, 69). Anti-GBM disease is estimated to have a very low frequency of less than 1 per million population per year (67), while a recent study in Ireland revealed a higher number reaching 1.64 per million population per year (70). Current treatments of the disease include plasma exchange to achieve quick removal of the pathogenic antibody together with immunosuppression to inhibit further production of the autoantibodies and to ameliorate end-organ inflammation (67, 68). Anti-GBM disease is much used as a model disorder for the study of mechanisms mediating autoimmunity.

Immune Complex-Mediated Glomerulonephritis

Immune complex-mediated GN consists the most heterogeneous group of the above described standardized classification of GN. These diseases are characterized by the deposition of polyclonal Ig that can be monitored by immunofluorescence microscopy. In many case the complement is simultaneously deposited along with the Ig (66). The group of immune complex-mediated GNs includes many well-defined diseases as IgA nephropathy, lupus nephritis, infection-related GN and fibrillary GN (71-74). Cases of GN resulting from infections or auto-immune diseases may as well be included in immune-complex GN (75-77). A proper diagnosis for the type of immune-complex GN requires a thorough study of the type, relative intensity and pattern of distribution of the various immune reactants (66). Even the characterization of an immune complex GN and one of the other groups of GN used to be troublesome in the earlier times. Of great historical interest stands the article from 1978 by Loughlin et al. in which the first cases of children with idiopathic nephritis due to immune complexes and with absence of the a-GBM antibody are described and the relevant doubts over previous reports of Goodpasture disease are stated (78). In every immune-complex GN typical patterns of glomerular injury can be found. Those patterns consist of mesangial proliferative,

endocapillary proliferative, exudative membranoproliferative, necrotizing, crescentic, sclerosis or even different combinations of the above (61, 79).

The murine model of Nephrotoxic Serum Nephritis

Nephrotoxic serum nephritis (NTS) is a murine model of immune-complex GN, which is characterised by pathology that closely resembles a membranoproliferative GN. It is a model which has been extensively studied in the recent years, leading to many new findings in the field of T cell mediated autoimmunity, as well as in the field of glomerulonephritis. Mice are subjected to NTS by the injection of sheep or rabbit anti-mouse glomerular basement membrane serum. To achieve a more robust phenotype, an immunization against sheep or rabbit IgG is often perform prior to the aGBM serum injection.

Mice subjected to NTS develop proteinuria from the early timepoint of 7 days after disease induction. Additionally, severe proliferative and inflammatory glomerular changes are noted. Those include the formation of crescents, development of glomerulosclerosis and infiltration of leukocytes in the kidney.

Th1 and Th17 cells have been shown to play an important role in the pathogenesis of the disease, mainly by their function to recruit macrophages and neutrophils to the kidney (80-86). More in detail, the pathogenesis of the disease depends on both the innate and the adaptive immune system and the final phenotype is an outcome from the balance between pro-inflammatory and anti-inflammatory cells and their actions (37). Firstly, gamma delta (γδ) T cells activated by IL-23 migrate to the kidney and recruit neutrophils by the production of IL-17A. The neutrophils immediately start to act by causing glomerular damage. Mature dendritic cells, expressing CX3CR1 and CCR2, initially attract Th17 cells which express CCR6 and which further recruit neutrophils in the kidney. In a bit later timepoint DCs recruit also Th1 cells carrying CXCR3 (37, 87, 88). Th1 cells secrete IFNγ and activate macrophages. The stimulated macrophages then produce injurious mediators as TNF and nitric oxide. Interestingly, macrophages may as well play differential roles in the disease pathogenesis: as M1 pro-inflammatory cells that contribute into renal injury or as M2 anti-inflammatory cells, demonstrating a reparative phenotype (89-92).

As mentioned above, anti-inflammatory cells and their actions contribute as well in the development of NTS. At earlier stages, immature DCs inhibit the progression of the disease by recruiting invariant natural killer T (iNKT) cells, which produce IL-10 and IL-4 (37). Finally, at a later timepoint, regulatory T cells which migrate to the inflamed kidney inhibit the disease development, while the CCR7 expression on the Tregs is required for this inhibition to occur (88, 93-96).

The secondary lymphoid organs have been thoroughly studied regarding their role in the progression of NTS. Draining lymph nodes have been proven to play a crucial role in the pathogenesis of the disease. T cells, Tregs and DCs expressing CCR7 bind to their ligands CCL-19 and CCL-21 and migrate to the T cell zone of the lymph node during disease progression (88, 97). Additionally, mast cells positioned in the draining lymph node have been proven to regulate the disease activity by inhibiting its progression (98, 99). In contrast to the secondary lymph nodes, the spleen has been shown to play no direct role in the pathogenesis of NTS. Although Treg numbers increase also in the spleen of the mice subjected to NTS and the size and weight of the organ rises significantly, splenectomized mice demonstrated a similar disease phenotype with sham-operated mice (88, 100).

Glucagon like peptide-1 and glucagon like peptide-1 receptor

The gastrointestinal tract and incretin hormones

The gastrointestinal (GI) tract is composed by many different organs that work together as a system with the main function to digest nutrients. This is achieved by complex processes that include the secretion of digestive enzymes and the absorption of nutrients. Anatomically the GI tract can be divided to the upper and the lower GI tract. The upper GI tract includes the mouth, esophagus, stomach duodenum, jejunum and ileum, while the lower consists of the colon, rectum and anus. Epithelial barriers present in the GI system protect the organism from dangerous antigens and toxic and infectious agents while the enteric nervous system is a whole nerve network within the gut. The GI tract is also characterized by immune functions which are performed by the complex innate and adaptive mucosal immune system embedded in it, also known as the gut-associated lymphoid tissue (GALT) (101).

What is of greatest relevance to this dissertation though, are the specialized endoderm-derived epithelial cell, distributed broadly through the GI tract, named enteroendocrine cells (ECCs). Those cells secrete various hormones, making the GI tract one of the largest endocrine organs in the human body. The secretion of these hormones is triggered by nutrients, other hormones and neural signals (102). Table 2 summarizes the most important hormones secreted by the GI tract (102).

	Hormone	Secretion stimulus	Major Functions
Stomach	Gastrin	Food ingestion (mainly protein)	Stimulates gastric acid secretion and epithelial cell proliferation; iron homeostasis
	Ghrelin	Fasting, before meal	Increases appetite; stimulates gastric emptying, acid secretion and migrating motor complexes; protects from gastric stress; increases growth hormone release
	Leptin	Food ingestion, Vagal nerve stimulation, CKK, secretin	Reduces appetite; stimulated CKK and GLP-1 secretion; modulates nutrient absorption
Small or large intestine	Somatostatin	Intraluminal nutrients and acid, adrenergic stimulation, CKK, gastrin	Inhibits gastric acid and pancreatic secretion
	Secretin	Acid chyme, digested fat and protein	Stimulates pancreatic alkaline secretion; inhibits gastric motility and acid secretion; body fluid homeostasis/osmoregulation
	GIP	Food ingestion (mainly carbohydrates and fat)	Incretin hormone action, enhances glucose-stimulated insulin secretion
	CCK	Food ingestion (mainly protein and fat)	Reduces appetite; inhibits gastric emptying and acid secretion; stimulates gall bladder contraction and pancreatic digestive enzyme secretion
	Motilin	Interdigestive fasting period	Induces GI motor activity
	PYY	Food ingestion (mainly fat)	Reduces food intake; inhibits gastric emptying and secretions; suppresses intestinal motility and electrolyte secretion; inhibits pancreatic secretion
	GLP-1	Food ingestion (mainly carbohydrates and fat), bile acids on TGR5	Incretin effect; reduces food intake; inhibits gastric emptying, GI secretion, glucagon secretion
	GLP-2	Co-secreted with GLP-1	Stimulates cell growth in the gut mucosa amd protects against apoptosis; inhibits gastric emptying and acid secretion; enhances nutrient absorption and blood flow
	OXM	Food ingestion (mainly fat)	Reduces food intake; inhibits gastric acid and pancreatic secretion; enhances glucose-stimulated insulin secretion
	FGF19	Bile acids on FXR	Suppresses bile acid synthesis; stimulates hepatic protein synthesis and glycogenesis; suppresses gluconeogenesis

Table 2：Hormones secreted by the GI tract

Adapted from ref. (102)

From the hormones produced by the GI tract, glucose-dependent insulinotropic polypeptide (GIP) and glucagon like peptide-1 (GLP-1) consist the incretin hormones, a name given already in 1929 by La Barre who was the first to successfully purify the glucose-lowering elements from gut extracts (INtestine seCREtion Insulin) (103). GIP is produced by the K cells, which are single cells located to the mucosa in the duodenum and upper jejunum, while GLP-1 is produced by the L cells of the small and large intestine, found more excessively in the ileum and colon (104). GIP and GLP-1 are present in low plasma concentrations in fasting humans but their concentration increases within a few minutes after nutrient intake. A significant variation between individuals has been noted in the secretion of GIP and GLP-1 (104).

The incretin hormones are mainly known for their insulinotropic effect as they stimulate insulin secretion by the pancreatic β cells, a phenomenon called the incretin effect (105, 106). The incretin effect may be described by the discovery that oral glucose intake leads to more insulin secretion than intravenous glucose infusion even when the same plasma glucose concentration is achieved (104).

GIP and GLP-1 stimulate insulin secretion in a strictly glucose dependant manner. The binding of the hormones on their receptors located in the cell membranes of pancreatic β cells enhances cyclic AMP production which activates protein kinase A. This, together with hyperglycaemia, initiates the release of pre-formed insulin secretory granules from β cells (104, 106). Of note, recent findings indicate that the effect of GLP-1 in insulin secretion is more complicated than initially believed (107). The debate mainly triggered by the low circulating levels of biologically active GLP-1 led to further research and study of the hormone's action. Recently two novel mechanisms have been proposed for the insulinotropic action of GLP-1. One of the two is that insulin secretion is achieved through the vagus nerves by the activation of receptors in nodose ganglions (108). The second is that GLP-1 produced by the α cells of the pancreas activates receptors on the β cells in a paracrine manner (109-111). Nevertheless, these issues are awaiting more thorough characterization.

Glucagon like peptide-1 and its receptor

GLP-1 was firstly identified after the cloning of the cDNAs and genes encoding proglucagon (112, 113). It is produced via the processing of proglucagon by prohormone convertase 1 (PC1). Proglucagon may also be processed to glicentin, oxyntomodulin and GLP-2 in the gut L cells, and into glucagon in the pancreatic α cells (114-116). GLP-1 is first produced as an inactive form, namely GLP-1 (1-37), a 37-amino acid peptide. The bioactive GLP-1 is generated by post-translational modifications and exists in two circulating molecular forms, the 31 amino acid long peptide GLP-1 (7-37) and the 30 amino acid long GLP-1 (7-36) amide (114-116). The amidated GLP-1 is more abundant in the circulation after food intake (112, 113).

Both bioactive forms of GLP-1 are quickly degraded by dipeptidyl peptidase-4 (DPP-4) to GLP-1 (9-37) or GLP-1 (9-36) amide. This enzymatic deactivation of GLP-1, together with the rapid renal clearance due to the molecule's low weight and hydrophilic nature result in a very short half-life of less than 2 minutes (112, 114, 115, 117). The human plasma levels of active GLP-1 are 5-10 pmol/L in fasting state and elevate up to 15-50 pmol/L after eating (112).

DPP-4, also known as CD26, is a serine protease able to modify or inhibit the activity of oligopeptides or proteins. It cleaves dipeptides from their aminoterminus of oligopeptides or proteins containing an alanine or proline in position 2. GLP-1, which holds an alanine at position 2, therefore consists a substrate for DPP-4. DPP-4 is expressed in many different tissues including the kidney, lung, adrenal gland, liver, intestine, spleen, testis, pancreas and CNS. It is also found on the surface of macrophages, lymphocytes and endothelial cells. Finally, it exists as well as a soluble protein in the circulation (117, 118).

GLP-1 acts by binding onto a 463 amino acid hepta-helical G-protein coupled receptor called the GLP-1 receptor (GLP-1R). The expression profile of GLP-1R has been the topic of a long debate, as the proper mapping of the receptor had been since recently prevented due to the lack of a reliable antibody (119). This problem was finally overcome five years ago when Richards et al. (120) managed to generate a transgenic mouse model where 'humanized' Cre recombinase is expressed under the control of GLP-1R promoter. With this method it was proved that the GLP-1R is expressed widely in various tissues, including the pancreas, GI tract, liver, kidneys, brain, lung and heart. Particularly in the kidney, GLP-1R is expressed exclusively by pre-glomerular vascular smooth muscle cells and juxtaglomerular cells (120, 121).

GLP-1R agonism

To date, there is a number of GLP-1R agonists available and already in clinical use for patients with type 2 diabetes and obesity. Table 3 summarizes the six FDA approved GLP-1R agonists (122). Exenatide, a synthetic exedin-4, was the first GLP-1R agonist to be introduced to the market. Exedin-4 was discovered in the salivary gland venom of a lizard and proved to share 53% of its amino acid sequence with mammalian GLP-1. It is more stable than GLP-1 as it is resistant to degradation by DPP-4 but it can be cleared by the kidney by glomerular filtration (112, 113, 122). Exenatide has a half-life of 2-3 hours after subcutaneous injection. Liraglutide, another broadly used agonist of GLP-1R, is partly DPP-4 resistant and shares 97% homology with native GLP-1. Its half-life is approximately 13 hours, due to two modification in the sequence of native GLP-1 that reduce renal clearance and provide resistance to DPP-4. These modifications include an Arg34Lys substitution and a glutamic acid and 16-C free fatty acid addition to Lys26 (112, 122, 123).

GLP-1R agonist	**Trade name**	**Year of FDA approval**
Liraglutide	Victroza / Saxenda	2010 / 2014
Albiglutide	Eperzan	2014
Exenatide	Byetta / Bydureon	2005 / 2012
Dulaglutide	Trulicity	2014
Lixisenatide	Lyxumia	2016
Semaglutide	Ozempic	2017

Table 3 : GLP-1R agonists

Clinical trials with GLP-1R agonists

Since the first GLP-1R agonist has been approved in 2005, followed in a relatively short time by another five GLP-1R agonists, a number of clinical trials with these drugs have been performed and published. The data from these trials help us gain a good understanding on the clinical effects of GLP-1R agonists and open the possibilities for further uses of incretin-based therapies. Some of the largest clinical trial programs conducted in the last couple of decades are namely: AMIGO, DURATION, LEAD, SCALE, GETGOAL, HARMONY, AWARD, LEADER, SUSTAIN-6, ELIXA and EXSCEL (124, 125). A short description with the key characteristics of each trial program is given below.

The AMIGO trials, which consisted of three 30-week trial periods and included 1446 participant, evaluated the outcomes of the treatment with exenatide in type 2 diabetes. A reduction of 0,5 to 0,8% of A1c was noted when 5mcg exenatide were injected twice per day, while with 10mcg twice daily this reduction reached up to 1% accompanied by 1 to 2 kg weight loss and 20 mg/dL less fasting plasma glucose. In these initial studies, nausea was often reported in exenatide-treated participants (30 to 40%) and ever since it has been registered as a common side effect of GLP-1R agonists. Once-weekly exenatide, the extended-release exenatide that can be used once every 7 days, was tested in the DURATION trial program. The data of this trial program demonstrated a 1,3% to 1,9% reduction in A1c and a weight loss of 2 to 4 kg (126-128).

The clinical trial program LEAD was performed with the aim to study the effects of liraglutide in diabetes. Over a time period of 26 weeks, liraglutide (1,2 mg or 1,8 mg daily) resulted in a 0,9 to 1,4% reduction in A1c and in a weight loss of 1 to 3 kg. Fasting plasma glucose was reduced 30 to 50 mg/dL and a decrease in blood pressure was as well noted (124). Liraglutide effects were also studied in the SCALE clinical trials, but in this case with the focus on weight reduction. A daily dose of 3 mg liraglutide together with diet and physical activity for 56 weeks resulted in a weight reduction of 8,4 ±7,3 kg compared to 2,8 ±6,5 kg in the placebo group, in overweight/obese participants (124, 129, 130).

The GLP-1R agonists lixisenatide, albiglutide and dulaglutide were tested under the phase 3 control trials GETGOAL, HARMONY and AWARD respectively. All three agonists demonstrated a significant reduction in A1c and body weight, with the latter two also achieving reductions in fasting plasma glucose (124).

Finally, the remaining four clinical trials that have been listed above, were performed with a focus on cardiovascular outcomes. The LEADER trials included 9340 participants with type 2 diabetes that were either ≥50 years old with prior cardiovascular disease (CVD) or ≥60 years old with no prior CVD but at least one cardiovascular (CV) risk factor. The participants were followed for a median 3 years. They were treated with 1,8 mg liraglutide daily or with placebo. The primary outcome of cardiovascular death, nonfatal myocardial infarction or nonfatal stroke occurred in significantly fewer patients treated with liraglutide (13%) than with placebo (14,9%). Additionally, the deaths of cardiovascular causes as well as from other causes were significantly lower in the liraglutide group (131, 132). SUSTAIN-6 was similar in entry criteria to LEADER but with the main aim to test the safety of semaglutide over placebo, and therefore only 3297 participants were included. The trial lasted 104 weeks and the participants were treated with 0,5mg or 1mg semaglutide or placebo once a week. The most outstanding result of the study was the highly significant reduction in nonfatal stroke in the semaglutide group (133-135). During the ELIXA trials 6068 participants diagnosed with type 2 diabetes and within 180 days of an acute coronary event were subjected to a lixisenatide treatment of a daily dose of 20mcg or placebo. Lixisenatide did not alter the rate of major cardiovascular events but was associated with a reduction in new-onset macroalbuminuria when adjusted for baseline A1c (136, 137). The largest cardiovascular outcome trial has been EXSCEL, conducted with 14752 participants. Patients with type 2 diabetes aged ≥60 years old with prior CVD were treated with extended-release exenatide or placebo. No significant difference in the incidence of cardiovascular disease was found between the groups of the patients that received exenatide and placebo (125, 138).

To sum up, the above described clinical trials demonstrated that GLP-1R agonists are characterised by high glycaemic efficiency and by the ability to induce weight loss and to improve cardiovascular outcomes for patients at risk.

Further biological effects of GLP-1

Besides the long known insulinotropic effect of GLP-1, increasing evidence is gathering regarding its additional biological effects. GLP-1 interacts with many different tissues leading to various outcomes. Table 4 summarizes these effects.

GLP-1, as discussed earlier, is mainly known for demonstrating pancreatic effects by stimulating insulin secretion. This is nevertheless not the only manner by which GLP-1 regulates glucose metabolism. GLP-1 is known to be a powerful suppressor of glucagon secretion, though the mechanism of action for this effect is not yet clear. In a big part of the literature a direct action of GLP-1 on the α cells of the pancreas is supported (117, 139), while there is also evidence of an indirect inhibitory effect via the stimulation of somatostatin secretion (140). Finally, a third possibility is a different paracrine action, mediated by insulin (141). This last theory is not much supported the recent years since evidence of glucagon inhibition by GLP-1 in individuals with type 1 diabetes was found (142).

Glucagon like peptide-1 (GLP-1)	**Organ**	**Effect**
	Brain	Decreases appetite and food intake Increases satiety
	Heart	Multiple effects
	Pancreas	Increases insulin and somatostatin secretion Inhibits glucagon secretion
	Stomach	Inhibits gastric emptying and possibly gastric acid secretion
	Gut	Slows down transit
	Adipose Tissue	Promotes 'browning'
	Kidney	Enhances natriuresis and diuresis
	Bones	Increases stability
	Blood vessels	Enhances vasodilation

Table 4 : The role of GLP-1 in different organs

Adapted from ref. (76).

From the multiple extra-pancreatic effects of GLP-1, what is of greater interest and relevance to this dissertation is the effect of GLP-1 on the kidney. GLP-1 mediates natriuresis and diuresis, alters renal haemodynamic and decreases systemic blood pressure (143-145). Possible pathways that have been described by which GLP-1 may carry out its renal actions include the direct stimulation of its receptor in the kidney, as well as indirect pathways, through the mediation of atrial natriuretic peptide (ANP) release from the heart or the inhibition of the renin angiotensin system (RAS) activity via angiotensin II (ANG2) regulation (146).

What should not be neglected to be mentioned is the anti-inflammatory action of GLP-1 that has been shown in several models. Glp-1r activation can inhibit inflammatory responses induces both by infections and allergens, such as LPS-induced inflammation both *in vivo* and *in vitro*, lung inflammation and mucus hypersecretion (147). By the employment of Glp-1r knock out mice, GLP-1 has been proven to decrease T cell proliferation under the effect of mitogenic stimulation (148). Furthermore, the Glp-1r agonism suppresses various indices of inflammation in humans, such as ROS stimulation by mononuclear cells (MNC), intranuclear NFκB binding, and the expression of TNFα and other cellular proinflammatory mediators in MNCs (149). Additionally, GLP-1 can increase the number and function of regulatory T cells as shown in different mouse models (148, 150). *In vitro*, GLP-1 inhibits the secretion of various cytokines when used to treat human invariant natural killer T cells (151) while liraglutide suppressed cytokine expression in $CD4^+$ T cells both *in vitro* and *in vivo* (152).

Hypothesis and aims

Based on the anti-inflammatory capacity demonstrated by Glp1r agonism, we hypothesized a protective effect of Glp1r agonism on the T cell-dependent model of rapid progressive glomerulonephritis, namely the murine model of NTS. To investigate the role of the Glp1r in NTS, we approached the research question by setting three main aims: (i) to evaluate the phenotype and the development of NTS in Glp1r knock-out mice and compare the outcome to wild type mice, (ii) to study the effect of Glp1r agonist liraglutide on wild type mice when subjected to NTS and (iii) to focus on getting an insight into the mechanism of action of Glp1r agonists by treating murine T cells *in vitro* with liraglutide.

Materials and Methods

In vivo experiments

Mice and study design

C57BL/6J mice were obtained from Charles River Laboratories (Sulzfeld, Germany). Four male and three female heterozygotes mice, carrying the knock-out gene for the glp1r, were kindly provided by Daniel Drucker (Toronto, Canada). The mice were inter-crossed and back-crossed with C57BL/6J mice. All off springs were genotyped for further breeding. Genomic DNA was isolated from the tail tips of the mice using the DNeasy Blood and Tissue kit (QIAGEN, Hilden, Germany) and the genomic PCR was performed with the following primers: primer A GLP-1R 5' (5'-TAC ACA ATG GGG AGC CCC TA-3'), primer B GLP-1R 3' (5'-AAG TCA TGG GAT GTG TCT GGA-3'), primer C Neo1 (5'-CTT GGG TGG AGA GGC TAT TC-3'), primer D Neo2 (5'-AGG TGA GAT GAC AGG AGA TC-3') (1). As a result, whole-body Glp1r -/- mice on the C57BL/6 genetic background and littermate WT controls were generated. For respective experiments Glp1r -/- mice and littermate WT controls were subjected to NTS. 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 twelve- week old male mice were used for all experiments. Mice weight ranged between 19-24g without significant differences between groups and experiments. Mice were healthy without any bacterial or viral infections as evaluated quarterly by the animal facility (1).

Mice were injected daily intraperitoneally with 200µg/kg bodyweight liraglutide (Victoza, NovoNordisk, Bagsvaerd, Denmark) or vehicle (saline) starting on the day of immunization (1).

Induction of NTS

NTS was induced as described previously (1). Briefly, mice were pre-immunized subcutaneously with 100µl of 2 mg/ml rabbit IgG (Jackson ImmunoResearch Laboratories Inc., West Grove, PA, USA) dissolved in incomplete Freund's adjuvant (Sigma, St. Louis, MI, USA) and non-viable desiccated Mycobacterium tuberculosis H37a (Difco Laboratories,

Detroit, MI, USA). After 3 days, heat-inactivated rabbit anti-mouse GBM antiserum was injected via the tail vein.

Urinary albumin detection

Urinary albumin excretion was determined by a double-sandwich ELISA, as reported previously (1, 94). Goat anti-mouse albumin antibody A90-13A-5 (Bethyl Laboratories, Montgomery, TX, USA) was used at a concentration of 1 μg/ml to coat 96-well plates. After an overnight incubation at 4^{o}C, plates were blocked with 0,5% BSA (SERVA Electrophoresis GmbH, Heidelberg, Germany) in PBS with 0,05% Tween20 for half hour at room temperature. Mouse urine samples were diluted in serial dilutions ranging from 1:10^2 to 1:10^7. Urine samples and mouse albumin standard (Sigma-Aldrich) were pipetted in triplicates and a two-hour incubation at room temperature followed. An HRP-conjugated goat anti-mouse albumin antibody A90-134P-7 (Bethyl Laboratories) was applied as a secondary antibody and incubated for further two hours. TMB substrate solution (Thermofisher, San Jose, CA, USA) was used as a substrate and the reaction was stopped after fifteen minutes with 2M H_2SO_4. OD was measured at 450nm and final concentrations were calculated using a four-parameter logistic curve.

Urinary lipocalin-2 detection

Lipocalin-2/NGAL levels were determined using a commercially available mouse ELISA DuoSet kit (R&D Systems, Park Abingdon, UK), according to manufacturers' recommendations. Briefly, 96-well plates were coated with rat anti-mouse Lipocalin-2 capture antibody in a concentration suggested by the company and incubated overnight at room temperature. The next day the plates were blocked with PBS including 1% BSA for one hour at room temperature and then samples and standard curve dilutions were added on the plate in triplicates and incubated for two hours. Mouse urine samples were diluted in serial dilutions ranging from 1:10^3 to 1:10^6. A two-hour incubation with biotinylated rat anti-mouse Lipocalin-2 detection antibody followed and a 20-minutes incubation with Streptavidin-HRP (all provided by the kit). Finally, the Substrate solution provided by the kit was added for 20 minutes and the reaction was stopped with 2M H_2SO_4. Optical density was determined at 450nm and final concentrations were calculated using a four-parameter logistic curve.

Urinary creatinine detection

Urinary creatinine was quantified using a picric acid-based method (Sigma-Aldrich) or by liquid chromatography tandem mass spectrometry (LC-MS/MS) (1). For the first, urine samples were diluted in a 1:10 ratio in distilled H2O. Diluted samples and creatinine standard were pipetted on 96 well-plates in triplicates and incubated for twelve minutes at room temperature with alkaline picrate solution. OD was measured at 490 nm. Subsequently, acid reagent was added to all wells and after a five-minute incubation at room temperature OD values were measures at 490 nm. Creatinine values were calculated with the following equation:

Creatinine (mg/dl) = (INITIAL Sample OD – FINAL Sample OD) / (INITIAL Standard

OD – FINAL Standard OD) *0,3.

For the later, separations were achieved on a Hypercard column (ThermoFisher, San Jose, CA USA) at 50°C by isocratic elution with a mobile phase consisted of 0.25 % (v/v) formic acid and 5 % (v/v) methanol at a flow-rate of 1.0 mL/min. Detection was performed by positive electrospray ionization on a SCIEX QTRAP 4500 triple quadrupole instrument (U.S.A. Framingham, MA) (1).

Evaluation of renal histopathology

Kidneys were fixed in formalin, embedded in paraffin and cut in 4 μm sections with a rotation microtome. After deparaffinization and hydration in Xylene and Ethanol, the sections were stained with periodic acid's Schiff (PAS). Briefly, slides were dipped in Periodic Acid Solution (Sigma-Aldrich) for 5 minutes, washed and further dipped in Schiff's Reagent (Sigma-Aldrich) for 15 minutes. After washing, slides were stained with Gill's Haematoxylin III (Sigma-Aldrich), dehydrated and mounted in Roti-Histokitt II mounting medium (Carl Roth, Karlsruhe, Germany). A minimum of 50 glomerular cross sections was evaluated per sample (1). The PAS score evaluation was performed as described previously (153), following a semi quantitative scoring system with a scale from 0 to 3. According to the scoring system, 0=no PAS positive material, 1 < 1/3 PAS positive material, 2 = 1/3 - 2/3 PAS positive material and 3 > 2/3 PAS positive material. To evaluate cell proliferation in the different glomerular

compartments different scoring systems were applied. Mesangial hypercellularity was subclassified as mild (score 1) = 4-5 cells/mesangial area, moderate (score 2) = 5-6 cells/mesangial area and severe (score 3) = >6 cells/mesangial area (1). Endocapillary hypercellularity defined as hypercellularity due to increased number of cells within glomerular capillary lumina was subclassified as mild (score 1) = present in single glomerula, moderate (score 2) = <50% and severe (score 3) = >50% affected glomerula (1). 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 (1).

In order to monitor proliferating cells in the kidneys, deparaffinated kidney slides were unmasked using the R-Universal Epitope Recovery buffer (BioVendor, Brno, Czech Republic) and cooked in a 2100 Antigen Retriever (BioVendor) for twenty minutes. Before proceeding the slides were kept in the Antigen Retriever pot for another two hours, without opening the lid. Slides were subsequently stained with mouse anti-human/mouse/rat PCNA antibody (clone PC10, Biolegend, San Diego, CA, USA) using the three-layer immunoperoxidase staining (1). To detect the mouse primary antibody on mouse tissue the M.O.M. Immunodetection Kit (Vector Laboratories, Burlingame, CA, USA) was used according to manufacturers' recommendations. Slides were subsequently incubated with Vectastain ABC HRP kit (Vector Laboratories) for 45 minutes and dipped in 3-amino-9-ethylcarbazole substrate-chromogen for 6-8 minutes. Nuclei were stained with Gill's Haematoxylin III (Sigma-Aldrich) and slides were finally mounted in Aquatex mounting medium (Merck, Kenilworth, NJ, USA). The quantification of glomerular PCNA positive cells was performed by counting the positive cells in 50 glomeruli per sample and calculating the average number of positive cells per glomerulus (1). 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 (1).

Kidney sections were stained with picrosirius red staining (Sigma-Aldrich) to monitor fibrotic changes in the kidney (1). Briefly, slides were deparaffinated as described above and Gill's Haematoxylin III (Sigma-Aldrich) was applied for eight minutes to stain the nuclei. Picro Sirius Red was added and incubated for one hour. Slides were finally dehydrated in 100% ethanol, cleared with Xylene and mounted in a resinous medium. Samples were blinded before evaluation.

Evaluation of immunohistochemistry

Frozen kidney tissue was cut with a cryotome in 4 µm frozen sections and subjected to a three-layer immunoperoxidase staining for the detection of macrophage, T cell and neutrophil subpopulations in the kidney (1). The samples were brought to room temperature, fixed with acetone for eight minutes and blocked for twenty minutes in 20% fetal calf serum, 10% goat serum and 200 µl/ml Avidin (Vector Laboratories, Burlingame, CA, USA). The primary antibodies were diluted in PBS with 200 µl/ml Biotin (Vector Laboratories) and applied on slides for one hour. Macrophages, T helper cells, cytotoxic T cells and neutrophils were stained with rat anti-mouse anti-CD68 antibody (clone FA-11, Biorad), 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 respectively (1). Biotinylated goat anti-rat IgG (Jackson ImmunoResearch Laboratories) was used as a secondary antibody, diluted in PBS and applied for 45 minutes. Finally, slides were incubated with Vectastain ABC HRP kit (Vector Laboratories) for 30 minutes, washed with 0,1M Sodium-acetate buffer and dipped in 3-amino-9-ethylcarbazole substrate-chromogen for 6-8 minutes. Nuclei were stained with Gill's Haematoxylin III (Sigma-Aldrich) and slides were mounted in Aquatex mounting medium (Merck, Kenilworth, NJ, USA). Samples were blinded before evaluation. A semi quantitative scoring system for kidney-infiltrating macrophages was performed as follows: 0 = 0–4 cells stained positive; 1 = 5–10 cells; 2 = 10–50 cells; 3 = 50–200 cells; and 4 = >200 cells stained positive per low-power field (1). T cell and neutrophil quantification was performed by counting the number of positive cells in six adjacent high-power fields (Hpf) of renal cortex and medulla (1).

Immunofluorescence staining of kidney sections

In order to detect the deposition of autologous and heterologous IgG in kidneys of animals subjected to NTS, frozen kidneys were cut into 4µm frozen tissue sections on a cryotome and stained with direct immunofluorescence staining (1). Firstly, the slides were left to dry at room temperature for thirty minutes and subsequently they were fixed with cold aceton for five minutes. The samples were then blocked in 1,5% fetal calf serum diluted in PBS for five minutes. FITC-conjugated goat anti-mouse IgG (Jackson ImmunoResearch Laboratories) and FITC-conjugated goat anti-rabbit IgG (Jackson ImmunoResearch Laboratories) diluted in a 1:800 dilution were applied on the slides for 45 minutes for the detection of autologous and

heterologous IgG deposition in the kidneys, respectively. Slides were mounted in a mounting medium for fluorescence stainings (Vector Laboratories) and analysed under an LSM510 META microscope (Zeiss, Oberkochen, Germany) (1).

Assessment of autologous antibody responses

A semi-quantitative ELISA was used for the detection of anti-rabbit IgG. 96-well plates were coated with rabbit IgG (Jackson ImmunoResearch Laboratories) in carbonate/bicarbonate buffer at a concentration of 100 µg/ml and incubated overnight at 4°C. Plates were blocked with BSA for 30 minutes and incubated with serial mouse serum dilutions in duplicates for two hours. HRP-conjugated goat anti-rabbit IgG was used as a secondary antibody (Jackson ImmunoResearch Laboratories). TMB substrate solution (Thermofisher, San Jose, CA, USA) was used as a substrate and the reaction was stopped after ten minutes with 2M H_2SO_4. OD was measured at 450nm and OD values were directly compared between the groups.

Reverse transcription (RT) real-time polymerase chain reaction (PCR)

TRI Reagent (Sigma-Aldrich) was used according to standard protocol for the isolation of total RNA from kidneys, spleens and inguinal lymph nodes. The RNA amounts in each sample were measured using a NanoDrop One Microvolume Spectrophotometer (Thermofisher). Subsequently, 2µg of total RNA was reverse transcribed using Superscript III Transcription Kit (Invitrogen, Carlsbad, CA, USA) and random primers (Invitrogen) (1). Real-time PCR was performed in duplicates on a CFX96 Real-Time System (BioRad, Vienna, Austria) using TaqMan gene expression assays (Applied Biosystems, Foster City, DA, USA) 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) (1). *Hprt* was used as reference gene for kidney and spleen tissues, while for lymph node tissue *Rpl0* was used (1). The primers used for the housekeeping genes 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'; 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) (1). The data was evaluated using the 2ΔΔCT method (1).

Splenocyte restimulation

WT and Glp1r-/- mice were subjected to NTS for 14 days and the spleens were harvested and kept in PBS on ice. Splenocytes were isolated by mashing the organs through a 70µm cell strainer. The cells were counted with a CASY cell counter (Schärfe System, Reutlingen, Germany) and plated in 96 well plates in equal numbers with 200ng/ml LPS for 16 hours (1). For the stimulation with rabbit IgG, the plates were pre-coated with 100 µg/ml rabbit IgG and incubated overnight at 4ºC. The cells were cultured on the plates for 24 hours (1). The proliferation of the cells was evaluated with the EZ4U cell proliferation assay (Biomedica, Vienna, Austria) according to manufacturers' instructions. Briefly, 20 µl of the assay's substrate were added in 96-well plates with 200µl of cell suspension. After 5 hours incubation at 37 ºC, the optical density was measured at 450nm with 620nm as reference. The stimulation index was calculated as a ratio between OD values of stimulated cells to OD values of unstimulated cells.

Flow cytometry

Lymph nodes were harvested, weighted and smashed through 70µm cell strainers with cold PBS. The single cell suspension was stained with anti-mouse CD4, CD25 and FoxP3 antibodies (BioLegend, San Diego, CA, USA). Samples were analyzed with the LSII and FACS Calibur cytometers (both BD Biosciences, San Jose, CA, USA) (1).

Blood pressure measurement

The blood pressure was assessed by the non-invasive tail cuff measurement. The measurement was performed for four days in the evening with a Kent Scientific Corporation CODA Non-Invasive Blood Pressure System (Kent Scientific CODA, Torrington, CT, USA). The mean arterial pressure (MAP) was calculated with the following equation:

MAP = ((2xdiastolic pressure) + systolic pressure) / 3

In vitro experiments

Study design

T cells were isolated from spleens of C57BL/6J and Glp1r-/- mice, stimulated with aCD3/CD28 antibodies (eBioscience, San Diego, CA, USA) and treated with liraglutide (60µg/ml) for 72 hours (1). Additionally, T cells from C57BL/6J mice were polarized into a Th1 and Th17 phenotype and treated with liraglutide (60µg/ml) for 5 and 4 days respectively (1).

T cell isolation and stimulation

T cells were isolated from spleens of C57BL/6J mice or Glp1r-/- mice using the MagniSort Mouse T cell Enrichment Kit (Invitrogen) according to manufacturers' recommendations. The cells were plated on 96-well plates previously coated with anti-mouse CD3e antibody (clone 145-2C11) in a concentration of 5 µg/ml and incubated overnight at 4°C. The number of cells plated per well was $0.4x10^6$, and anti-mouse CD28 antibody was added to the wells in a concentration of 2 µg/ml (1). For the collection of supernatants, cells were plated on 24-well plates, at a density of $2x10^6$ cells per well. The application of aCD3/CD28 antibodies and liraglutide as well as the timepoints was performed on the same matter as described above. As cell culture medium, RPMI 1640 with 10% FCS, 1% PenStrept and 0,1% 2-mercaptoethanol was used.

Assessment of cell proliferation

Cell proliferation was assessed with the EZ4U cell proliferation assay (Biomedica, Vienna, Austria) (1). This assay is based on the conversion of tetrazolium salt to coloured formazan, a process requiring functional mitochondria, and therefore living cells. 20µl of substrate provided by the kit were added to 200µl sample and after 5 hours of incubation at 37°C, absorbance was measured at 450nm with 620nm as reference. For pan T cells, the stimulation index was calculated as a ratio between OD values of stimulated cells to OD values of

unstimulated cells (1). For polarized Th1 and Th17 cells, the raw data values from OD measurements were used (1).

Quantification of cytokine levels in cell supernatant

The supernatant of T cells was collected and stored at -80°C. IFN-γ, Il-6, Il-4 and TNF-α were determined using commercially available ELISA kits (BD, San Jose, CA, USA) (1). Briefly, 96-well plated were coated with accordant capture antibody at a concentration of 4µl/ml and incubated overnight at 4°C. The next day plates were blocked in PBS with 10% FCS for one hour and later on with the diluted samples and standard for two hours. Finally, the suitable detection antibody together with HRP were added on the plates both at a concentration of 4µl/ml. TMB substrate solution (Thermofisher) was used as a substrate and the reaction was stopped after fifteen minutes with 2M H_2SO_4. OD was measured at 450nm and final concentrations were calculated using a four-parameter logistic curve. Il-17 was determined using the IL-17A Mouse Uncoated ELISA Kit (Thermofisher). The exact protocol provided by the company together with the kit was followed.

Assessment of T cell glycolysis

Glycolysis of T cells was evaluated using the EnzyChrom Glycolysis Assay Kit (BioAssay Systems, Hayward, CA, USA). In brief, concentration of L-lactate in samples was calculated based on an L-lactate standard curve and the reduction of a formazan dye by pyruvate and NADH, which are generated by the lactate dehydrogenase catalyzed oxidation of L-lactate. 5µl of sample or standard were added on 96-well plates and 95µl of working reagent including two enzymes, NAD/MTT and assays buffer, all provided by the kit was added to each well. After incubation at room temperature for half hour, optical density was read at 565nm and final concentrations were calculated using a linear regression fit.

Reverse transcription (RT) real-time polymerase chain reaction (PCR) for cells

For the isolation of RNA from cells the RNeasy Mini Kit (QIAGEN, Hilden, Germany) was used. The integrity of the RNA was checked on a 1% agarose gel and the amounts of RNA in the samples were measured with a NanoDrop One Microvolume Spectrophotometer (Thermofisher). Subsequently, 2µg of total RNA was reverse transcribed using Superscript III Transcription Kit (Invitrogen, Carlsbad, CA, USA) and random primers (Invitrogen) (1). Real-time PCR was performed in duplicates on a CFX96 Real-Time System (BioRad, Vienna, Austria) using TaqMan gene expression assays (Applied Biosystems, Foster City, DA, USA) for *Glut-1* (Mm00441473_m1). *Rpl29* was used as a reference gene and was assessed using SYBR Green Mastermix (Invitrogen). The primers used were: forward 5'-CGC GGG TTA CCG TGA GTG T-3'; reverse 5'-TGT CTG CAC CTC GCG ACC-3'.

Th1 and Th17 polarization

Spleens from C57BL/6J mice were harvested and kept in cold PBS on ice. The organs were then mashed over 70µm cell strainers and subsequently passed through 40µm cell strainer. Cells were isolated using the $CD4^+CD62L^+$ T cell isolation kit (Miltenyi Biotec, Bergisch Gladbach, Germany). For the Th1 polarization cells were plated on 12-well plates which had been coated with anti-mouse CD3e antibody (clone 145-2C11) in a concentration of 2,5µg/ml and incubated overnight at 4°C. Anti-mouse CD28 (eBioscience), anti-mouse IL4 (BioLegend, San Diego, CA, USA), recombinant mouse IL2 (BioLegend) and recombinant mouse IL12 (BioLegend) were added to the cells in the following concentrations respectively: 3µg /ml, 10 µg /ml, 5ng/ml and 10ng/ml. Cells were incubated for 5 days (1). The polarization of the $CD4^+CD62L^+$ T cells into a Th17 phenotype was achieved by keeping the in culture for 4 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 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 (1). The proliferation of the cells was evaluated with the EZ4U cell proliferation assay (Biomedica) (1). 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, USA and Thermofisher) (1).

Assessment of *Glp1r* expression in T cells and different T cell subpopulations

Spleens and inguinal lymph nodes of C57Bl/6J and Glp1r -/- mice were harvested and kept in cold PBS on ice. Tissues were mashed over 70µm cell strainers and the cell suspension was further processed with the MagniSort Mouse T cell Enrichment Kit (Invitrogen) for the isolation of pan T cells. The cells were stimulated with aCD3/CD28 stimulation (as described above) and kept in culture for 72 hours or used directly for RNA isolation. To acquire RNA from Th1 and Th17 polarized cell, spleens were harvested from C57Bl/6J mice, kept in cold PBS on ice and used for the isolation of $CD4^+CD62L^+$ T cells and further polarization to Th1/T17 as described above. After the polarization protocol, cells were used for RNA isolation. RNA was isolated from the cells using the RNeasy Mini Kit (QIAGEN, Hilden, Germany) (1). After checking the RNA integrity by running it on a 1% agarose gel and measuring the amounts of RNA in the samples using a NanoDrop One Microvolume Spectrophotometer (Thermofisher), 2 µg of total RNA was reverse transcribed using Superscript III Transcription Kit (Invitrogen, Carlsbad, CA, USA) and random primers (Invitrogen). With the use of the following primers for *Glp1r* and *β-Actin*: forward 5'GGC CAT GTG TAC CGG TTC TG3'; reverse 5'GGT GCA GTG CAA GTG TCT GA3'; forward 5'GAA GTG TGA CGT TGA CAT CCG3'; reverse 5'TGC TGA TCC ACA TCT GCT GGA3', respectively, PCR was performed (1). 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 (1).

Statistical analysis

All statistical analyses were performed using GraphPad Prism 7.0 (GraphPad Software, La Jolla, CA, USA). Results are presented as mean ± SEM. The normal distribution of the data was determined by the Kolmogorov-Smirnov test with Dallal-Wilkinson-Lilliefors correction or the Shapiro-Wilk normality test. 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 (1).

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 (1).

Results – Findings

The development of NTS in Glp1r-/- mice

Glp1r-/- mice and wild type (WT) mice were subjected to NTS and sacrificed after 14 or 21 days, in order to evaluate the role of Glp1r in NTS (1). Increased albuminuria, indicating the development of NTS, was noted after 7, 14 and 21 days from NTS induction in both groups, but with no significant differences between the groups in any of the above time points (Fig. 1A) (1). Furthermore, kidney pathology, as monitored by the study of histological changes in the kidneys after 14 and 21 days of NTS, did not differ between Glp1r-/- and WT mice (Fig. 1B, Fig. 2) (1). The infiltration of immune cells in the kidneys of the mice was thoroughly examined. After 21 days of NTS there was a significantly higher amount of $CD68^+$ and $CD4^+$ T cells in the kidneys of Glp1r-/- mice in comparison to WT mice; a difference that was not present on day 14 after disease induction (Fig. 1 C, E, G) (1). The infiltration of $CD8^+$ T cells was equal in nephritic kidneys of the two groups throughout the observation period (Fig. 1D) (1). A significant increase was observed in the renal infiltration of polymorphonuclear granulocytes (PMN) in Glp1r-/- mice after 14 days of NTS. This difference was lost on day 21 of the disease (Fig. 1 F, G) (1).

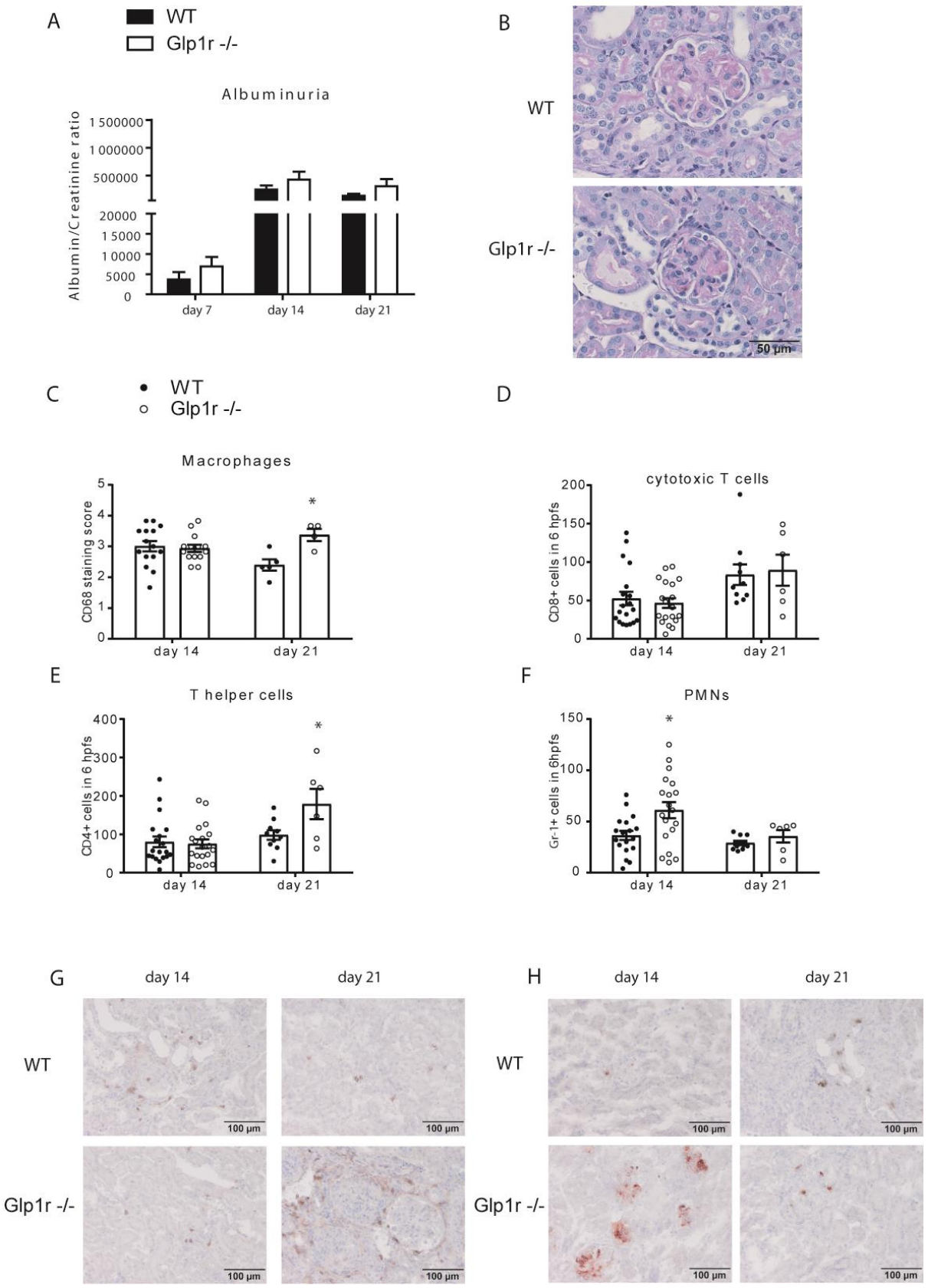


Figure 1: NTS phenotype in Glp1r-/- mice

After 7 (n=13 per group), 14 (WT n=19, Glp1r -/- n=18) and 21 days (WT n=9, Glp1r -/- n=6) of NTS, WT and Glp1r-/- mice were monitored for the disease phenotype. (A) Albuminuria, given as urinary albumin/creatinine ratios (µg/mg), was assessed at indicated time points after NTS induction. Representative pictures of PAS-stained kidney sections from WT and Glp1r-/- mice after 14 days of NTS are given. The renal infiltration of (C) $CD68^+$ macrophages, (D) $CD8^+$ cytotoxic and (E) $CD4^+$ T helper cells as well as (F) polymorphonuclear granulocytes (PMN) was evaluated in WT and Glp1r-/- mice at indicated time points after NTS induction. Representative pictures from kidney sections stained for (G) $CD4^+$ T cells and (H) PMNs after 14 and 21 days of NTS are shown. Magnification 200x. hpf= high power field.*$p<0.05$. Reproduced from (1) with permission of publisher Elsevier.

A thorough histological study of kidney slides stained with PAS revealed no difference between Glp1r-/- and WT mice kidneys on day 14 and day 21 of NTS. Glomerulosclerosis as presented by PAS score was equal between the groups on the two time points studied (Fig. 2A) (1). Crescent and intraluminal cast formation was as well evaluated but no differences were noted (Fig. 2 B, H) (1). Finally, cell proliferation in the different glomerular compartments, glomerular intraluminal thrombi, segmental capillary necrosis and tubular injury were assessed but did not differ between Glp1r-/- and WT mice (Fig. 2 C-G) (1).

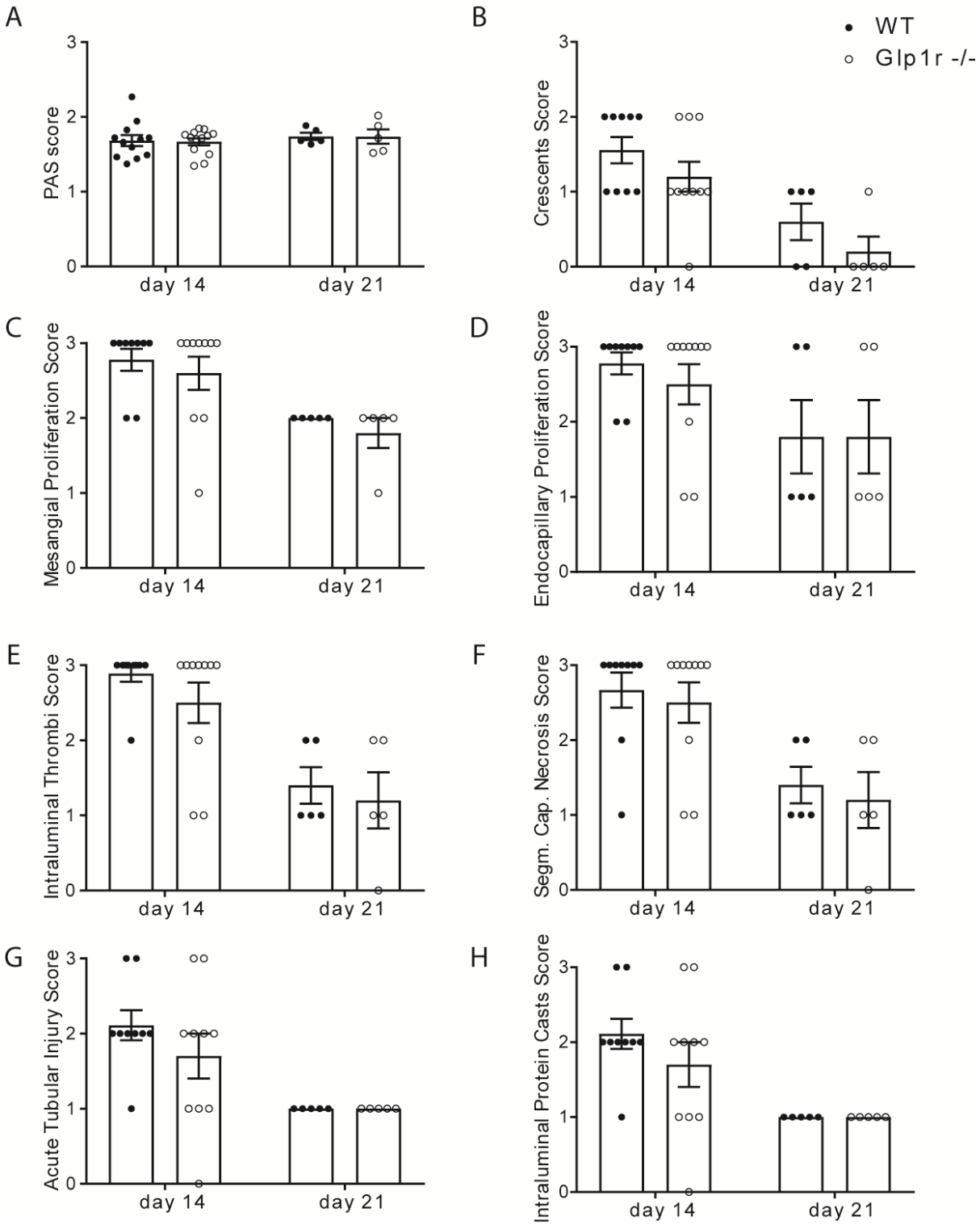


Figure 2: Histomorphological evaluation of renal pathology of Glp1r-/- mice after NTS

WT and Glp1r -/- mice were subjected to NTS for 14 days (WT n=9, Glp1r -/- n=10) and 21 days (WT n=5, Glp1r -/- n=5). PAS stained kidney sections were evaluated. Kidney sections were quantified for PAS positive deposits (A) and for crescent formation (B), mesangial (C) and endocapillary proliferation (D), as well as intraluminal thrombi (E), segmental capillary

necrosis (F), acute tubular injury (G) and intraluminal protein casts score (H). Reproduced from (1) with permission of publisher Elsevier.

The linear deposition of mouse and rabbit IgG on the glomerular basement membrane of mice after 14 days of NTS induction was studied with the use of immunofluorescence staining and confocal microscopy. No difference was detected in deposited mouse or rabbit IgG between Glp1r-/- and WT mice kidneys on day 14 of NTS (Fig. 3) (1).

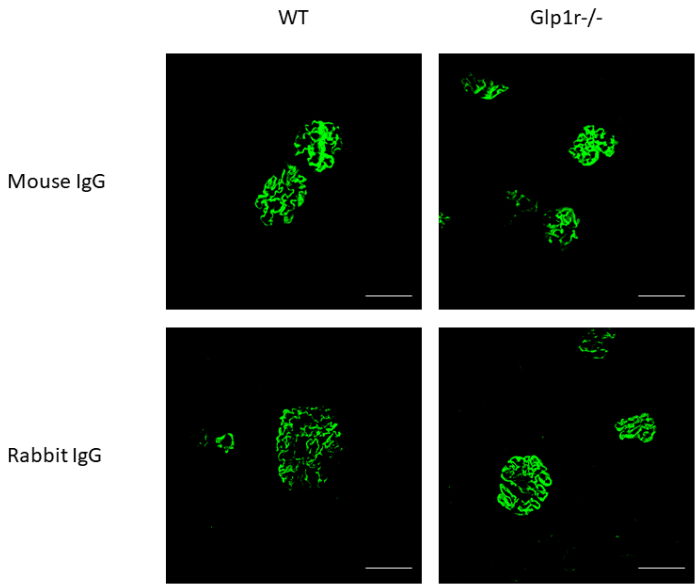


Figure 3: Deposition of mouse and rabbit IgG on the glomerular basement membrane of Glp1r-/- mice after NTS

After 14 days of NTS, kidney sections from WT and Glp1r-/- mice were evaluated for the deposition of autologous and heterologous IgG. Representative pictures from the direct immunofluorescence staining are given. Magnification 400x. Reproduced from (1) with permission of publisher Elsevier.

Glp1r-/- mice demonstrate an upregulation in the expression of inflammatory genes in lymph nodes and spleens after NTS induction

A significant increase in the mRNA expression of *Il-10*, *Ifn-γ* and *Tnf-α* in the lymph nodes of Glp1r-/- in comparison to lymph nodes of WT mice after 14 days of NTS was found (Fig. 4A) (1). Besides those cytokine encoding genes, the expression of the marker of Th17 cells *Rorγt*, as well as the master regulators of Th1 and Th2 cells *Tbet* and *Gata3* were upregulated in the lymph nodes of Glp1r-/- mice (Fig. 4A) (1). The profiling of the expression of the same genes by qPCR was performed also for the spleens from those mice. The evaluation revealed an upregulation in the expression of *Ifn-γ, Rorγt, Tbet* and *FoxP3* in the spleens of Glp1r-/- compared to WT mice (Fig. 4B) (1). The lymph nodes from those mice were also used for flow cytometry to evaluate the $CD4^+CD25^+FoxP3^+$ T reg population of Glp1r-/- mice after NTS induction. There was no difference found between the lymph nodes from Glp1r-/- and WT mice after 14 days of disease in absolute or relative numbers (Fig. 5) (1).

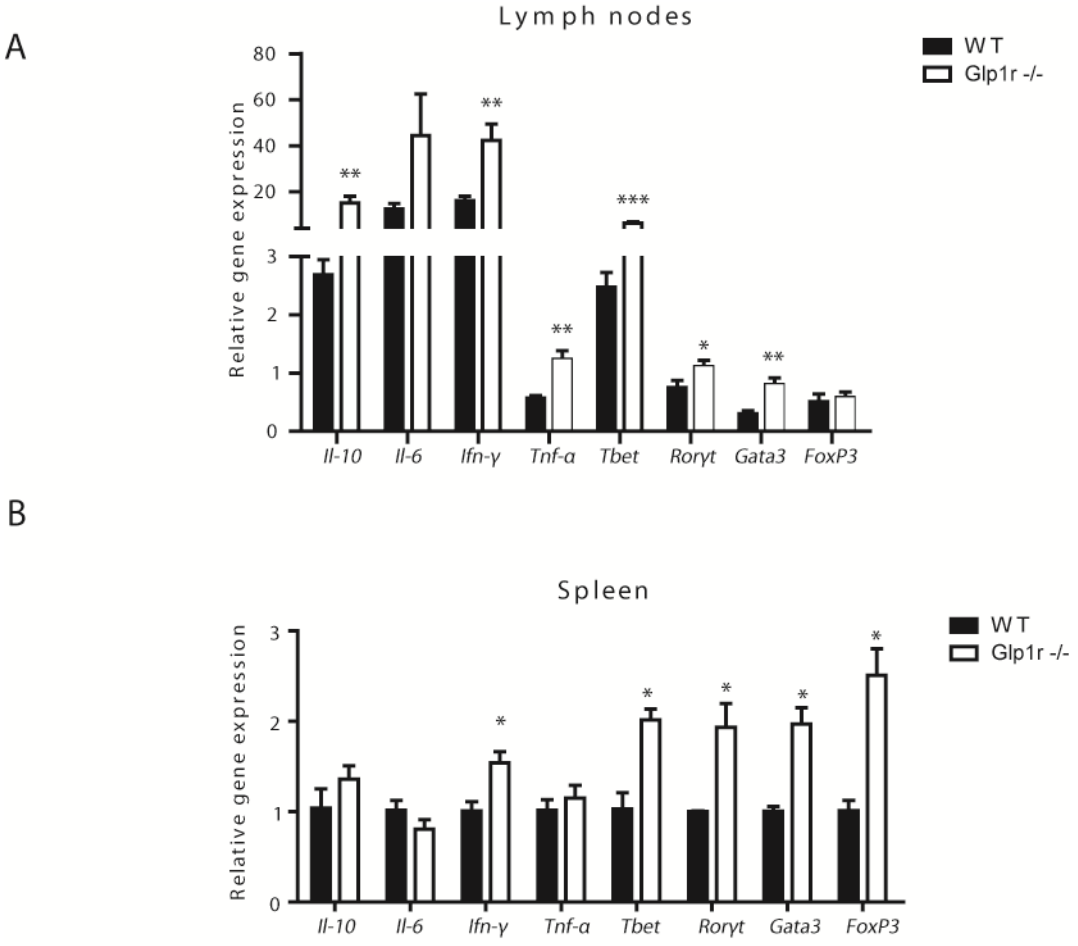
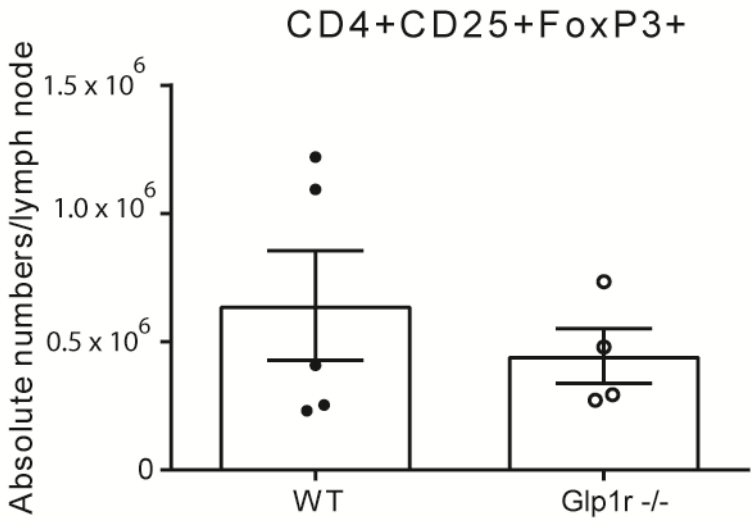


Figure 4: Systemic inflammation in lymph nodes and spleens of Glp1r-/- mice

WT and Glp1r -/- mice were subjected to NTS for 14 days and the inguinal lymph nodes and spleens were studied for the expression of various inflammatory genes. (A) The mRNA expression profile of genes related to inflammation in lymph nodes of WT (n=8) and Glp1r-/- mice (n=10). 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). The fold increase compared to the mean mRNA expression in diseased WTs is provided. *$p<0.05$, **$p<0.01$, and ***$p<0.001$. Reproduced from (1) with permission of publisher Elsevier.

A



B

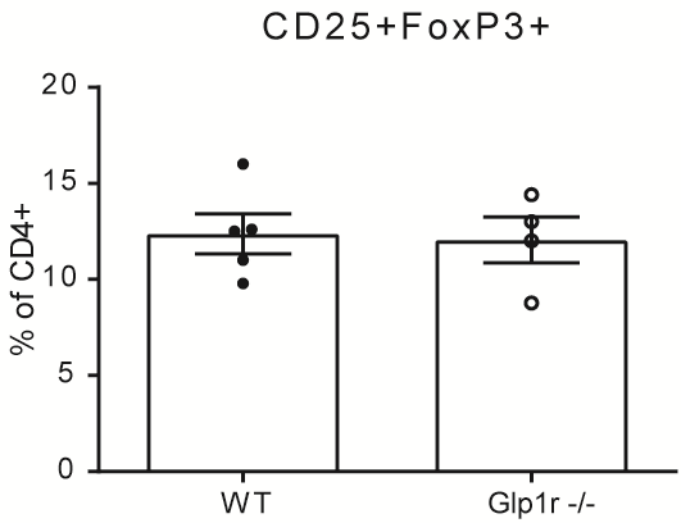


Figure 5: $CD4^+CD25^+$ Treg population in the lymph nodes of Glp1r-/- mice

NTS was induced in WT (n=5) and Glp1r-/- mice (n=4) for 14 days. Flow cytometry was used to evaluate the $CD4^+CD25^+FoxP3^+$ Tregs. The results are shown in (A) absolute and (B) relative numbers. Reproduced from (1) with permission of publisher Elsevier.

Interestingly, cytokine expression profiling by qPCR in lymph nodes of healthy Glp1r -/- mice and wild type controls revealed significant differences. mRNA levels of Th1, Th2 and

regulatory T cell markers were lower in the lymph nodes of Glp1r-/- mice. No difference was noted on the expression of Th17 marker *Rorγt* (Fig. 6) (1).

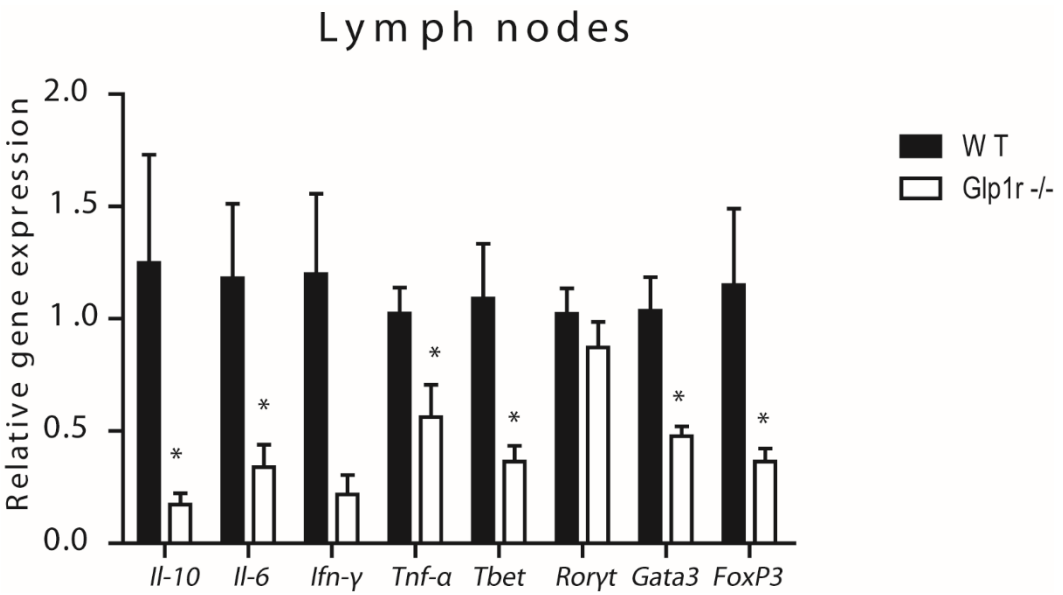


Figure 6: Expression of inflammatory genes in the lymph nodes of healthy Glp1r-/-

The inguinal lymph nodes of healthy WT (n=5) and Glp1r-/- mice (n=3) were analysed for the mRNA expression of the indicated genes. The fold increase compared to the mean mRNA expression in WT mice is provided. *$p<0.05$. Reproduced from (1) with permission of publisher Elsevier.

Splenocytes from Glp1r-/- mice subjected to NTS proliferate more after *in vitro* stimulation

Splenocytes were isolated from WT and Glp1r-/- mice after 14 days of NTS, cultured *in vitro* and stimulated with rabbit IgG or LPS. With both stimulation manners the cells from the Glp1r-/- mice revealed a significantly higher stimulation index than the splenocytes from WT mice (Fig. 7) (1).

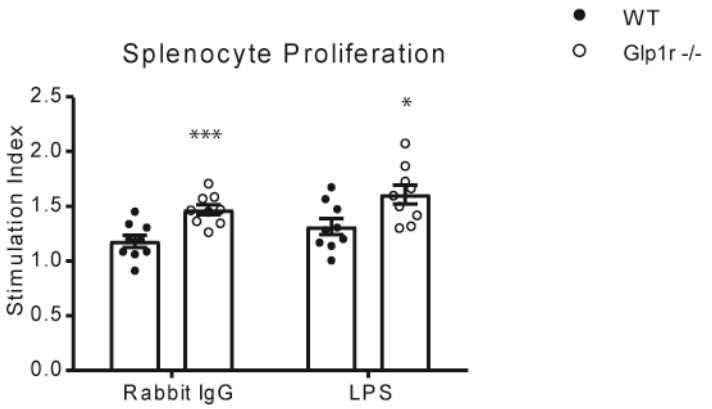


Figure 7: Glp1r-/- splenocytes showed increased proliferation in vitro upon stimulation

WT (n=9) and Glp1r -/- mice (n=9) were subjected to NTS and after 14 days their splenocytes were isolated. The splenocytes were stimulated *in vitro* by plate-coated rabbit IgG or by LPS. Proliferation of the cells 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 ***$p<0.001$. Reproduced from (1) with permission of publisher Elsevier.

Glp1r-/- mice subjected to NTS do not demonstrate a difference in natriuresis compared to WT mice

After 14 days of NTS, WT and Glp1r -/- mice were placed in metabolic cages over a time period of 16 hours. Glp1r -/- mice secreted a significantly lower urine volume than WT mice (Fig. 8A). The urine samples were collected and the urinary sodium was evaluated. When calculated as total urinary sodium over 16 hours, the sodium secretion is significantly lower in the Glp1r -/- mice than in WT mice (Fig. 8B). Nevertheless, the urinary sodium/creatinine ratio as well as the fractional sodium in the urine did not differ between the two groups (Fig. 8C-D).

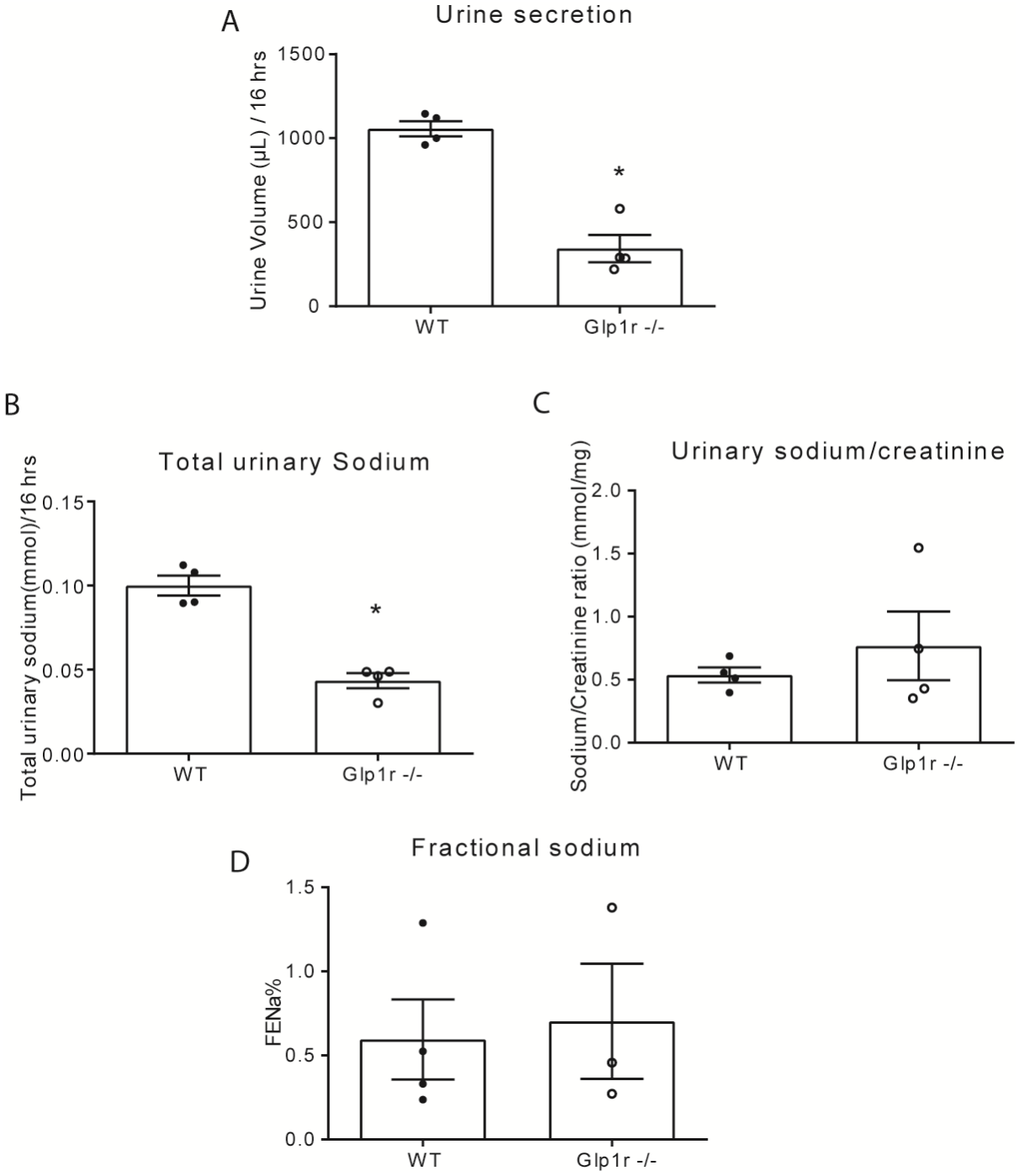


Figure 8: Urinary secretion and natriuresis in Glp1r-/- mice

WT (n=4) and Glp1r -/- mice (n=4) were subjected to NTS for 14 days and the urinary secretion and natriuresis were evaluated. (A) The urine volume produced over 16 hours was assessed (B) The total urinary sodium secreted in 16 hours is given as mmol. (C) Urinary sodium secretion is also given as urinary sodium/creatinine ratio (mmol/mg) and as fractional sodium (D). *$p<0.05$

Blood pressure does not differ in healthy Glp1r-/- mice compared to healthy WT mice

Healthy WT and Glp1r -/- mice were followed for four consequent days regarding their blood pressure. No significant difference was noted between the two groups (Fig. 9).

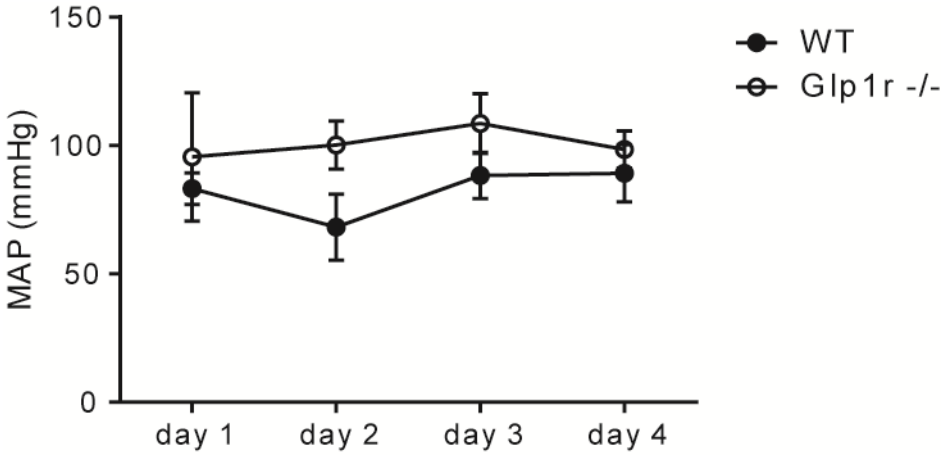


Figure 9: Blood pressure in healthy Glp1r-/- mice

Healthy WT (n=5) and healthy Glp1r -/- mice (n=4) were monitored for the blood pressure over a period of four days. The mean arterial pressure was calculated and is shown in mmHg.

Liraglutide treatment protects mice from NTS

To further understand the role of GLP-1R in NTS and investigate a potential clinical use of it we treated mice with liraglutide, a GLP-1R agonist, and subjected them to NTS. On day fourteen after NTS induction there was a significant reduction in albuminuria, as well as urinary NGAL levels by the liraglutide treatment (Fig. 10A-B) (1). In accordance with these differences, kidney histology revealed more severe renal damage in vehicle treated mice than in liraglutide treated mice. The quantification of PAS-positive deposits showed a significant decrease in the liraglutide group (Fig. 10C, E) (1), while the evaluation of crescent formation in the kidneys revealed lower scores for the liraglutide treated group (Fig. 10D) (1). A detailed evaluation of the kidney sections stained for PAS showed a general amelioration on all histomorphological aspects studied in the kidneys of mice treated with liraglutide (Fig. 11) (1). More specifically, kidneys from mice treated with liraglutide demonstrated significantly

decreased glomerular injury in terms of mesangial (Fig.11A) and endocapillary (Fig. 11B) proliferation, intraluminal thrombi (Fig. 11C) and segmental capillary necrosis (Fig. 11D). Tubular injury was also decreased in the liraglutide group (Fig. 11D) as well as the scores for intraluminal protein casts (Fig. 11E).

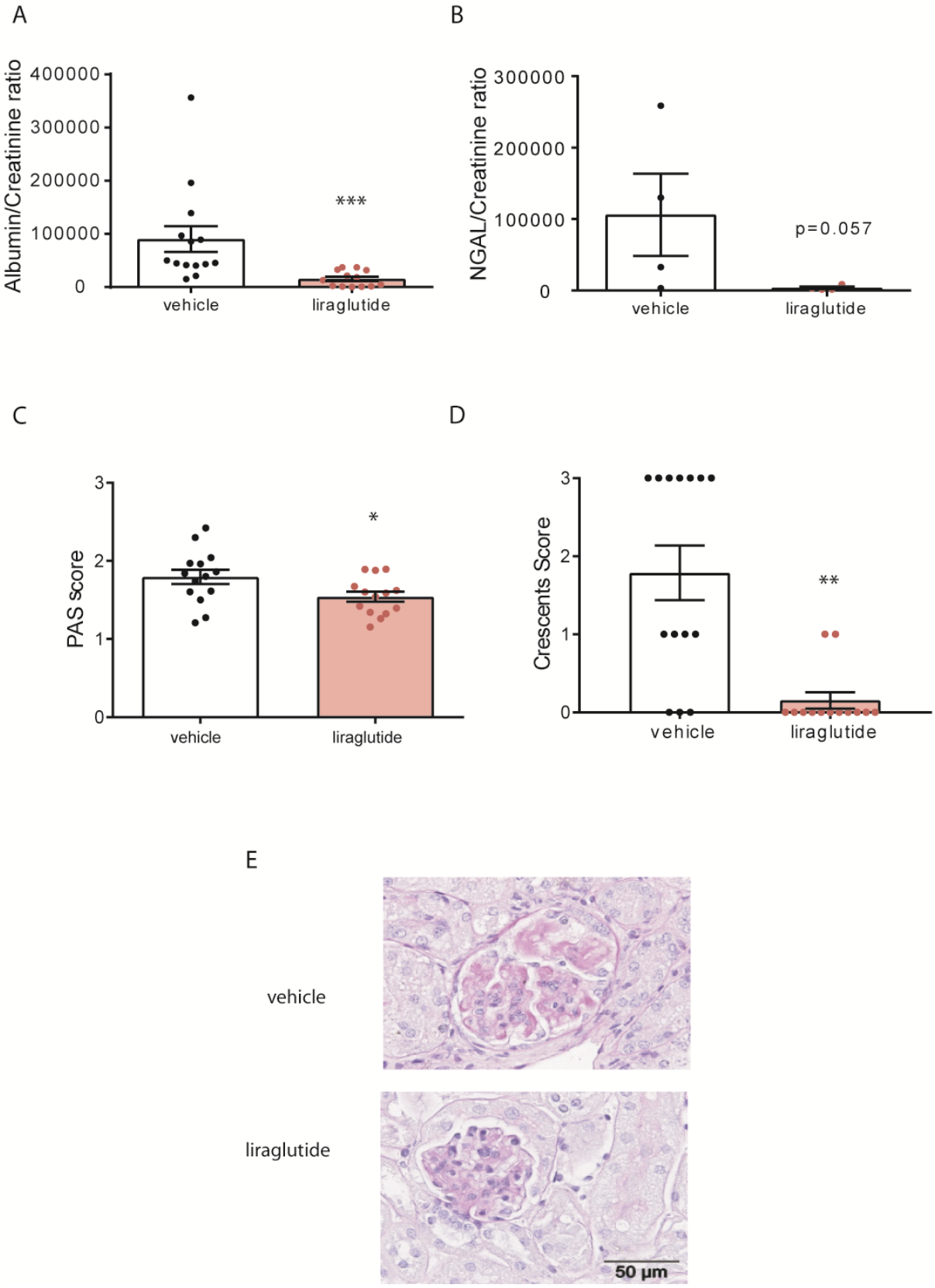


Figure 10: Mice treated with liraglutide demonstrated an ameliorated NTS phenotype

C57BL/6J 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 albumin/creatinine ratios (µg/mg) were evaluated on day 14 after NTS induction. (B) Urinary NGAL/creatinine ratios (pg/mg; n=4 per group) were evaluated also on day 14 after disease initiation. (C) Kidney sections were quantified for PAS positive deposits and for crescent formation (D). (E) Representative pictures of kidney sections after 14 days of NTS from mice treated with vehicle or liraglutide stained for PAS are shown. Magnification 400x. *$p<0.05$, **$p<0.01$. Reproduced from (1) with permission of publisher Elsevier.

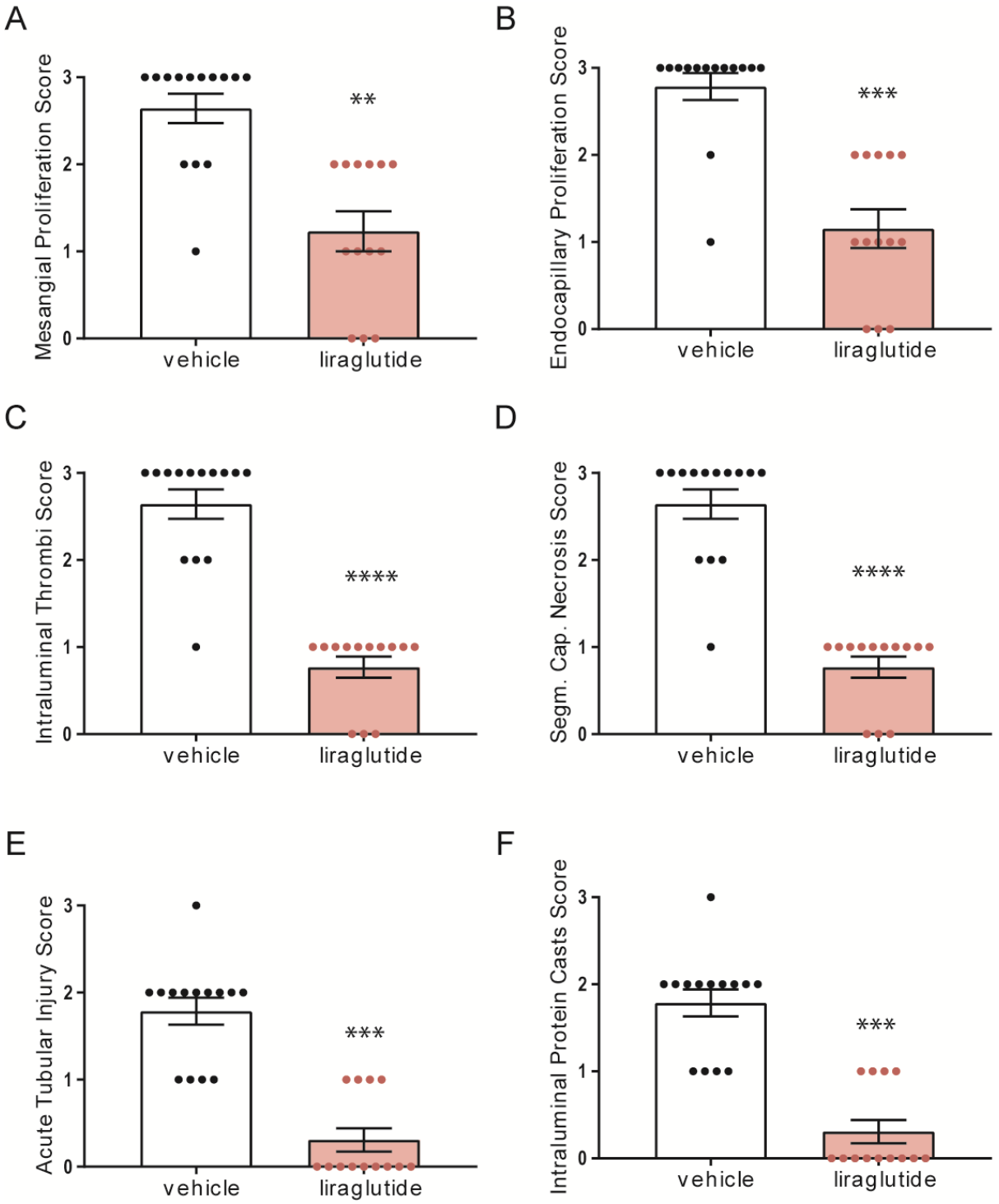


Figure 11: Histomorphological evaluation of renal pathology of mice treated with liraglutide

C57BL/6J mice were subjected to NTS for 14 days and treated with vehicle (n=14) or liraglutide (n=13) from the day of immunization. Kidneys were harvested and processed for PAS-staining. Stained kidney sections were quantified for mesangial (A) and endocapillary proliferation (B), intraluminal thrombi (C), segmental capillary necrosis (D), acute tubular injury (E) and intraluminal protein casts score (F). **p<0.01, ***p<0.001 and ****p<0.0001. Reproduced from (1) with permission of publisher Elsevier.

The infiltration of $CD4^+$ T cells, $CD8^+$ T cells and $CD68^+$ macrophages was significantly decreased in the kidneys from mice treated with liraglutide as compared to vehicle treated mice 14 days after NTS induction (Fig. 12B-E) (1). Regarding infiltrating PMNs, a trend towards less cells in the kidneys of the liraglutide group was detected (Fig. 12A) (1). In line with the differences in infiltrating immune cells, the expression of several inflammation related genes in the kidney was downregulated in mice treated with liraglutide. In detail, *Il-6, Il-10, Tnf-α, Tbet* and *FoxP3* had a lower relative expression in the kidneys of GLP-1R agonist treated mice than vehicle mice. No significant difference was noted in the expression of *Ifn-γ*, *Rorγt* and *Gata3* between the groups (Fig. 12F) (1).

Proliferating cells in kidneys of mice subjected to NTS for 14 days and treated with liraglutide or vehicle were detected by a PCNA staining. Both in the glomeruli and in the periglomerular region, proliferating cells were significantly decreased in the kidneys of liraglutide treated mice in comparison to controls (Fig. 13) (1).

Finally, we did not detect a difference in deposited mouse and rabbit IgG on the glomerular basement membrane of mice treated with liraglutide, 14 days after NTS induction (Fig. 14) (1).

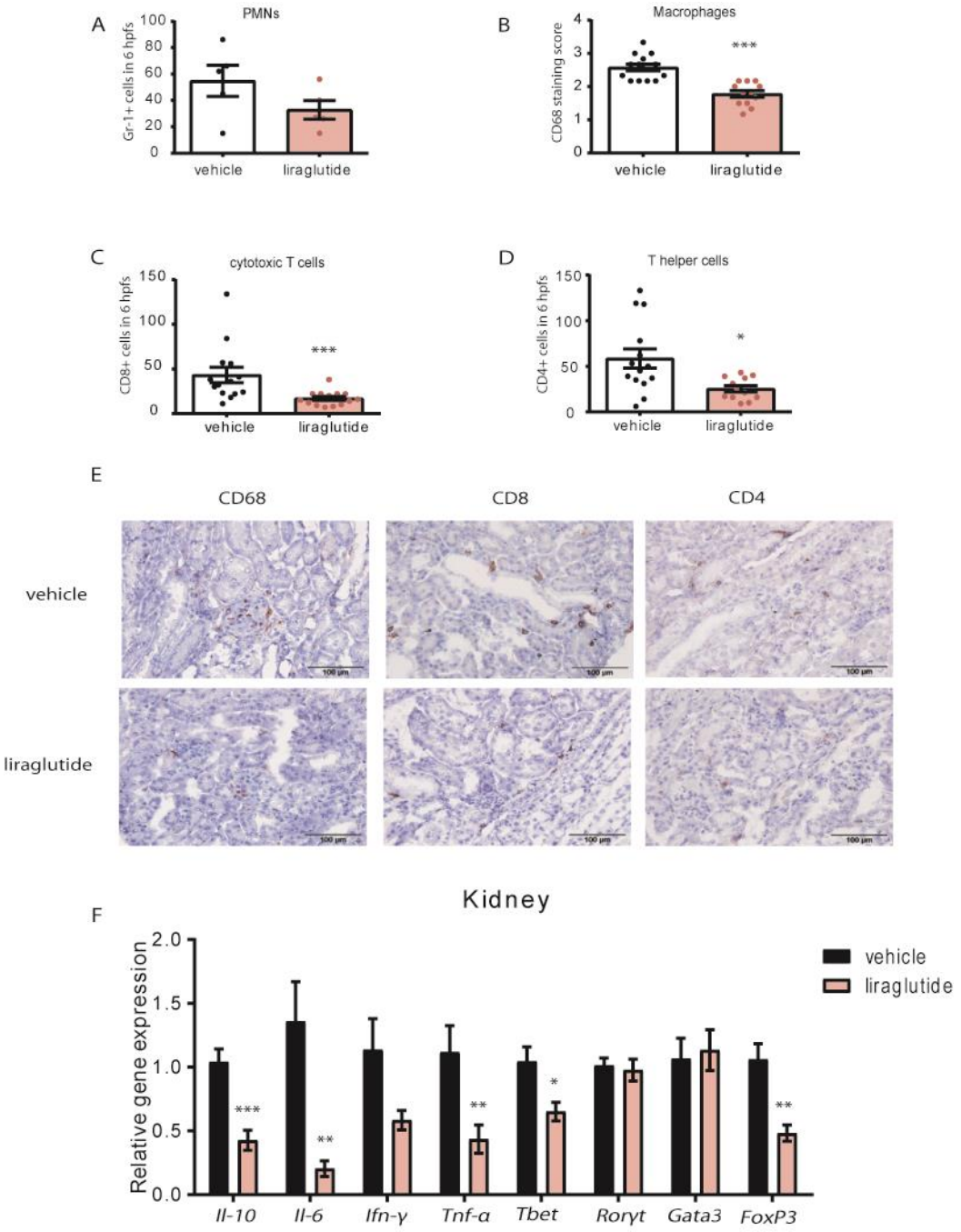


Figure 12: Effect of liraglutide on renal inflammatory cell infiltration and inflammatory gene expression in the kidney

C57BL/6J mice were subjected to NTS for 14 days and treated with vehicle (n=14) or liraglutide (n=13) from the day of immunization. The renal infiltration of (A) polymorphonuclear granulocytes (PMN), (B) $CD68^+$ macrophages, (C) $CD8^+$ cytotoxic and (D) $CD4^+$ T helper cells was evaluated. (E) Representative pictures from kidney sections stained for $CD68^+$, $CD8^+$ and $CD4^+$ cells after 14 days of NTS are shown. Magnification 200x. (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. *p<0.05, **p<0.01 and ***p<0.001. Reproduced from (1) with permission of publisher Elsevier.

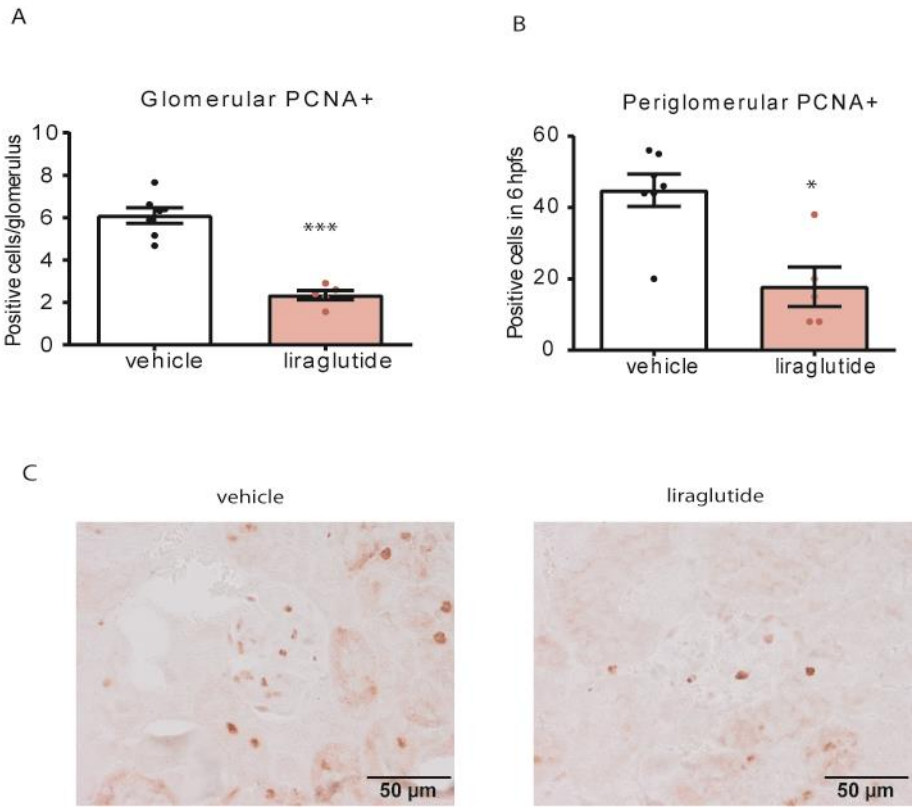


Figure 13: Liraglutide decreases proliferating cells in nephritic kidneys

Kidneys from C57BL/6J mice subjected to NTS for 14 days and treated with vehicle (n=6) or liraglutide (n=5) were stained for proliferating cells by performing a PCNA stain. (A) Positive cells in glomeruli and (B) the periglomerular region were counted. (C) Representative pictures for PCNA stain in the kidneys are shown. Magnification 400x. *p<0.05, **p<0.01, and ***p<0.001. Reproduced from (1) with permission of publisher Elsevier.

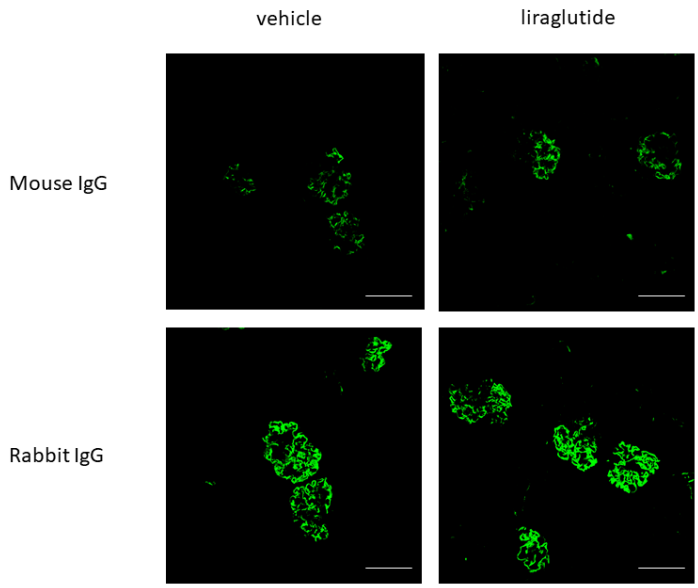


Figure 14: Deposition of mouse and rabbit IgG on the glomerular basement membrane of mice treated with liraglutide

Kidney sections from mice treated with vehicle or liraglutide and subjected to NTS for 14 days were stained with direct immunofluorescence staining and the deposition of autologous and heterolous IgG was evaluated. Representative pictures of the staining are shown. Magnification 400x. Reproduced from (1) with permission of publisher Elsevier.

Liraglutide treatment decreases the expression of fibrosis marker Col1A1 in the kidneys of mice subjected to NTS

The kidneys from Glp1r-/- mice and WT mice after 14 days of NTS, as well as kidneys from C57BL/6J mice treated with liraglutide or vehicle and subjected to NTS for 14 days were monitored for fibrotic changes associated with inflammation. The staining of kidney slides from all the above groups with Picro Sirius Red revealed no degree of fibrosis (Fig. 15B, D) (1). Interestingly though, when the mRNA expression of *Col1A1*, a marker of fibrosis, was checked in those kidneys, a significant downregulation was observed in the liraglutide group in comparison to the vehicle treated group (Fig. 15C) (1). No difference was found in the expression of *Col1A1* between the kidneys of WT and Glp1r-/- mice (Fig. 15A) (1).

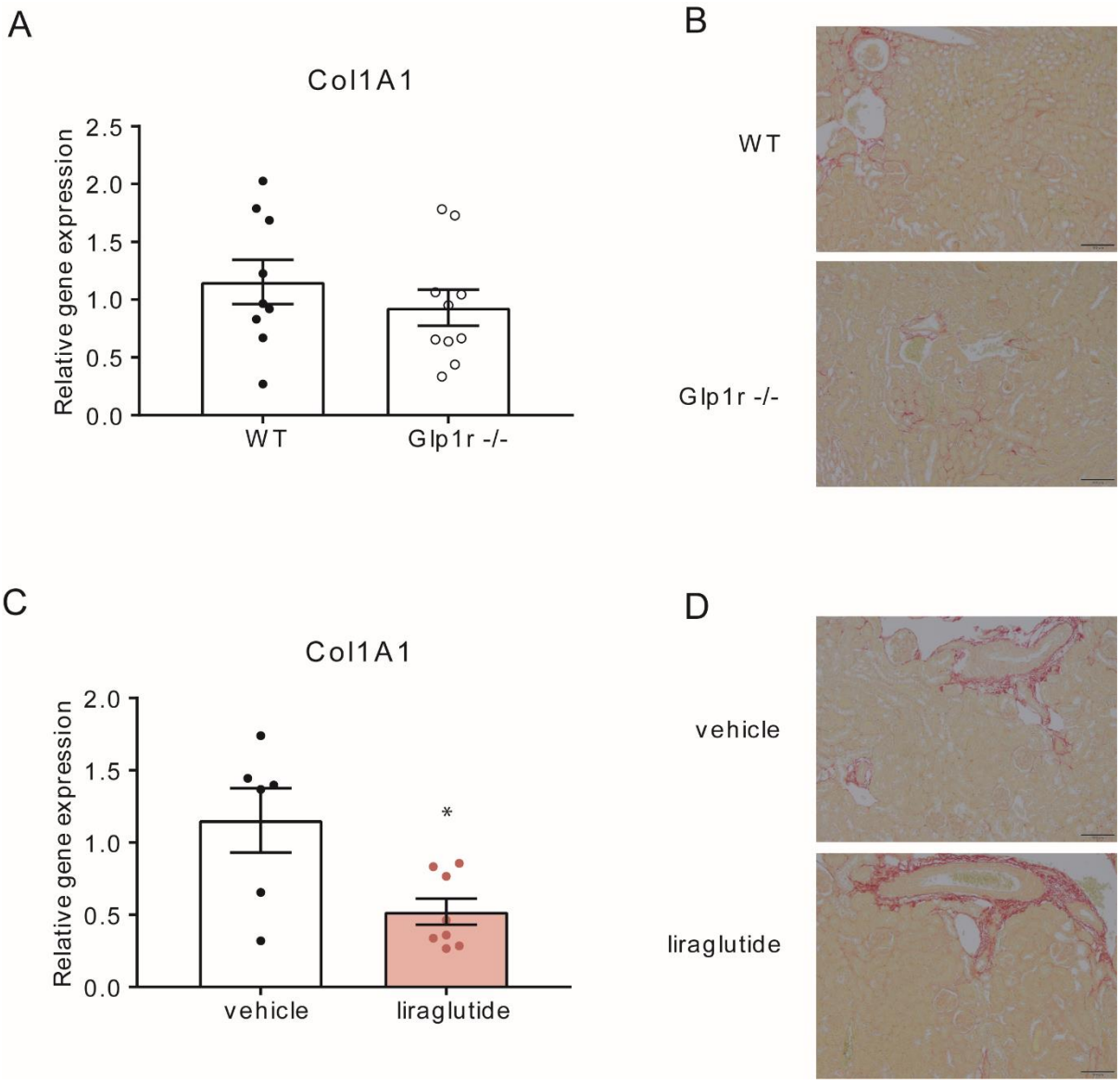


Figure 15: Liraglutide has a protective effect against renal fibrosis during NTS

WT and Glp1r -/- mice were subjected to NTS for 14 days (WT n=9, Glp1r -/- n=10). (A) The mRNA expression of *Col1A1* gene was evaluated in the kidneys of the mice. The fold increase compared to the mean mRNA expression in diseased WTs is provided. (B) Representative pictures of kidney sections of WT and Glp1r-/- mice after 14 days of NTS stained with Picro Sirius red staining. Magnification 200x. WT mice were subjected to NTS for 14 days and treated with vehicle (n=6) or liraglutide (n=8) from the day of immunization. (C) The mRNA expression of *Col1A1* gene was evaluated in the kidneys of the mice. The fold increase compared to the mean mRNA expression in vehicle-treated mice subjected to NTS is provided. (D) Representative pictures for Picro Sirius red stain in the kidneys are shown. Magnification 400x. *$p<0.05$. Reproduced from (1) with permission of publisher Elsevier.

Liraglutide does not influence the phenotype of NTS in Glp1r-/- mice

In order to investigate whether liraglutide protects again NTS acting via the Glp1r, Glp1r-/- mice were subjected to NTS for 14 days and treated with vehicle or liraglutide from the day of disease induction. No differences were noted in albuminuria between vehicle and liraglutide treated mice (Fig. 16A) (1). The study of infiltrating immune cells in the kidneys of those mice also showed no difference between the group (Fig. 16B-E) (1).

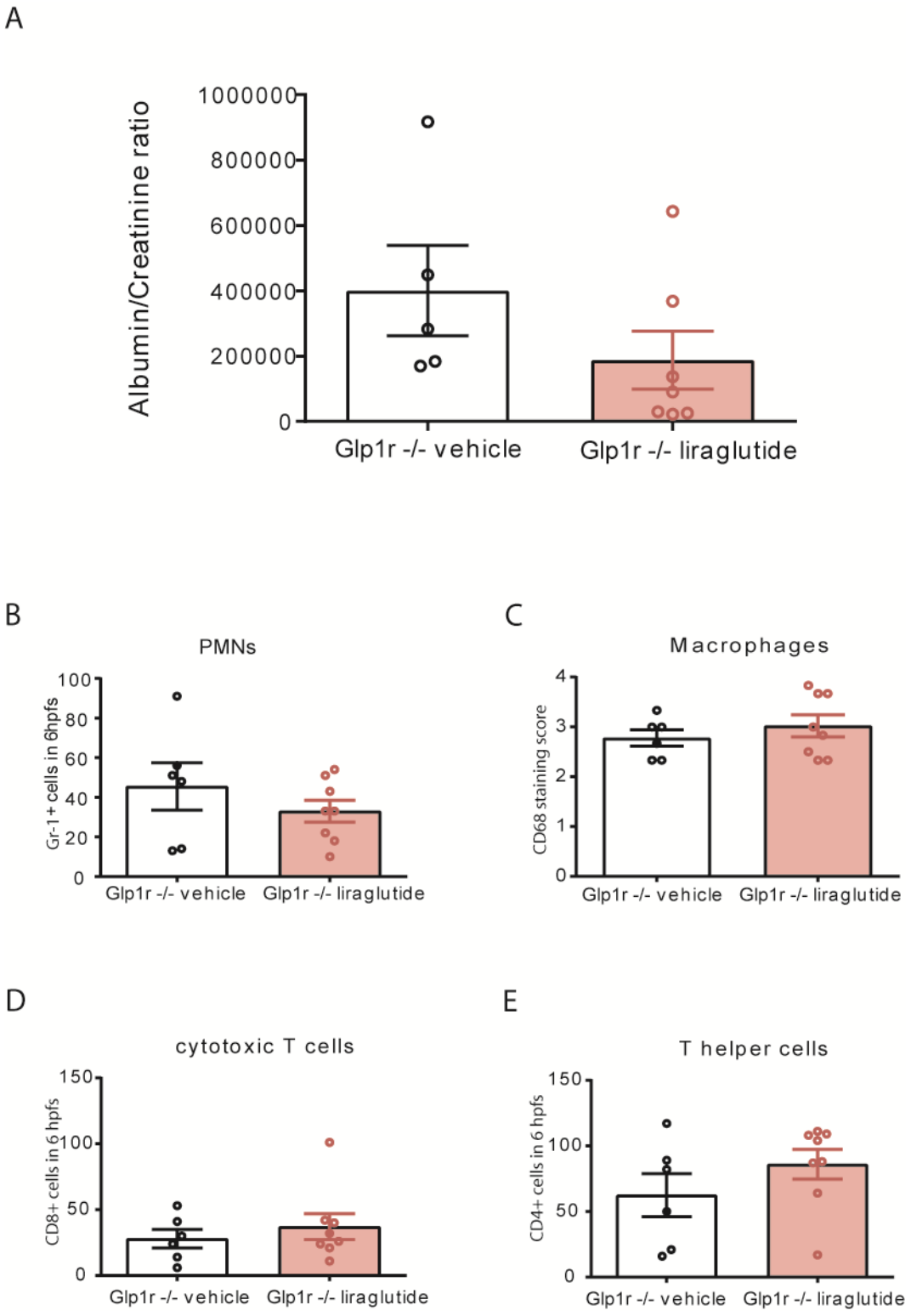


Figure 16: Liraglutide does not affect the NTS phenotype in Glp1r-/- mice

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) Albuminuria was evaluated on day 14 after NTS induction. The results are given as the ratio of urinary albumin to urinary creatinine (µg/mg). Kidney sections were analyzed for the infiltration of (B) PMNs, (C) $CD68^{+}$ macrophages, (D)

$CD8^+$ cytotoxic T and (E) $CD4^+$ TH cells. Reproduced from (1) with permission of publisher Elsevier.

Liraglutide treated mouse T cells proliferate less upon stimulation in comparison to untreated T cells

T cells were isolated from the spleens of C57BL/6J mice, stimulated with aCD3/CD28 and treated with liraglutide in a concentration of 60µg/ml for 72 hours. The proliferation of the cells and the cytokine levels in the supernatant were assessed. Liraglutide had a significant inhibiting effect of T cell proliferation (Fig. 17A) (1). To prove whether this effect of liraglutide on T cells is signalled via the Glp1r, we performed the same experiment with T cells isolated from Glp1r-/- mice. Additionally, we included a third group of T cells isolated by littermate WT controls, untreated. No differences were found in the proliferation between any of those three groups (Fig. 17B) (1). The study of the cytokine levels in the supernatant of liraglutide treated T cells revealed a significant decrease of Il-6 in comparison to the levels in the supernatant of untreated T cells (Fig. 17G) (1). 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. 17 C-F, H) (1).

To investigate which of the various T cell subsets are affected by the action of liraglutide, we polarized T cell from C57BL/6J mice to Th1 and Th17 phenotypes and treated them with liraglutide. In both subsets liraglutide significantly inhibited the proliferation of the cells (Fig. 17I) (1).

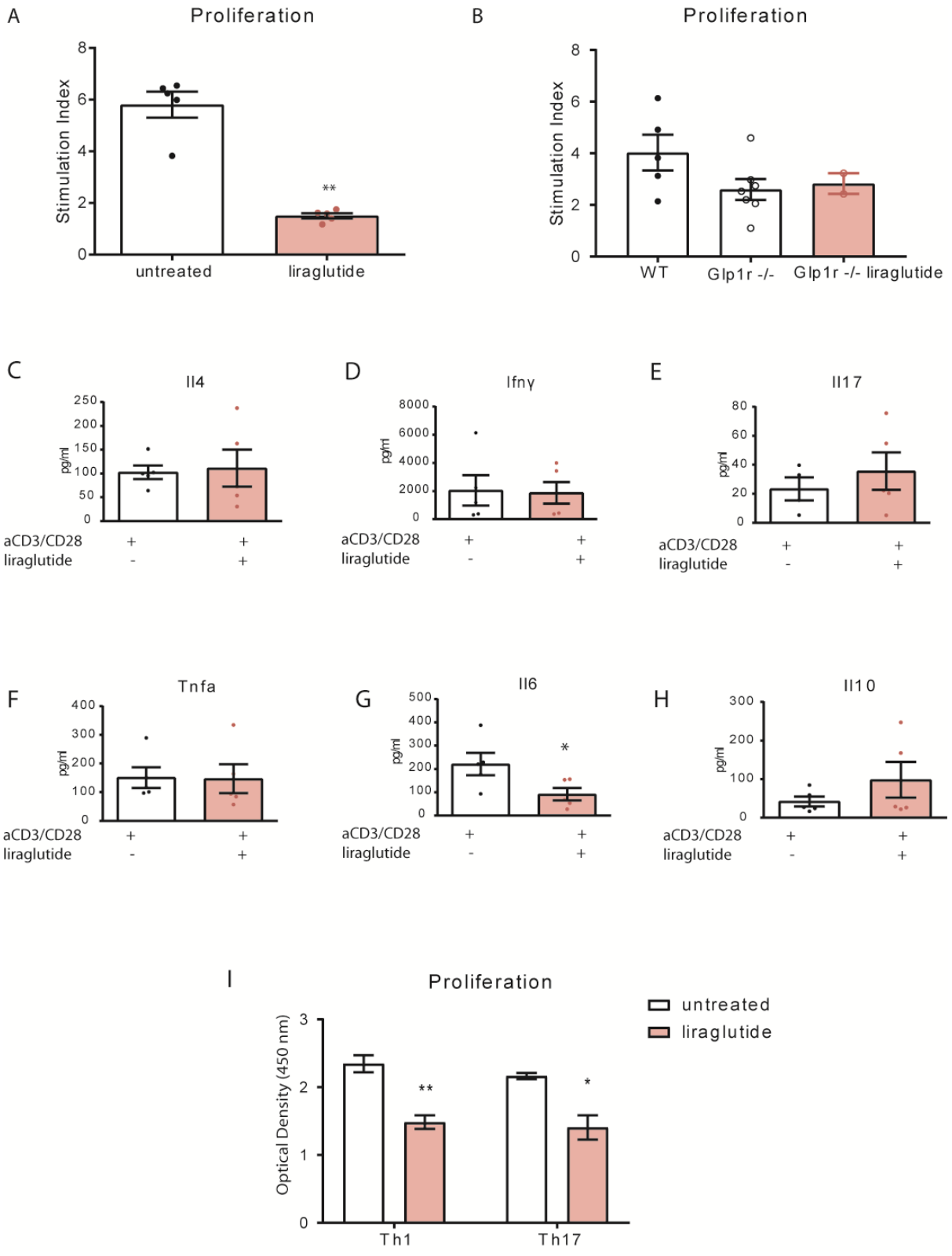


Figure 17: Liraglutide inhibits the proliferation of stimulated T cells and decreases Il-6 production

C57BL/6J mice were used for the isolation of T cells. The cells were then stimulated with aCD3/CD28 and treated with liraglutide in a concentration of 60µg/ml (n=5) or kept untreated

(n=5). The cells were kept in culture under these conditions for 72 hours. The same procedure was followed for T cells isolated from WT littermate controls (n=5), Glp1r -/- mice and treated with vehicle (n=7) and Glp1r-/- mice and treated with liraglutide (n=2). (A-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 ***$p<0.001$. Reproduced from (1) with permission of publisher Elsevier.

Liraglutide inhibits glycolysis in stimulated mouse T cells

The effect of liraglutide was then studied on T cell metabolism. A dramatic inhibition of glycolysis was noted in stimulated T cells treated with liraglutide (Fig. 18A) (1). In line with this finding, the mRNA expression of *Glut-1*, a marker of glycolysis, was significantly downregulated in T cells treated with the Glp1r agonist (Fig. 18B) (1).

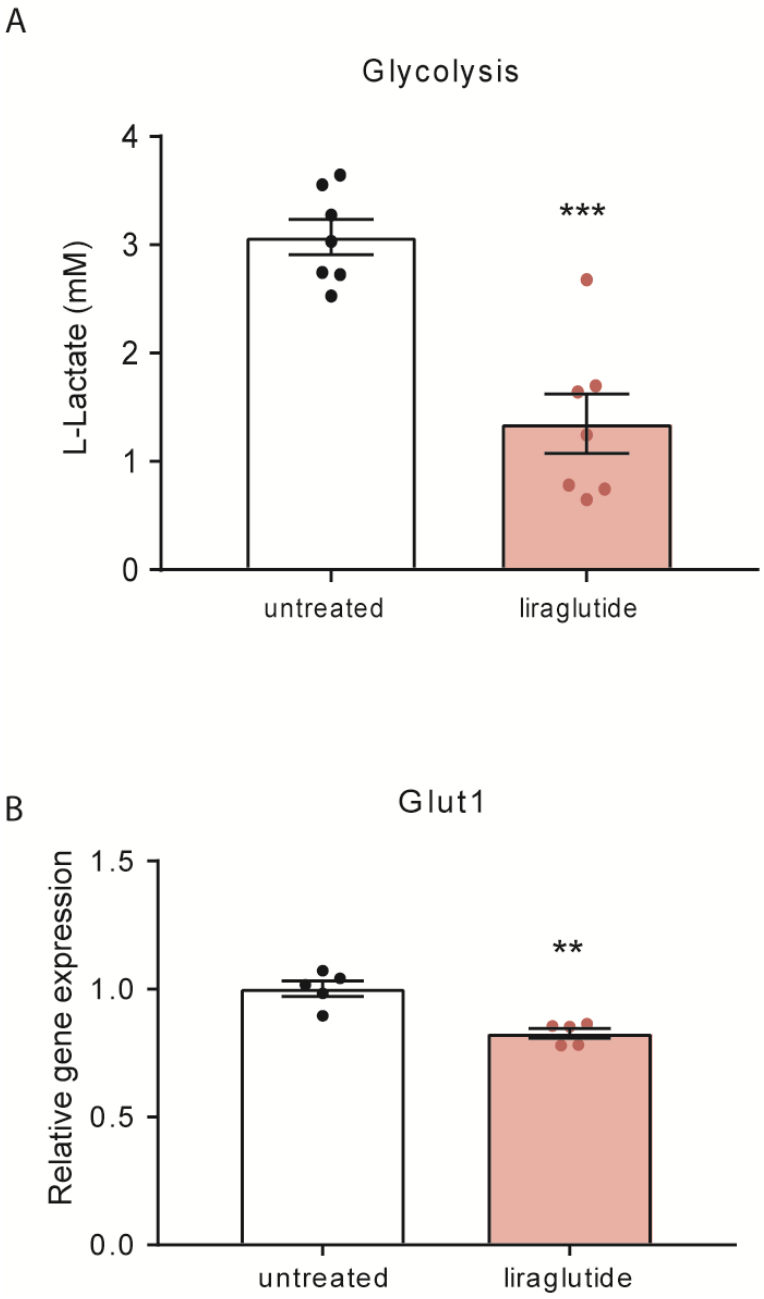


Figure 18: Effect of liraglutide on T cell glycolysis

C57BL/6J mice were used for the isolation of T cells. The cells were then stimulated with aCD3/CD28 and treated with liraglutide in a concentration of 60µg/ml (n=57or kept as untreated controls (n=7). The cells were kept in culture under these conditions for 72 hours (A) Glycolysis of cells was evaluated by measuring the production of L-lactate (mM). (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. Reproduced from (1) with permission of publisher Elsevier.

T cells transcribe the *Glp1r* mRNA

Due to lack of reliable antibodies against the Glp1r (1), a full expression profile of this protein is yet not available. In order to confirm the presence of the Glp1r in T cells, and different T cell subpopulations, we isolated RNA from pan T cells isolated from WT and Glp1r-/- mice directly or after CD3/CD28 stimulation, performed PCR and run the product on an agarose gel. As anticipated, T cells from WT mice proved to transcribe the *Glp1r* (Fig. 19) (1).

Additionally, after polarization to Th1 and Th17 phenotype, the cells still transcribed the receptor (Fig. 20A) (1). To confirm the success of the polarization, the supernatant of the cells was used to evaluate the protein levels of Ifn-γ and Il-17 (Fig. 20 B-C) (1).

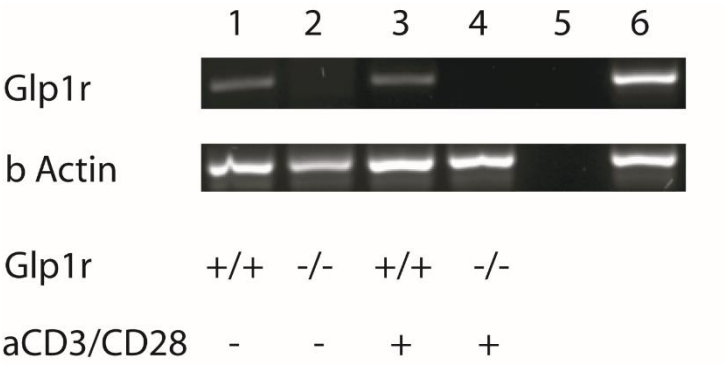


Figure 19: *Glp1r* is transcribed in T cells

T cells were isolated from C57Bl/6J mice (lane 1 and 3) and from Glp1r -/- (lane 2 and 4) mice. The cells were stimulated with aCD3/CD28 for 72 hours (lane 3 and 4) or directly subjected to RNA isolation (lane 1 and 2). PCR was performed to detect *Glp1r* and *β-Actin*. The samples were loaded on a 1% agarose gel as follows. Lane 1: C57Bl/6 T cells; lane 2: Glp1r -/- T cells; lane 3: C57Bl/6 T cells stimulated; lane 4: Glp1r -/- T cells stimulated; lane 5: negative control; lane 6: positive control (mouse brain tissue). Reproduced from (1) with permission of publisher Elsevier.

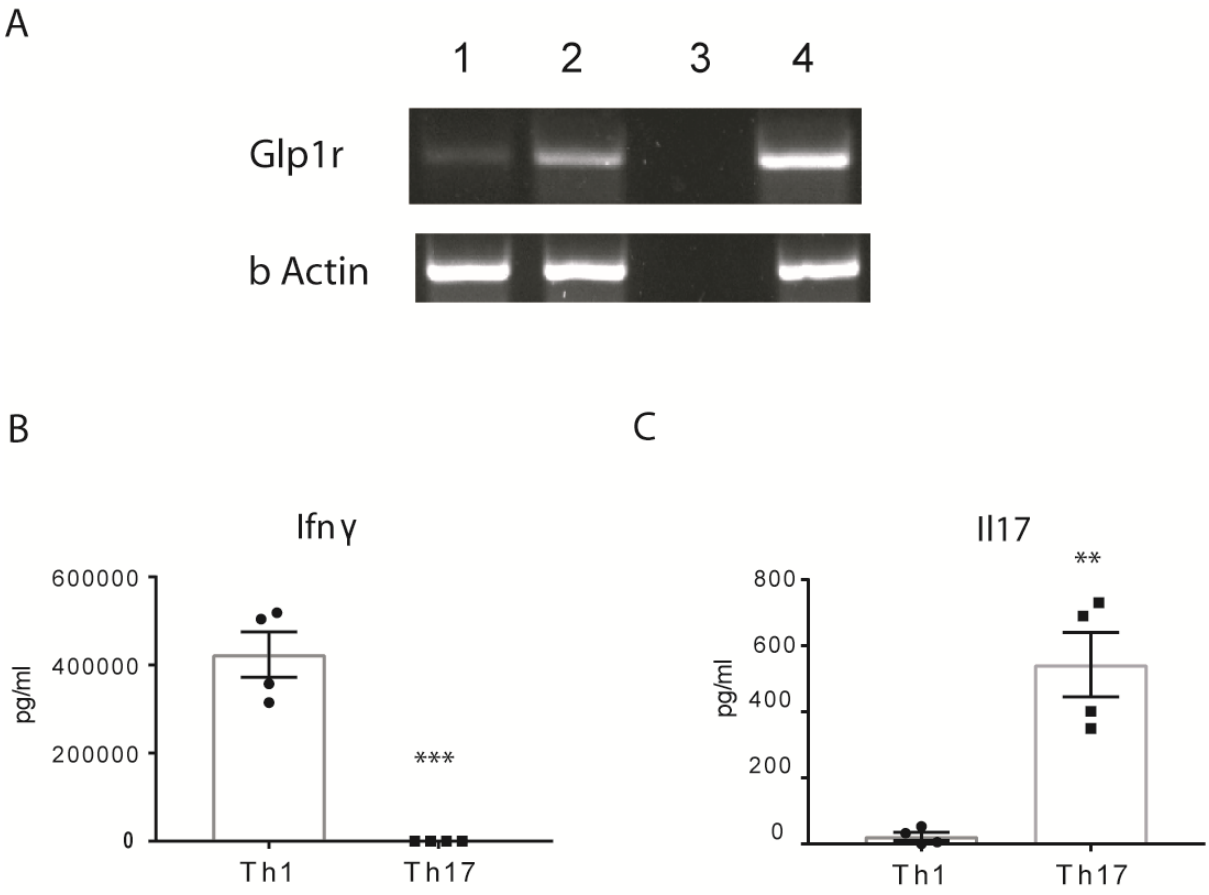


Figure 20: Glp1r expression in Th1 and Th17 cells

(A) RNA was isolated from Th1 (lane 2) and Th17 (lane 2) polarized cells. PCR was performed to detect *Glp1r* and *β-Actin*. The samples were loaded on an agarose gel in the following order. Lane 1: C57Bl/6 Th1 cells; lane 2: C57Bl/6 Th17 cells; lane 3: negative control; lane 4: positive control (mouse brain tissue). The supernatant of the cells was collected and the levels of (B) Ifn-γ and (C) Il17 were measured by ELISA. Reproduced from (1) with permission of publisher Elsevier.

Discussion

During the last couple of decades, more and more drugs from the group of Glp1r agonists are emerging and being approved in the USA and in Europe. Glp1r agonists are broadly used for the treatment of type 2 diabetes, while there is gradually increasing evidence for their beneficial effects beyond blood glucose lowering (144, 154, 155). Our data provide evidence that Glp1r agonism by liraglutide ameliorates the course of NTS, a T cell mediated kidney disease, by suppressing the proliferation of T cells. In addition, we hereby provide evidence of the multiple *in vivo* actions of liraglutide that go beyond blood glucose lowering (1).

The renal-protective effect of Glp1r agonists

Two large-randomized controlled trials using Glp1r analogues that have taken place in the last few years have shown a renal-protective effect of these drugs (136, 156), attributing a significant clinical relevance to our results. More specifically, LEADER (Liraglutide Effect and Action in Diabetes: Evaluation of Cardiovascular Outcome Results), a double-blind trial in type 2 diabetes patients with high CV risk that were daily treated with liraglutide or placebo, showed in the follow up study 3,8 year later that the renal outcome was observed in 268 patients out of 4668 in the liraglutide group and in 337 out of 4672 patients treated with placebo. In the double-blind trial named SUSTAIN-6 (Trial to Evaluate Cardiovascular and Other Long-term Outcomes With Semaglutide in Subjects With Type 2 Diabetes) during which patients with type 2 diabetes received semaglutide once per week for 2 years, 3,8% of the patients in the treated group developed new or worsening nephropathy, while the percentage was up to 6,1% in the patients treated with placebo (157). These outcomes are mainly attributed to a decreased rate of new onset of persistent macroalbuminuria (158-160). Nevertheless, the mechanism responsible remains unclear and the possibility of an anti-inflammatory effect by Glp1r agonism playing a crucial role is often discussed (143). Based on the above, we consider the NTS model to be ideal to address our research questions as it is a model with rapid development of macroalbuminuria that depends on pro-inflammatory responses in normal glycemic conditions (88). Therefore, throughout our experiments we were able to have a view focused

on the anti-proteinuric and anti-inflammatory capacity of Glp1r agonism, leaving out its effect on blood glucose levels.

The anti-inflammatory and immunoregulatory role of Glp1r agonism

Already in 2010, Hadjiyanni et al. showed that peripheral lymphocytes of Glp1r -/- mice hyperproliferate in comparison to cells from wild type mice, when treated with mitogenic stimuli (148), indicating so an anti-inflammatory and immunomodulatory role for Glp1r agonism. In agreement with their observations, in our hands Glp1r agonism by liraglutide suppressed the proliferation of T cells *in vitro* (1). This outcome was further supported by our *in vivo* experiments, where fewer proliferating cells were detected in the glomeruli and in the periglomerular region of animals subjected to NTS when treatment with liraglutide was applied (1). Of note, these are locations where mainly immune cells infiltrate during disease (37, 96). Moreover, splenocytes isolated from Glp1r-/- mice after 14 days of NTS and stimulated *in vitro* with rabbit IgG proliferated more in comparison to controls. The anti-inflammatory and immunomodulatory role of Glp1r agonism was further supported by our finding that in lymph nodes and spleens of Glp1r-/- mice after 14 days of NTS, Th1 cytokine transcripts were significantly increased. Since that was not the case for Th17-related transcripts, such as their transcription factor Rorγt, which remained unaltered, we were initially led to the hypothesis that the noted effect influences more Th1 than Th17 cells. Nevertheless, when we polarized T cells to Th1 and Th17 cells, both populations expressed Glp1r mRNA and when treated with liraglutide *in vitro* both T cell populations demonstrated a decreased proliferation. This finding suggests that Th1 and Th17 cells are equally inhibited by Glp1r agonism with liraglutide.

We then questioned whether the discovered effect of liraglutide on proliferation is signaled via the Glp1r. Therefore, we isolated T cells from Glp1r-/- mice, treated them with liraglutide and studied their proliferating ability. Since no difference was found in the proliferation of the cells when treated with the Glp1r agonist in comparison to controls, we can safely conclude that liraglutide inhibits the proliferation of T cells by acting via the Glp1r.

Glp1r agonism and Tregs

Glp1r agonism has been discussed to apply its anti-inflammatory effect by increasing the number of peripheral Tregs (148, 150, 161). Treatment with Exenatide, a Glp1r agonist, has been shown to increase the frequency and function of Tregs in the spleens of NOD ice with diabetes (150). In agreement with this finding, male Glp1r-/- mice demonstrate a lower percentage of peripheral Tregs in their lymph nodes, although no differences can be found in the Tregs from the spleen or in the peripheral Tregs from female Glp1r-/- mice (148). As it is well proven that Tregs play a crucial role in limiting disease activity in NTS (86, 93, 95, 96), we considered it of great importance to study the number of Tregs in Glp1r-/- mice and liraglutide-treated compared to respective controls after NTS induction. Nevertheless, no differences were observed in the frequency of these cells in the lymph nodes of the studied groups (1). This finding suggests that the effect of liraglutide on NTS phenotype may not be attributed to Tregs. Although our data offer only evidence for the number of Treg cells in the lymph nodes and not directly for their actual function, we consider it to be adequate evidence. It has been proven already fourteen years ago that when CD4+CD25+Tregs are transferred into mice before the induction of NTS, the cells have the ability to migrate to the lymph nodes, and surprisingly not the inflamed kidney, and from there limit the disease activity (94). Additionally, CCR7 knock-out mice that were shown to be more susceptible to NTS, demonstrated as well a significantly lower number of Tregs in their lymph nodes, further indicating the way of action of the Tregs and supporting that their correct localization to the draining lymph nodes is necessary for the disease inhibition to occur (96).

The effect of liraglutide on immune cell glycose metabolism

There is increasing evidence supporting the importance of glucose metabolism on the function of immune cells (162). When clonal proliferation is initiated, Th1 and Th17 cells have been shown to switch their glucose metabolism to glycolysis, while this is not the case for Tregs, for which lipid oxidation via AMPK and oxidative phosphorylation are the predominant metabolic programs (162). In our hands, T cells stimulated *in vitro* and treated with liraglutide produced significantly less L-lactate in comparison to untreated stimulated T cells (1). These data suggest

a change in the metabolic program of T cells in the presence of liraglutide, which leads to the inhibition of glycolysis. Further experiments are needed in order to fully understand the mechanism that leads to this blockade of glycolysis, though a possibility is that it is mediated by the downregulation of the glucose transporter Glut1, a major glucose transporter located primarily in the cell membrane and on the cell surface. Contradictory effects have been described for Glp1r agonism in other cells types such as pancreatic β cells and cardiomyocytes (163-165). More in detail, exenatide was shown to induce Glut1 translocation in atrial human myocardium and based on that the authors assume an insulin-independent enhance in glucose uptake (164). Almost a decade earlier, an increase in myocardial glucose uptake in rats induced by Glp1r had been observed, accompanied by the increase of Glut1 protein expression levels in the cell membrane (165). In pancreatic b cells the application of exenatide over 18 hours promoted significant metabolic reprogramming of the cells with upregulation in glycolytic metabolism (163). These findings do not come in contrast to ours, since they are on different cell types, but they do bring up further questions on the mechanism of action of Glp1r agonism on glucose metabolism. Further experiments would help us interpret the results better and understand the mechanism that lies behind the effect we discovered. In the same experimental setup in which we noted the decrease in L-lactate production, a more detailed study in glycolytic metabolism and function could give us important input. The rates of glucose uptake and consumption as well as a profiling for the mRNA and protein expression of different glycolytic enzymes would be highly informative.

The unexpected phenotype of Glp1r-/- mice

After a thorough study of the phenotype of NTS in Glp1r-/- mice we came to the rather puzzling conclusion that Glp1r-/- mice do not present an aggravated phenotype of the disease, as we would have expected based on the significant reduction of NTS severity by liraglutide (1). The finding that healthy Glp1r-/- mice display decreased lymph node cytokine transcripts indicates an altered immune system, when compared to wild type mice, which suggests the possibility of a delayed development of NTS in these mice. We therefore believe that this could be the reason for the lack of a more severe NTS phenotype in Glp1r-/- mice. This theory is further supported by the findings by Hadjiyanni et al., who showed that although peripheral T cells

from Glp1r-/- mice are hyperproliferative, Glp1r-/- thymocytes demonstrate a severely decreased proliferation (148).

Further evidence for the effects of Glp1r agonism

Our study is not the first one to suggest that Glp1r agonism can improve T cell mediated diseases. Data have already been published about the effect of exendin-4 or liraglutide in T cell mediated murine disease models like experimental autoimmune encephalitis and the model of non-obese diabetic mice (161, 166, 167). Taken together these data and our findings open the field for new applications for Glp1r agonism in inflammatory, T cell-mediated diseases. Nevertheless, due to the lack of clinical data and the increased risk for side effects caused by Glp1r agonists, such as ketosis, the clinical applications have to be critically addressed. Furthermore, our data and the anti-inflammatory effect of liraglutide that has been described may give a new explanation to the reno-protective effect that has been described in large randomized control trials with patients suffering from type 2 diabetes (132, 136, 156). Immune cells, including also T cells, have been discussed to play a role in the development of insulin resistance and in diabetic nephropathy, based on data from pre-clinical studies (168, 169). Interestingly, Tregs were found to significantly improve insulin sensitivity and diabetic nephropathy both in obese patients with insulin resistance and in the murine model of *db/db* mice (168). Furthermore, interesting outcomes immerged from a large multinational, randomized phase 3 clinical trial which enrolled patients with type 2 diabetes and stage 4 chronic kidney disease called Bardoxolone Methyl Evaluation in Patients with Chronic Kidney Disease and Type 2 Diabetes (BEACON). Although the BEACON trial had to be terminated earlier due to the preliminary analyses showing that patients randomized to bardoxolone, an immune-modulatory drug, experienced significantly higher rates of heart failure events, a post-hoc analysis meant to study the changes in kidney function showed that bardoxolone preserved kidney function and possibly delayed the onset of end-stage renal disease (170, 171).

Glp1r agonism and the RAS system

Glp1r agonism has been shown to suppress the activation of the renin-angiotensin-aldosterone system and resolve in increased natriuresis and decreased blood pressure. Exendin-4 has been shown to have a natriuretic effect in mice that is mediated via the Glp1r (172), while liraglutide reduces blood pressure and promotes aortic relaxation in wild type but not in Glp1r-/- mice (173). This inhibition of the activation of the renin-angiotensin-aldosterone system that is proven by these studies, could be partly the reason for the renal improvement noted in mice subjected to NTS and treated with liraglutide. In humans, a network meta-analysis from 2015 suggested that liraglutide and Exenatide induced a modest but significant reduction in blood pressure compared to placebo in patients with type 2 diabetes (174). Another interesting study was performed by Lovshin and coworkers and focused on the haemodynamic effect of liraglutide in hypertensive patients with type 2 diabetes. They showed that the administration of liraglutide for 3 weeks resulted in significantly increased 24-hour and nighttime urinary sodium excretion but 24-hour systolic blood pressure was not altered (175). Based on this finding and since in our experiments we also treated our mice for a limited period with liraglutide, we hypothesize that the renal protective effect of liraglutide in NTS is not caused by a lowered blood pressure. The fact that the knocking-out of the Glp1r in mice does not affect their blood pressure when they are healthy further supports this view. In terms of natriuresis, an initial evaluation of total sodium secretion in the urine of Glp1r -/- mice subjected to NTS showed significantly lower values than in WT mice, but after a more detailed study of our results and with the calculation of sodium/creatinine ratio and of fractional sodium in the urine, we conclude that the above mentioned difference is due to the reduced urine volume in Glp1r -/- mice. Though the significantly lower urine secretion in Glp1r -/- mice subjected to NTS brings up further questions, in the above described experimental setup, no difference in natriuresis was detected between WT and Glp1r -/- mice. Nevertheless, the possibility that an increase in natriuresis and an effect in blood pressure might play a role in the effects of Glp1r agonism cannot be yet excluded, before further experiments are performed. A thorough study with C57BL/6J mice subjected to NTS, treated with liraglutide or vehicle and monitored for natriuresis and blood pressure for the whole period of the disease progression, would help to bring light to the arising questions. Daily blood pressure measurements from the day of immunisation till the evening before sacrificing on day 13 would give us concrete evidence on whether the noted effect of liraglutide is related with alterations in blood pressure or not.

Additionally, sodium secretion in the urine should be measured during specific interim timepoints (for example day 0, day 7 and day 14 of disease) to monitor natriuresis in the liraglutide treated mice. Of great importance would be to nail down the activation of the RAS system during the disease progression. Commercial ELISA kits for the assessment of renin and/or aldosterone in plasma samples of different timepoints could be used and angiotensin in peripheral blood could be measured with mass-spectrometry. It would also be highly informative to perform the same measurements and evaluations for WT and Glp1r -/- mice subjected to NTS. This would add up to our already obtained data on natriuresis in these mice on day 14 of NTS.

Conclusion

Taken together, the present study provides evidence that Glp1r agonism by liraglutide ameliorates the NTS phenotype by suppressing the proliferation of T cells. Our data can further explain the renal-protective effects of Glp1r agonism that have been observed in large clinical trials with type 2 diabetes patients. Furthermore, we believe that our findings provide the possibility to develop novel therapeutic options for T cell-mediated diseases.

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