PhD Thesis

The Role of Prostaglandin E2 Receptor 4 (EP4) in the Murine Model of Nephrotoxic Serum Nephritis

Submitted by
Dr.med.univ.
Ida ARINGER

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Division of Nephrology
Department of Internal Medicine
and
Institute of Experimental and Clinical Pharmacology

under the Supervision of
Assoz.Prof. PD. Dr. Kathrin ELLER
Univ.-Prof. Dr. Akos HEINEMANN

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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 at the Medical University of Graz".

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I have to thank all co-authors who contributed to this work. All co-authors have explicitly agreed to the use of their data in this thesis.

Katharina Artinger,1 Alexander H. Kirsch,1 Corinna Schabhüttl,1,3 Katharina Jandl,2,4 Thomas Bärnthaler,2 Agnes A. Mooslechner,1 Sereina A. Herzog,5 Moritz Uhlig,1 Andrijana Kirsch,6 Saša Frank,6 Miriam Banas,7 Marion Pollheimer,8 Philipp Eller,4 Alexander R. Rosenkranz,1 Akos Heinemann,2 and Kathrin Eller1

1 Clinical Division of Nephrology, Department of Internal Medicine, Medical University of Graz, Graz, Austria; 2 Division of Pharmacology, Otto Loewi Research Center, BioTechMed Graz, Medical University of Graz, Graz, Austria; 3 Intensive Care Unit, Department of Internal Medicine, Medical University of Graz, Graz, Austria; 4 Ludwig Boltzmann Institute for LungVascular Research, Graz, Austria; 5 Institute for Medical Informatics, Statistics and Documentation, Medical University of Graz, Graz, Austria; 6 Gottfried Schatz Research Center, Molecular Biology and Biochemistry, Medical University of Graz, Graz, Austria; 7 Clinical Division of Nephrology, Department of Internal Medicine, University Hospital Regensburg, Regensburg, Germany; and 8 Institute of Pathology, Medical University of Graz, Graz, Austria

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EIDESSTATTLICHE ERKLÄRUNG

Ich erkläre hiermit ehrenwörtlich, dass ich diese Arbeit selbstständig verfasst habe, und dass jene Personen und Organisationen, die an dieser Arbeit beteiligt waren, namentlich genannt sind. Benutzte Quellen wurden kenntlich gemacht, und ich
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Advanced glycation end products (AGE)

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CXC- chemokine receptor (CXCR)

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F

Fetal calve serum (FCS)

Focal segmental glomerulosclerosis (FSGS)

G

Glomerular basement membrane (GBM)

Glomerular filtration rate (GFR)

Glomerulonephritis (GN)

Granulomatosis with polyangiitis (GPA)

Granulocyte colony stimulating factor (G-CSF)

H

Haemolytic uremic syndrome (HUS)

High power field (HPF)

Human leukocyte antigen (HLA)

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I

Intercellular adhesion molecule-1 (ICAM-1)

Immunoglobulin (Ig)

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Intravenously (i.v.)

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L

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M

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Reverse transcription (RT)

Rheumatoid arthritis (RA)

S

Serum amyloid A protein (SAA)

Sodium–glucose cotransporter 2 (SGLT2)

Standard error of the mean (SEM)

Subcutaneously (s.c)

Sjogren'ssyndrome (SS)

Systemic Lupus erythematosus (SLE)

Systemic Lupus International Collaborating Clinics (SLICC)

T

Transforming growth factor-beta (TGF-β)

Transforming growth factor-β1(TGFβ1)

T helper cell (TH)

T-helper 1 cell (TH1)

T-helper 2 cell (TH2)

T-helper 17 cell (TH17)

T-helper 9 cell (TH9)

Regulatory T cells (Treg)

Follicular helper T cell (Tfh)

Soluble TNF receptor (sTNFr)

Thromboxane A2 (TXA2)

Thrombotic thrombocytopenic purpura (TTP)

Thrombotic microangiopathy (TMA)

Toll-like receptors (TLRs)

Tubulointerstitial nephritis with uveitis (TINU)

Tumor-necrosis factor alpha (TNFα)

Regulatory T cell (Treg)

U

Unilateral ureteral obstruction (UUO)

V

Vascular endothelial growth factor (VEGF)

ABSTRACT IN GERMAN

Es ist bekannt, dass Prostaglandin E2 (PGE2) entzündliche und vaskuläre Signale moduliert. Selektive Agonisten oder Antagonisten von einzelnen PGE2 Rezeptorsubtypen (E-Typ-Prostanoid-Rezeptoren, EP) könnten vielversprechende neue therapeutische Optionen bei immunvermittelten Erkrankungen wie der Glomerulonephritis sein. Daher haben wir den therapeutischen Effekt eines selektiven EP4 Rezeptor Agonisten und Antagonisten im Mausmodell der nephrotoxischen Serumnephritis (NTS) untersucht. Es erfolgte eine *in-vivo*-Behandlung mit zwei verschiedenen Dosierungen eines EP4-Agonisten (ONO AE1-329 [280 oder 1000 ug/kg Körpergewicht/Tag]), sowie Antagonisten (ONO AE3-208 [10 mg/kg Körpergewicht/Tag]) oder Vehikel für 10 oder 14 Tage nach NTS Induktion. Darüber hinaus wurde EP4 in murinen distalen Tubuluszellen stimuliert oder blockiert. *In vivo* führte die höhere Dosis des EP4-Agonisten zu einem verbesserten NTS-Phänotyp. Es zeigte sich eine verminderte renale Immunzellinfiltration; dies könnte auf eine Minderperfusion der Niere im Rahmen der rezidivierenden hypotensiven Episoden durch die höhere Dosis des EP4-Agonisten zurückgeführt werden, im Sinne einer ischämischen Präkonditionierung. Darüber hinaus erhöhte es signifikant die *in vivo* und *in vitro* Proliferation von distalen Tubuluszellen. Diese Wirkungen waren dosisabhängig, da die Behandlung mit dem Niedrigdosis-Agonisten zu weniger ausgeprägten Hypotonie-Episoden und einem mit Vehikel-Kontrollen vergleichbaren Phänotyp führte. Interessanterweise hatte die Behandlung mit EP4-Antagonisten keine Auswirkungen auf den Blutdruck und verbesserte ebenfalls den NTS-Phänotyp signifikant. Außerdem wurde der verbesserte NTS-Phänotyp auch beobachtet, wenn die Behandlung 4 Tage nach der NTS-Induktion begonnen wurde. Es verringerte die Expression (C-x-c-Motiv Chemokin-Ligand) in Tubuluszellen, wodurch sich die Neutrophilen-Infiltration in das Interstitium der Niere vermindert zeigte. Zusammengefasst sind die blutdrucksenkenden Effekte des EP4-Agonisten ein limitierender Faktor für einen therapeutischen Einsatz. Im Gegensatz dazu, hat der EP4-Antagonist keine Wirkung auf den Blutdruck und verbessert den NTS-Phänotyp durch Verringerung der CXCL1- und CXCL5-Produktion in tubulären Zellen gefolgt von einer reduzierten renalen Infiltration von neutrophilen Granulozyten.

ABSTRACT IN ENGLISH

The lipid molecule and cyclooxygenase (COX) product prostaglandin E2 (PGE2) acts on four different G protein-coupled receptors namely EP1 to 4. EP4 receptors are expressed on different immune cells, resident kidney cells (epithelial cells of the glomerulus, mesangial cells, afferent arteriole, collecting duct, proximal and distal tubular cells) and endothelial cells. These cells play a crucial role in the pathophysiology of immune-mediated diseases such as glomerulonephritis. Thus, selectively targeting the receptors of PGE2 (E-type prostanoid receptors) might be an attractive new therapeutic option in the treatment of glomerulonephritis. Therefore, we tested EP4 receptor agonists and antagonists in a murine model of nephrotoxic serum nephritis (NTS), which reflects immune-complex nephritis in humans. *In vivo* treatment with two different doses of an EP4 agonist ONO AE1-329 [280 or 1000 µg/kg bw/day], antagonist ONO AE3-208 [10 mg/kg bw/day] or vehicle was performed for 10 or 14 days of NTS. EP4 was stimulated or blocked on murine distal convoluted tubular epithelial cells *in vitro*. *In vivo*, the higher dose of the EP4 agonist led to an improved NTS phenotype due to recurrent hypotensive episodes resulting in ischemic preconditioning. This was accompanied by decreased renal infiltration of immune cells. Furthermore, it significantly increased tubular cell proliferation *in vivo* and *in vitro*. These effects were dose-dependent since treatment with the low-dose agonist resulted in less pronounced episodes of hypotension and a comparable phenotype to vehicle controls. Interestingly, EP4 antagonist treatment did not have any effects on blood pressure and significantly improved the NTS phenotype. Remarkably, the improved NTS phenotype was also observed when treatment was started 4 days after NTS induction. It decreased tubular chemokine (C-X-C motif) ligands CXCL1 and CXCL5 expression and reduced interstitial neutrophil influx into the kidney. Taken together, the hemodynamic effects of EP4 agonism limit its therapeutic use in NTS. In contrast, EP4 antagonism has no effect on blood pressure and improves the NTS phenotype, most likely by decreasing CXCL1 and -5 production in tubular cells and thereby reducing renal neutrophil infiltration.

1. INTRODUCTION

1.1. CHRONIC KIDNEY DISEASE (CKD)

Definition

The 2017 'Kidney Disease – Improving Global Outcome (KDIGO) clinical guidelines on the diagnosis and management of chronic kidney disease (CKD)' define CKD according to the presence of kidney damage and the reduced level of kidney function, irrespective of the type of kidney disease, which persist more than 3 months and have implications for health (2). A minimum period of time of 3 months, during which these abnormalities persist, was introduced in order to distinguish acute from chronic kidney disease. CKD is classified by cause, glomerular filtration rate (GFR) and category of albuminuria. GFR varies according to sex, race, body size, serum creatinine and decreases with age. The GFR is the best overall estimation of primary filtrate produced during a given period of time (3). GFR in CKD is classified into six stages (CKD G1, 2, 3a, 3b, 4 and 5) (see table 1). It must be mentioned that in the stage CKD G3 there is a separation between G3a and G3b due to the fact that the risk of cardiovascular mortality and hospitalization rate increases especially in stage CKD G3b and higher (4).

Table 1 - CKD Stages

GFR category	GFR (ml/min/1.73 m^2)
G1	>90
G2	60–89
G3a	45–59
G3b	30–44

G4	15–29
G5	<15

Adapted from ref. (4)

Moreover the degree to which the glomerular filter leaks albumin has repeatedly been shown to be a predictor of various adverse endpoints, including worsening renal function, dialysis incidence, cardiovascular events and death (4). Albuminuria is classified according to the urinary albumin-to-creatinine ratio (ACR), which is subdivided in three stages: A1 (< 30 mg/g), A2 (30 mg/g – 300 mg/g) and A3 (> 300 mg/g) (2,5). It is known that the amount of albumin in the urine is strongly associated with the risk of adverse clinical outcomes (6). Independent of the cause, CKD is diagnosed when GFR is below 60 ml/min per 1.73 m^2 body surface area for ≥3 months and urine ACR ≥ 30 mg/g (2). In end stage renal disease (ESRD) or CKD Stage 5 (GFR below 15 ml/min per 1.73 m^2), renal function has decreased to a degree that uremic toxins are no longer cleared and accumulate, which ultimately is lethal. At this point, depending on age, comorbidities, resources, and patients' preferences, renal replacement therapy (i.e. hemodialysis, peritoneal dialysis, or kidney transplantation) or conservative management of supporting medication may be initiated. It is estimated that around 10% of the western population is affected by CKD (7).

Causes

In developed countries, the main causes of CKD and responsible of 50% of the ESRD are type 2 diabetes mellitus (DM) and arterial hypertension. In addition glomerulonephritis is responsible for about eleven percent, hereditary disease especially the autosomal dominant polycystic kidney disease are responsible for about eight percent and interstitial kidney disease (partly due to medication) are responsible for about six percent of ESDR (7–10).

Epidemiology and impact of chronic kidney disease

CKD stage G3b-5 affects more than 36 million people in the European Union (EU). More than 350 000 patients are being treated with dialysis and there are approximately 200 000 prevalent recipients of renal allografts (5,11). CKD is linked to a high prevalence of other comorbidities. In consequence, patients with CKD have markedly higher health care utilization compared to

people with normal renal function (2,12). Patients with CKD G5 cost up to 80,000 Euro per year and dialysis treatments consume 2% of health care budgets in Europe (5,11).

1.2. ACUTE KIDNEY INJURY (AKI)

Acute kidney injury (AKI) describes a short-onset decrease in GFR. Since GFR cannot be routinely measured in clinical practice, AKI has been defined as a recent increase in serum creatinine by ≥ 0.3 mg/dl within 48 hours or > 50% from baseline within 7 days. Once AKI has been diagnosed, the severity can be further staged using AKI stages (13).

Table 2 - AKI Stages

Stage	Serum creatinine	Urine output
1	1.5-1.9 times baseline OR ≥ 0.3 mg/dl increase	< 0.5 ml/kg/h for 6-12h
2	2.0-2.9 times baseline	< 0.5 ml/kg/h for ≥ 12h
3	3.0 times baseline OR Increase in serum creatinine to ≥ 4.0 mg/dl OR Initiation of renal replacement therapy OR Patients <18 years, decrease in eGFR to >35 ml/min per 1,73 m^2	< 0.3 ml/kg/h for ≥ 24h OR Anuria for ≥ 12h

Adapted from ref. (13)

AKI can lead to life-threatening hyperkalaemia, severe metabolic acidosis and unmanageable hypervolemia with respiratory insufficiency, which may make renal replacement therapy (RRT) necessary. The optimal time point to initiate RRT has still remained unclear (5,14–16). AKI can occur in the presence of pre-existing CKD or may occur in patients without underlying kidney disease. Especially elderly and patients with preexisting CKD are more prone to develop AKI. When AKI is detected, nephrotoxic drugs like nonsteroidal anti-inflammatory drugs (NSAIDs) and certain antibiotics may need to be discontinued, or better, should not be prescribed in patients with CKD and those who are susceptible to AKI (14). AKI on top of

preexisting CKD leads to impaired recovery and can worsen preexisting CKD (17). Pre-renal or renal AKI frequently have similar causes (14,15,17,18), and prolonged prerenal AKI may lead to renal AKI due to ischemic tubular damage.

Table 3 - AKI causes

Postrenal acute kidney injury	Renal acute kidney injury	Prerenal acute kidney injury
Obstructive nephropathy (e.g. urolithiasis, retroperitoneal fibrosis, neoplasia, papillary necrosis, neurogenic bladder dysfunction, prostatic hyperplasia, endometriosis)	Parenchymal kidney diseases like glomerulonephritis TMA (e.g. HUS, TTP) Nephrotoxic (e.g. NSAIDs) myeloproliferative or lymphoproliferative diseases Pyelonephritis	Decreased kidney perfusion due to volume depletion, shock, sepsis Vasodilative medications

Adapted from ref. (14,15,17): TMA (thrombotic microangiopathy); HUS (hemolytic uremic syndrome); TTP (thrombotic thrombocytopenic purpura); NSAIDs (non-steroidal anti-inflammatory drugs)

1.3. GLOMERULONEPHRITIS (GN)

Definition

Glomerulonephritis is a subclass of glomerular diseases, which are characterized by immune-mediated glomerular damage. (12,19) Glomerulonephritis must be distinguished from non-immune mediated glomerular disease (e.g. diabetic glomerulosclerosis or amyloidosis). Glomerulonephritis is classified into primary and secondary glomerulonephritis. While primary glomerulonephritis is an isolated, inflammatory disease which affects only the glomerulus (e.g. IgA nephropathy (IgAN)), secondary glomerulonephritis represents a renal manifestation of a systemic disease (e.g. lupus nephritis in the setting of systemic lupus erythematosus (SLE), or membranoproliferative glomerulonephritis in the setting of hepatitis C virus (HCV) infection) (12,20,21).

Symptoms

Patients suffering from GN may have hematuria, proteinuria, edema, azotemia, oliguria or anuria and high blood pressure. Patient with glomerular disease can develop a nephritic or nephrotic syndrome (21) .

Classification

Primarily based on kidney biopsy findings in immunofluorescence microscopy (IF), immunohistochemistry (IHC) or electron microscopy (EM) and pathogenic type, GNs can be distinguished as followed (Table 3) (12).

Table 4 - GN types and differentiation

Descriptive	Disease related	Morphologic
RPGN, acute nephritic Syndrome, chronic GN	Goodpasture Syndrome, LN, postinfectious GN	MN, MPGN, MCD, FSGS, IgAN.

Adapted from ref. (12,19): RPGN (Rapid progressive glomerulonephritis); LN (lupus nephritis); MN (Membranous nephropathy); MPGN (membranoproliferative glomerulonephritis); MCD (minimal change disease); FSGS (focal segmental glomerulosclerosis); IgAN (IgA nephropathy)

Rapid progressive glomerulonephritis (RPGN)

Rapid progressive glomerulonephritis (RPGN) summarises a heterogeneous clinical picture of haematuria, red-cell casts, proteinuria, decreased GFR, oliguria, oedema and hypertension. An important criterion to distinguish between a glomerular disease or extra glomerular kidney damage are dysmorphic red blood cells in the urine. In the setting of glomerular haematuria, red-cell casts or dysmorphic red cells (acanthocytes) will be identified on urine microscopy (21). Acanthocytes are a specific type of dysmorphic erythrocyte in the urinary sediment, which are considered pathognomonic for glomerular bleeding (21,22). Although research within the past decades led to a better understanding of the pathogenesis and to new therapeutic options in RPGN, this entity and the ensuing loss of kidney function is leading to reduced life quality and expectancy . Due to its fast progression and the risk of CKD development, treatment regimens of RPGN so far are aggressive including immunosuppressive therapy such as glucocorticoids, cyclophosphamide and rituximab (21,23). The main goal is to stop the acute autoimmune process, and thereby loss of kidney function (24).

Kidney biopsy is recommended and helps to accurately diagnose the disease and to choose the most appropriate treatment. Histological characteristics of RPGN are glomerular epithelial cell proliferation, increased number of mesangial cells, expansion of the mesangial matrix and rupture of the glomerular capillary loops. This severe glomerular injury gives rise to glomerular crescents, which are defined as at least two cell layers in Bowman's space (21,23). In case of an acute disease course immunohistochemistry or immunofluorescence staining to detect either granular, linear, or pauci immune deposits, is of great importance. Moreover,

complement factor 3 (C3) deposits in the mesangial and peripheral capillary loops can be detected in immunofluorescence microscopy (see figure 1 A and 7 C). Depending on the cause of RPGN, decreased C3 serum levels, anti-nuclear antibodies (ANA) or anti-neutrophil cytoplasmic antibodies (ANCA) can be observed (21). To differentiate the cause of RPGN, histological evaluation of the patterns of immune complex deposits is crucial (24). Mesangial immune complex deposition is observed in IgA glomerulonephritis and lupus nephritis class I and II. Subendothelial immune complex deposition is consistent with lupus nephritis class III and IV, whereas subepithelial immune complex deposition is leading to podocyte destruction and ultimately to a progressive membranous nephropathy (12).

Table 5 - RPGN is classified into the following types

Primary RPGN		
Type 1: GBM	Type 2: immune-complex mediated GN	Type 3: Glomerulonephritis associated with vasculitis (Pauci-Immune GN, ANCA associated)
Goodpasture-Syndrom	Postinfectious GN, LN, IgAN PSHN, DDD	GPA, MPA, EGPA, ANCA-negative vasculitis
linear deposits of immunoglobulin, crescents	granular deposits of immunoglobulin	few or no immune deposits, crescents
Secondary RPGN		
infective endocarditis, sepsis, hepatitis B infection with vasculitis and/or cryoglobulinemia, systemic necrotizing vasculitis, IgG and immunoglobulin M nephritis, malignancy, rheumatoid vasculitis		

Adapted from ref. (24) GBM (anti-glomerular basement membrane); LN (Lupusnephritis); IgAN (IgA nephropathy); PSHN (Purpura Schönlein-Henoch nephritis); DDD (Dense-deposit disease); GPA (granulomatosis with polyangiitis); MPA (microscopic polyangiitis); EGPA (eosinophilic granulomatosis with polyangiitis)

1.4. NEPHRITIC VS. NEPHROTIC SYNDROME

Patient with glomerular disease can develop nephritic or nephrotic syndrome. While some underlying diseases are predominantly associated with nephritic or nephrotic syndrome, it is important to highlight that there is significant overlap and heterogeneity in the initial presentation (e.g. membranoproliferative glomerulonephritis can present clinically with predominant nephritic or nephrotic syndrome) (20).

Table 6 - nephritic vs nephrotic syndrome

Nephritic Syndrome:	**Nephrotic Syndrome:**
Haematuria >5 RBC per HPF and the presence of at least one acanthocyte, red cell cast or mixed red cell/white cell cast Azotaemia Mild to moderate proteinuria (usually less than 1.5 g/day) Leukocyturia in the absence of urinary tract infection	Protein excretion >3.5 g/24 hours Hypoalbuminemia Oedema Hyperlipidaemia
Most common diseases:	
Post-infectious glomerulonephritis Glomerulonephritis related to hepatitis B or C IgAN LN class I-IV Vasculitis PICG GBM disease (Goodpasture's disease)	MN MPGN MCD FSGS IgMN LN class V DN Amyloidosis

Adapted from ref. (20,25,26):IgAN (IgA nephropathy); LN (lupus nephritis class); PICG (pauci-immune focal necrotizing glomerulonephritis); GBM (anti- glomerular basement membrane); MN (membranous nephropathy); MPGN (membranoproliferative glomerulonephritis); MCD (minimal change disease); FSGS (focal segmental glomerulosclerosis); IgMN (IgM nephropathy); LN (lupus nephritis); DN (diabetic nephropathy)

1.5. GLOMERULAR DISEASES

1.5.1. Post infectious glomerulonephritis (PSGN)

PSGN is associated with viral, bacterial or protozoal infections, which usually occur approximately 2 to 3 weeks prior to nephritis onset. This kind of glomerulonephritis is more frequently encountered in males between the age of 2 and 10 years. In the histological evaluation one can find mesangial and endocapillary proliferation of endothelial cells, and infiltration of immune cells (e.g. monocytes, eosinophils) into the kidney. In fulminant disease, glomerular crescents may be observed. Moreover IgG deposition and complement factor C3 deposition can be detected (21). Therefore, serum complement factor C3 and the total complement activity CH50 are decreased in the first two weeks. Values commonly return to the normal range after 8 to 12 weeks (24). Clinically, patients present with haematuria and nephritic syndrome accompanied by arterial hypertension (20). Symptoms usually improve

within weeks without any complications. If a *Streptococcus* infection can be proven, antibiotic therapy with penicillin, alternatively erythromycin in cases of penicillin allergy is recommended (24).

1.5.2. Glomerulonephritis related to hepatitis C

There are also a number of renal diseases (membranoproliferative glomerulonephritis (MPGN) type 1 with and without cryoglobulins, panarteritis nodosa and membranous nephropathy), which may occur as an extrahepatic manifestation of HCV. Overall, these entities are rare and are responsible for <10% of all GFR impairments in HCV patients (27). Nevertheless untreated hepatitis C with GFR impairment points to a MPGN, which is the most common underlying cause of type 1 MPGN in adults. HCV-associated GN are caused by deposition of immune complexes from viral antigens and immunoglobulins in the glomerulus. HCV-associated MPGN can be accompanied by a systemic autoimmune disease called mixed cryoglobulinemia. Patient with mixed cryoglobulinemia type II present with cutaneous vasculitis, arthralgia and abdominal pain (27–29).

1.5.3. IgA nephropathy

The mesangioproliferative immune complex IgA nephropathy (IgAN) is the most widespread glomerulonephritis, which despite the fact that it commonly presents with slowly progressing CKD, frequently leads to ESRD. It is caused by an intrinsic antigen (galactose-deficient IgA1) and circulating anti-glycan antibodies. A clinical hallmark of this disease is hematuria, which is commonly combined with a respiratory or gastrointestinal tract infection. This can be an important clinical hallmark, since it allows to distinguish the synpharyngitic haematuria in IgAN, from the delayed onset of haematuria in pauci-immune focal necrotizing glomerulonephritis (PICGN). In the histological evaluation, mesangial deposition of IgA can be detected by an immunofluorescent staining. For histological evaluation of IgA nephropathy the MEST-C score, i.e. mesangial hypercellularity (M), endocapillary hypercellularity (E), segmental glomerulosclerosis (S), tubular atrophy or interstitial fibrosis (T) and crescents (C), is used (12,21,23,28,30). Since the glomerular histopathology of IgA nephropathy is identical to the changes in purpura Schönlein Henoch (PSH), these two diseases can only be distinguished via the extra renal symptoms of PSH (31). According to the KDIGO guidelines (32), angiotensin-converting enzyme inhibitors (ACEIs) or angiotensin-receptor blockers (ARBs) are strongly recommended if proteinuria is above 1g / day. In the absence of treatment success after 3-6 months, the use of glucocorticoids for a total 6 months should be considered if GFR is above 50 ml / min / 1.73 m² (in terms of therapy control at a GFR <50ml / min / 1.73 m² no

data are available). If crescents can be detected in more than 50% of the glomeruli and if there is a rapid deterioration in renal function, the KDIGO guideline recommends immunosuppressive therapy with steroids and cyclophosphamide (30,32). It is important to note that the use of immunosuppressants has recently been evaluated in randomized controlled trials (33,34). Immunosuppression was either shown to not delay the decline in renal function (34) or to be associated with a significant burden of adverse effects (33). Therefore, the treatment of IgAN has changed since publication of the most recent guidelines and therefore strict conservative treatment approach should be emphasized and is recommended.

1.5.4. Systemic Lupus erythematosus (SLE) class I-IV

According to the classification of the European League Against Rheumatism (EULAR) and the American College of Rheumatology (ACR) 2017 the diagnosis of SLE requires following criteria: the proof of erythema, non-scarring alopecia, detailed history of polymorphous light eruption (PLE), serositis, arthritis, hematologic manifestation with haemolytic anaemia, leukopenia or thrombocytopenia, positive ANA, positive anti-dsDNA, Anti-Sm, Antiphospholipid antibody, low complement or lupus nephritis (LN). In contrast, biopsy-proven lupus nephritis (LN) alone is enough to meet SLE criteria (35–39). The most common renal manifestation of SLE is immune complex glomerulonephritis. In SLE with renal involvement, autoantibodies bind to autoantigens that are deposited in the glomerulus (21,40). A subset of double-stranded DNA (dsDNA)-specific antibodies cross-react with annexin II on the cell surface in the cytoplasm and in the nucleus of mesangial cells as well as epithelial cells in the glomeruli, causing autoimmune reactions and infiltration of immune cells. Granular mesangial, subepithelial and subendothelial IgG, IgA, IgM, C3, C4 and C1q positive deposits are found in LN. Due to the complex pathophysiological mechanism, which is still not entirely understood, and the variety of immune complexes formed on different kidney cell types, the progression of renal disease in SLE can differ (41–44). Therefore only few patients with SLE develop RPGN and ESRD (23). SLE nephropathy is classified by histopathological criteria as follows: Class I: Minimal mesangial lupus GN, Class II: Mesangioproliferative lupus GN, Class III: Focal lupus GN (<50% of glomeruli) active / sclerosed, segmental or global, Class IV: Diffuse Lupus GN (> 50% glomeruli) active / sclerosed, segmental or global, class V: Membranous Lupus GN, Class VI: Sclerosed LGN (> 90% of glomeruli) (45). So far there is no evidence that treatment of class I, II and VI LN is clinically meaningful and the extra-renal manifestations should be therefore treated specific. This is in contrast to LN class III and IV. The KDIGO guideline recommends prompt immunosuppressive treatment for focal (class III) and diffuse (class IV) LN. All immunosuppressive regimens contain varying doses of steroids. In case of mild to

moderate SLE disease course treatment regimens include the disease-modifying drugs hydroxychloroquin (HCQ) and MTX and short courses of NSAIDs for symptomatic control. To avoid sun-induced skin lesions sun protection is recommended. Additionally cyclophosphamide or mycophenolate mofetil acid (MMF) can supplement the already established steroid therapy. For patients who do not respond to azathioprin (AZA), MMF or calcineurin inhibitor (CNI), B-cell antibodies like rituximab and belimumab may be considered (37).

1.5.5. Pauci-immune focal necrotizing glomerulonephritis (PICGN)

Pauci-immune focal necrotizing glomerulonephritis shows no or paucity of linear immunoglobulin deposition within glomeruli. In this disease, autoantibodies bind to neutrophil cytoplasmic antigens specific for either myeloperoxidase (MPO) or proteinase 3 (PR3). After binding to MPO or PR3, mediated by Fcγ IIa and IIIb and Fab 2 receptors, neutrophils and monocytes are activated, which feeds a vicious cycle causing direct cellular injury and necrosis (46–48). The complement factor C5a activates neutrophils. In a further step the alternative complement pathway is activated and C3 is generated (49,50). Anti-neutrophil cytoplasmic antibodies (ANCAs) can activate neutrophils and promote neutrophil infiltration into the kidney. Furthermore, ANCAs induce the release of neutrophil extracellular traps (NETs), which consists of chromatin fibres and autoantigens including MPO and PR3. NETs promote cell damage and kidney injury and may also be involved in perpetuating the ANCA autoimmune response (51–54). It is assumed that T cells infiltrate the glomeruli and the tubular-interstitial area. Circulating MPO- specific and PR3-specific T helper cells type 1 (TH1) and type 17 (TH17), as well as increased $CD8^+$ T cells directly reflecting the risk of disease relapse (12,20,55,56). ANCA subtypes can be detected via immunofixation or enzyme-linked immunosorbent assay (ELISA). However, about 10% of the PICGN patients are ANCA negative. While ELISA allows to distinguish the ANCA epitope (MPO versus PR3), immunofluorescence staining of ethanol fixed human neutrophils with patient serum allows to determine a perinuclear (p-ANCA) or cytoplasmic (c-ANCA) pattern (57). The predominant epitope in cANCA-associated vasculitis is PR3, while MPO ANCA are usually found in p-ANCA-associated disease. Histologically glomerular crescents, necrosis of capillary loops, immune cell infiltration into the interstitium and necrotic arteries can be examined (58). The PICG can be restricted to the kidney or kidney injury can be part of a systemic autoimmune vasculitis like the microscopic polyangiitis (MPA) associated with p-ANCA, granulomatosis with polyangiitis (GPA), which is associated with c-ANCA, or Churg-Strauss syndrome (CSS) (59). According to the KDIGO guidelines cyclophosphamide and corticosteroids may be used as

initial treatment. Also rituximab in combination with corticosteroids may be used as an initial treatment in patients without severe disease. In case of systemic participation of other organs beside the kidney, further therapy regimes should be considered (see systemic vasculitis) (32).

1.5.6. Systemic vasculitis

The systemic vasculitis can be subdivided in (12,21):

- ⇨ Granulomatosis with polyangiitis (GPA) (Wegener's granulomatosis)
- ⇨ Microscopic polyangiitis (MPA)
- ⇨ Eosinophilic granulomatosis with polyangiitis (EGPA, formerly Churg- Strauss syndrome)
- ⇨ Polyarteritis nodosa
- ⇨ Henoch–Schönlein purpura

Most commonly the IgG class-switched antibody ANCA can be detected in patients suffering from GPA, MPA and Churg–Strauss syndrome. The histological picture of Henoch–Schönlein purpura is comparable to the one of primary IgA nephropathy (21).

GPA MPA are responsible for most of RPGN cases. Clinically the patient can present with fever, weight loss, arthralgia, sinusitis, rhinitis, otitis, ocular inflammation, haemoptysis and bleeding leading to microcytic anaemia. The dermal manifestation of vasculitis is presenting mainly in the lower extremities. Concerning the renal manifestation of the disease, patients develop haematuria with acantocytes, oliguria and proteinuria. If vasculitis is systemic, the one year mortality rate is high (21,56). GPA are usually c-ANCA / PR3-ANCA positive and in the MPA usually p-ANCA / MPO-ANCA can be detected. In EGPA, renal involvement is often associated with ANCA detection. Patients without renal manifestation are almost always ANCA negative (60).

Therapy consists of aggressive immunosuppressive therapy with cyclophosphamide, cortisone and rituximab and treatment with rituximab or azathioprine in disease remission. It is unclear whether plasmapheresis is an additional therapy option, it might be only beneficial for those patients who already need dialysis and suffer from alveolar haemorrhage at the time of diagnosis. First data from the PEXIVAS study showed no additional benefit of plasmapheresis on top of standard immunosuppressive therapy in patients with aggressive ANCA vasculitis (ERA-EDTA Abstract), but full data have not been published so far. Thus, it is still under investigation which treatment regime is the best to limit side effects of drugs but improves or prevents organ damage. To put it in other words safer and more targeted therapies are urgently needed, therefore EUVAS trials are ongoing, investigating the immunosuppressive drugs and their treatment duration in systemic vasculitis (23,61).

1.5.7. Anti- glomerular basement membrane (GBM) disease (Goodpasture's disease)

Antibodies are formed against the α3 chain of type IV collagen (basement membrane collagen), leading to a ribbon-like staining of IgG over the anti-glomerlular basement membrane (GMB). These antibodies do not only bind to the basement membrane of glomeruli, but can also bind to that of pulmonary alveoli, thereby leading to pulmonary haemorrhage (21). Beside proteinuria, haematuria and dysmorphic erythrocytes seen in the urinary sediment, Goodpastures disease cause RPGN. In the patient serum anti-GBM antibodies can be detected, while C3 and C4 are frequently not out of range. This disease is life-threating and requires immediate treatment (20). Therapeutic regimen includes immunosuppression and plasmapheresis. By exchanging the patient plasma, the circulating pathogenic autoantibodies are removed (21).

1.5.8. Membranous nephropathy (MN)

Macroscopically immune complex deposition between the GBM and the podocytes can be observed. These deposits destruct the podocyte barrier function causing a nephrotic syndrome (62,63).

In 2009 Beck et al. discovered the M-type phospholipase A2 receptor 1 (PLA2R1), which changed the diagnostics and understanding of MN tremendously (64). Nevertheless, the function of PLA2R1 in podocytes is still under investigation (65). In approximately half of the serum of the patients with primary membranous nephropathy phospholipase A2 receptor (PLA2R)-specific antibodies, usually of the IgG4 subclass, can be detected. In another fourth of cases the disease is secondary as a result of another disease like neoplasia, infections (hepatitis B, C), unwanted drug effect (NSAIDs, penicillamine) and systemic autoimmune disease like systemic lupus erythematosus (SLE). A distinction should be made between primary and secondary MN, considering the different therapeutic approaches depending on the cause of MN (12,20,62,63).

Detection of IgG and its subclasses in kidney biopsy makes secondary MN more likely (66). Even though IgG4 may not activate the complement system, complement factor C3 staining is positive in active disease and can be detected within the immune deposits (67).

Levels of PLA2R titres in patients' serum reflects remission and outcome (68–70). In PAS staining thickening of capillary walls can be detected. Thickening of the GBM can be as well seen in a Grocott's Methenamine silver staining (71). Moreover, IgG immunohistochemistry staining can reveal granular subepithelial, glomerular capillary wall deposition of IgG (71). If

beside IgG4 also IgG1, 2, or 3, IgA, IgM and C1q can be detected, a secondary MN should be considered. Positive C4d and negative C1q staining hints towards a primary MN and an activation of the lectin pathway of complement (72). If subendothelial, mesangial electron-dense deposits can be detected via electron microscopy, then a secondary membranous nephropathy is likely (62).

Moreover, Tomas et al. found anti-thrombospondin type 1 domain-containing 7A (THSD7A) antibodies, present on podocytes, in 3%–5% of patients with primary MN, who were anti-PLA2R negative (73). Noteworthy, THSD7A antibodies also trigger IgG4 antibody response (71).

In secondary MN the underlying disease should be treated. Patients with a primary MN should receive supportive therapy including control of proteinuria, renin-angiotensin blockade, management of hypertension, control of hyperlipidaemia and anticoagulation (62). Up to 30% of patients with a primary MN have spontaneous remission of nephrotic syndrome and overall favorable long-term kidney and patient survival. (74). However, another 30% present with disease progression to ESRD (75,76). Those patients should be treated aggressively (77,78). Therefore, after conservative management for 3–6 month without disease remission an immunosuppression therapy should be started (62,71).

1.5.9. Membranoproliferative glomerulonephritis (MPGN)

Patients with membranoproliferative glomerulonephritis (MPGN) may present with a heterogeneous clinical picture suffering from asymptomatic haematuria, a nephrotic syndrome, acute nephritic syndrome or even with RPGN rapidly progressing to ESRD. Patients suffering from MPGN can present with a chronic or relapsing disease course (79–81). MPGN is mostly associated with secondary causes like viral infection (hepatitis B, hepatitis C), autoimmune diseases SLE, Sjogren'ssyndrome (SS) and rheumatoid arthritis (RA) and monoclonal gammopathy of undetermined significance (MGUS). MPGN is either an immune complex– or complement-mediated disease (81). Using histological silver staining, double-contour, thick glomerular capillary walls, endocapillary proliferation, capillary wall remodelling, thickening of GBM, mesangial expansion and hypercellularity can be detected. This histological pattern refers to the alternative term mesangiocapillary glomerulonephritis (80). The activation of the classical as well as alternative pathway of complement induces deposition of immune complexes and C3 activation in the glomerulus (82,83). If the alternative pathway of the complement system is dysregulated, C3 and terminal complement components (C5b-C9) can be detected in an immunofluorescence staining pointing towards a C3 glomerulopathy or MPGN II. C3 glomerulopathy sums up a heterogeneous group of disorders with various

underlying pathophysiology. If neither complements nor immunoglobulins can be detected, but MPGN pattern of injury can be seen histologically, a thrombotic microangiopathy (TMA) is more likely (81).

Based on deposit localisation as assessed by electron microscopy, four different MPGN types have been historically distinguished. MPGN type I is classified by subendothelial deposits, MPGN type II (or dense deposit disease (DDD)) has intramembranous deposits in the GBM, and in MPGN type III subendothelial and subepithelial deposits can be detected. The fourth MPGN type is the secondary MPGN (81). This classification was replaced by a new differentiation based on the composition of the glomerular deposits evaluated by a immunofluorescence staining (84). If immunogloboulins and C3 staining can be found the MPGN is called immune complex-associated MPGN (IC-MPGN) (84).

In cases of secondary MPGN (e.g. Hepatitis C/B, SLE, RA) the underlying disease should be treated. Patients with MGUS and MPGN should receive myeloma based therapies. In those patients with severe disease progression of MPGN, who do not have any recognized underlying cause, aggressive immunosuppressive therapy, including oral cyclophosphamide or MMF, rituximab plus low-dose corticosteroids are indicated (32,81).

1.5.10. Systemic Lupus erythematosus (SLE) class V

Diffuse membranous lupus glomerulonephritis was classified as class V (44). Patients with class V LN, normal kidney function and no extrarenal manifestations should be treated with antiproteinuric and antihypertensive drugs. Those patient with class V LN and renal impairment should be treated with corticosteroids plus an additional immunosuppressive agent like cyclophosphamide, CNI, MMF or azathioprine (32).

1.5.11. Minimal change disease (MCD)

The diagnosis of MCD requires kidney biopsy with electron microscopy (ELMI) evaluation. The ELMI is the only way to see the podocyte changes causing proteinuria. The mechanism leading to podocyte damage is not entirely resolved but it is assumed that T-cell mediated autoantibodies might play a role. Nevertheless, also drugs like NSAIDs can cause this disease. Patients suffering from MCD present with a nephrotic syndrome. More than 90% of adults reach complete remission (20,85). Regarding the KDIGO guidelines initial treatment with corticosteroids should be followed by a slow taper over a total period up to 6 months after achieving remission. In those cases with severe corticosteroid-related adverse effects like steroid-induced or uncontrolled diabetes, psychiatric deterioration, severe osteoporosis,

immunosuppressive therapy including oral cyclophosphamide, MMF or CNIs should be considered (32) .

1.5.12. Focal segmental glomerulosclerosis (FSGS)

FSGS is a common histologic diagnosis in adults with nephrotic syndrome and one of the most common causes of ESRD. Histologically a focal and segmental sclerosis of the glomerulus can be seen. Damaged podocytes can be detected in electron microscopy. The histological picture resembles the related disorder of minimal change disease (MCD) (86,87). The picture seen in FSGS may occur in any GNs in the terminal phase and sums up a group of clinical–pathologic syndromes sharing a common glomerular lesion (86). FSGS are divided into subtypes, of which the collapsing variant is associated with the worse prognosis and the tip lesion variant with the best prognosis (88). Pathophysiological focal and segmental destruction and sclerosis of glomerular capillary loops takes place either when nephron loss causes increased chronic glomerular pressure in the remaining nephrons or podocyte damage itself is causing described histological changes (86).

In the African American population FSGS has the highest prevalence and is the most common reason for nephrotic syndrome. Moreover, renal survival is worse in African Americans compared to Caucasians. It is speculated that the increased prevalence of FSGS in African American is due to genetic (e.g. variants in apolipoprotein L1 (APOL1), or myosin heavy chain 9 (MYH9)) and socioeconomic/environmental factors (87,89).

80% of cases are primary (idiopathic) FSGS. The diagnosis is made in a clinical pathological context. The differentiation between idiopathic and secondary FSGS is based on the clinical picture and is of great relevance considering the different therapeutic approaches (86,90). Whereas idiopathic FSGS needs immunosuppressive therapy, secondary FSGS is mainly symptomatically treated with RAAS blockade, ACE-inhibitor or ARB. Therefore, the underlying disease causing the secondary FSGS must be detected and treated (86,90). As a first-line therapy in idiopathic FSGS, glucocorticoids are recommended. In case of non-response, relapses or contraindications for steroids a second-line therapy including cyclophosphamide or CNI is suggested. Patients with steroid-resistant FSGS, who do not tolerate cyclosporine, may receive combined immunosuppressive therapy including MMF and high-dose dexamethasone (32).

Table 7 - Idiopathic versus secondary FSGS

Idiopathic FSGS	Secondary FSGS
Clinical Picture	
(sub-) acute renal insufficiency that can progress to rapidly progressive renal dysfunction Nephrotic syndrome haematuria with acanthocytes no indication of secondary aetiology	mild renal insufficiency, slowly progressing Slow progression of proteinuria over years
Aetiology	
Specific cause unknown mediated by circulating permeability factors	Familial or genetic (mutations in specific podocyte genes) Virus-associated (Human immunodeficiency virus type 1, parvovirus B19, simian virus 40, cytomegalovirus, Epstein–Barr virus) Drug-induced (Heroine, interferons, lithium, pamidronate, sirolimus, CNI, anabolic steroids) Adaptive (low birth weight, renal agenesis, renal dysplasia, reflux nephropathy)

Adapted from ref. (86,87)

1.5.13. IgM Nephropathy

A predominant deposition of IgM in a diffuse, granular, global mesangial distribution was first described 1978 by Cohen et al. (91) and Bhasin et al. (92). This is in contrast to FSGS, where IgM is not diffuse but segmentally and focally distributed in sclerotic areas (93,94).
Furthermore, C3 deposition can be detected on immunofluorescence staining (95,96). Patients suffering from IgM nephropathy present with protein- and haematuria (96). The pathogenesis of IgM nephropathy is still unclear. The classical complement pathway seems to play a role due to the fact that in the majority of the cases C3, C1q and C4 deposits can be seen in renal biopsies (61). The histological picture as well as the treatment options resembles MCD and FSGS. Therefore it is still a matter of discussion, whether IgM nephropathy is a separate disease. Nevertheless, patients with IgM nephropathy are less likely to respond to immunosuppressive agents than those with MCD, thus IgM deposits clearly signifies a worse prognosis (90,93). Zhang et al. evaluated in a retrospective (2004-2014) study treatment

outcomes in 106 FSGS patients with and without IgM/C3 deposition. Presence of IgM and C3 deposit indicated a worse renal outcome in patients with primary FSGS. They hypothesised that IgM deposition activates the complement system and therefore might be involved in disease progression (97).

Myllyma et al. postulated that, based on the evaluation of 110 patients (children and adults) with IgM nephropathy without systemic diseases, there are two subgroups of IgM nephropathy. One group with a predominance of men presenting with nephrotic syndrome, whereas the second group are mainly women with micro haematuria. Moreover IgM nephropathy patients with arterial hypertension and steroid resistance seems to have a worse renal survival rate (96).

1.5.14. Diabetic nephropathy

Poorly managed diabetes mellitus type I or II induces the activation of the local renal RAAS and increases the production of Angiotensin II, which exerts a constriction of the efferent, and dilation of afferent, arterioles. Followed by glomerular hyperfiltration and hypertension. Metabolic and haemodynamic changes that occur in diabetes lead to ultrastructural alterations causing glomerular damage, hypofiltration and ESRD. Histologically a thickening of the GBM, mesangioproliferation and podocyte damages and ultimately glomerular sclerosis can be observed (98–100). The latter is caused by intraglomerular hypertension induced by renal vasodilatation, or ischemic injury (101). Especially podocyte injury causes proteinuria. The podocyte-specific insulin receptor and the key glucose transporters, including GLUT4 and GLUT1 may contribute to diabetic nephropathy which have been elucidated in podocyte insulin receptor knock-out (KO) mice. Mice lacking the insulin receptor on podocytes developed albuminuria, GMB thickening, mesangial matrix expansion and glomerulosclerosis (102,103).

The prevalence of EDRS in type 2 diabetes is lower than in type 1 diabetes. In contrast to type 2 diabetes mellitus nephropathy, type 1 diabetic nephropathy is typically developing 10 to 15 years after disease onset, where proteinuria correlates with, and can predict, disease progression. If after 20 to 25 years after disease onset no proteinuria can be detected the chance of renal disease deterioration is only about one percent per year in Caucasians (104,105). It has been shown in an *in vitro* study that by an increase in the mesangial cell glucose concentration, mesangial cell matrix production (106,107) and mesangial cell apoptosis is stimulated (108,109). It is assumed that an overexpression of glucose transporters cause enhanced glucose entry into the cells (107).

Moreover it is hypothesised that tissue protein glycosylation leads to reversible early glycation products and later, irreversible advanced glycation end products (AGEs) are formed. The

tissue accumulation of AGEs and crosslink with collagen is associated with renal and microvascular complications (110). There is a direct correlation between circulating AGE levels and diabetic renal injury (111).

Furthermore, an elevation in protein kinase C and upregulation of heparinase expression may contribute to increased glomerular basement membrane permeability and consequently albuminuria (112–114).

Vascular endothelial growth factor (VEGF) may be as well involved in the pathophysiology of diabetic nephropathy (98,115). VEGF mediates endothelial injury (116) and in a diabetic mouse model it could be demonstrated that by blocking VEGF the renal phenotype could be improved and albuminuria was diminished (117).

Above that, inflammatory molecules and pathways are contributing to the development and progression of diabetic nephropathy including toll-like receptors, chemokines (monocyte chemoattractant protein-1, also known as CCL2), adhesion molecules (intracellular adhesion molecule-1 and vascular adhesion molecule-1), enzymes (cyclooxygenase-2, nitric oxide synthase), growth factors (VEGF, TGF-β), nuclear factors (NF-κB) and pro-inflammatory cytokines (interleukin-1, -6, -18, tumour necrosis factor-α) (98,115,118–120).

Beside controlling glucose and blood pressure patients benefit from restricting glomerular hypertension and hyperfiltration by RAAS blockade with ACE inhibitors or ARB. Additionally it is assumed that by blocking Angiotensin II also prevents fibrosis (20,121,122). Moreover, sodium–glucose cotransporter 2 (SGLT2) inhibitors may have as well reno-protective effects in diabetic nephropathy (123).

1.5.15. Amyloidosis

The most common histopathological diagnosis with renal involvement in plasma cell diseases includes cast nephropathy, monoclonal immunoglobulin deposition disease (MIDD), light chain deposition disease (LCDD) and the immunoglobulin (Ig) - associated amyloidosis, mostly a light chain amyloidosis (AL) (124). In case of the diagnosis of monoclonal gammopathy (MG) with renal involvement, but without diagnosis of multiple myeloma (MM), the term of monoclonal gammopathy with renal significance (MGRS) was shaped (124). Approximately 10% of amyloidosis is linked to multiple myeloma (20).

The glomerular injury is caused by deposition of insoluble amyloid monoclonal light chains, which can be limited either to the blood vessels or to the tubules. Patients may present with nephrotic syndrome. Depending on the location of the deposition of the amyloid fibrils the degree of renal disease varies (125). In general amyloidosis can be systemic and can cause progressive organ dysfunction. The following amyloidosis are associated with renal injury: AL,

chronic inflammatory disorders with serum amyloid A protein (SAA or AA), fibrinogen A α-chain (AFib), or leukocyte chemotactic factor 2 (ALect2), and apolipoprotein A-I amyloidosis (AApoA1) (126). Immunoelectrophoresis should be performed routinely to rule out myeloma and renal primary amyloidosis (25).

2. Immune system and the kidney

In addition to its excretory function, the kidney also acts as an endocrine organ producing several hormones like erythropoietin, renin or is activating vitamin D. Those hormones are directly or indirectly affecting immune responses (12). The healthy kidney contains several types of immune cells e.g. DCs, macrophages and lymphocytes (12,127,128). DCs as well as mast cells are located in the interstitial area between tubular epithelial cells and the peritubular capillaries (19,129), while macrophages are detected in the renal medulla and capsule. Neutrophils can be recruited to the kidneys by both DCs and tubular epithelial cells under inflammatory conditions, such as bacterial kidney infections (i.e. pyelonephritis) and DCs also produce pro-inflammatory cytokines in renal ischaemia (12).

Degenerated cells express damage-associated molecular patterns (DAMPs) in contrast to invading pathogens expressing pathogen-associated molecular patterns (PAMPs). Both are signals for the innate immune cells to express membrane-bound Toll-like receptors (TLRs) (130). Kidney cells, like tubular epithelial cells and endothelial cells express a subset of TLRs (TLR1 to TLR6) and inflammasome components; therefore it is hypothesised that this renal cells can respond to DAMPs (12). Due to apoptosis, necrosis and fibrosis in the kidney, DAMPs are released into the extracellular space, where they activate pattern-recognition receptor (PRRs). PRR expression is limited to immune cells like DCs, as well as macrophages which express numerous PRRs to identify PAMPs and DAMPs. PRR ligand binding activates the cell, which results in secretion of pro-inflammatory mediators that promote renal immunopathology. PRR activation in mesangial cells causes proliferation in mesangioproliferative forms of glomerulonephritis like lupus nephritis, IgA nephropathy and hepatitis C virus-associated glomerulonephritis. PRR activation of endothelial and epithelial cells like podocytes and tubular epithelial cells causes cell damage and proteinuria (12) .

2.1. THE IMMUNE SYSTEM AND CKD

In the end stage of immune-mediated CKD healthy kidney tissue is replaced with fibrotic tissue. NK cells and neutrophils are first responders to injured tissue and together with DCs, monocytes and macrophages infiltrate inflamed kidney tissue. DCs are the main actors in kidney inflammation. Resident kidney cells like epithelial and endothelial cells express TLRs and inflammasome components, what makes them respond to DAMPs and induce innate immune responses. Consequently this leads to a release of pro-inflammatory cytokines and promotes even more destruction of the kidney tissue (12,131–136). The T-cell priming during renal inflammation takes place in the local renal lymph nodes (137). Innate immune cells and T cells can interact with resident kidney cells to promote kidney damage. If renal inflammation

remains unresolved, progression of CKD might be the result. Moreover, in nephropathies caused by diabetes, hypertension or toxic kidney injury inflammatory cells play a pivotal role in the pathogenesis (12).

In conclusion, non-infectious triggers in the kidney induce innate immune responses that might result in inappropriate immunopathology. Distinct immunological pathways contribute to certain types of renal inflammation and these result in differing diseases (e.g. aHUS, GBM, IgAN) (12). CKD causes alterations in the immune system, like chronic systemic inflammation. This includes increased systemic concentrations of pro-inflammatory cytokines and acute phase proteins, such as the pentraxins and acquired immunosuppression with dysfunctional phagocytes, B and T cells (12). While systemic inflammation contributes to bone loss and accelerated atherogenesis, immunosuppression and immune dysregulation result in a higher infection rate in CKD patients, which accounts for a higher morbidity and mortality in CKD patients (12).

2.2. THE IMMUNE SYSTEM AND AKI

AKI seems to modulate the immune system and is associated with an increased risk for developing infection (131,138).

The reasons for AKI are diverse and they all lead to renal epithelial cell damage, which activates stress response pathways. This in turn promotes secretion of cytokines and vasoactive factors. These activated renal epithelial and necrotic cells release DAMPs which activate resident macrophages and DCs and perpetuate the inflammatory response. DAMPs or PAMPs trigger an inflammasome response that causes influx of innate immune cells, predominantly neutrophils, macrophages and natural killer (NK) cells. The initiated immune response is essential for the repair process to clear debris and necrotic tissue (139,140). However, the early infiltrating innate immune cells might also cause severe damage. In a second wave T cells are infiltrating the kidney. Moreover, it was demonstrated in an AKI mouse model that the lack of $CD4^+$ and $CD8^+$ T cells is leading to an improved renal phenotype (141,142). Nevertheless, especially DC-derived TNFα contributes to kidney injury. The neutrophils and M1 macrophages are as well supporting and promoting the pro-inflammatory milieu, thus leading to tissue damage and consequently to tissue remodeling (glomerular sclerosis, fibrosis) (131,143). An important hallmark of immune cell infiltration is adhesion to and transmigration through the endothelium. Accordingly, endothelial surface molecules such as intercellular adhesion molecule-1 (ICAM-1), P-selectin, and E-selectin and integrin are all upregulated in ischemic renal injury and promote neutrophil migration to the site of inflammation. These factors contribute to a quick influx of neutrophils into the renal interstitium

after ischemic renal injury. IL6 is one of the major cytokines recruiting neutrophils and leading to dysfunction of endothelial cells and therefore causes increased endothelial permeability. Moreover, IL6 triggers IL8 production, which causes recruitment and activation of even more neutrophils. Chronic inflammation leads to kidney tissue remodeling and subsequent development of CKD (131,143–147).

2.3. COMPLEMENT SYSTEM AND THE KIDNEY

The complement system is part of the innate humoral defence against pathogens and damaged cells (148). Besides its lytic pathway, it also plays a role in opsonisation of pathogens and abnormal cells, thereby triggering their clearance by phagocytes (149).

There are three different complement pathways. The classical which is activated by antigen-antibody complexes, the alternative complement pathway which is activated by fragments of microbial cell walls, and the lectin pathway which is activated by the interplay between microbial carbohydrates with mannose-binding protein (150). The main duty of the classical complement pathway is the destruction of antibody labelled cells/bacteria. Apoptotic cells are major activators of the classical pathway (151). All three pathways merge at the C3 cleavage and then generate the membrane attack complex C5b-9 that ultimately lyses the target cell (152). Another task of the complement system includes clearance of soluble immune complexes and cell debris. Clearance of cell debris plays an important role in prevention of autoimmunity development. Hence, it is critical for the homeostatic maintenance of the tissue (153,154).

Antigen-antibody complex deposition might be causative for kidney disease. It is hypothesized that these autoimmune kidney disease are linked to complement derived effector mechanisms in which plasma complement is activated through the classical or lectin pathways (155).

The complement system is tightly regulated. Several complement inhibitors regulate the degree of complement activation (156). Abnormally high C3 activation and deposition on endogenous cells can for example lead to renal diseases like C3 glomerulopathies and deposition of C3 on small vessel walls might cause immune complex-mediated inflammation. In electron microscopy, C3 deposition on the GBM can be detected and consistently, low serum levels of C3 can be measured. C3 glomerular deposition is attributed to the activation of the alternative pathway (AP) of complement. In patients with IgA nephropathy the driving force for inflammation is the activation of complement in the kidney. In the induction of inflammation in autoimmune diseases the classical pathway with its immune complexes seems to be most relevant (157–160).

The complement regulatory proteins complement factors I (FI) and H (FH), which are responsible for complement activation in the fluid phase, can be associated with secondary C3 deficiencies. Patients with decreased FI and FH are more prone to infections. FH is also responsible for the protection against deposition of complement and therefore protects from complement-induced diseases like hemolytic uremic syndrome (HUS) (161).

2.4. DENDRITIC CELLS (DC) AND MACROPHAGES IN THE KIDNEY

The myeloid derived antigen presenting DCs present a link between the innate and adaptive immune system (162). Upon activation, DCs upregulate expression of B7 co-stimulatory molecules (CD80 and CD86), which are crucial for subsequent lymphocyte activation. When DCs encounter an antigen, they travel to the local draining lymph nodes where they present processed antigen in the context of major-histocompatibility-complex II (MHCII). This process leads to specific T cell activation (163).

There are resident macrophage populations in almost all tissues. They are highly effective phagocytic cells, which are able to engulf and kill invading microorganism (67,164,165). Macrophages belong to the innate immunity but they closely interact with the adaptive immune system. They are one of the major cells recognizing PAMPs (162,166). There are different ways of macrophage activation such as by TLRs, IFN-γ, IL-4/IL13 and depending on the activation signal, they are activated and become M1 (classical) or M2 (alternative) macrophages, reflecting TH1 or TH2 polarization seen in T cells. Lipopolysaccharide (LPS), produced by microbes and cytokines such as TNF, GM-CSF or IFN-γ are leading to the M1 macrophage phenotype, whereas the IL-4 and IL-13 are inducing the M2 macrophage polarization (162,167–169).

It is postulated that the mouse kidney contains a couple of mononuclear phagocytic subpopulations including cells which show DC and macrophage functions to some degree (170). Cells with predominant DC functionality are mainly detected in the cortical tubulointerstitium and can hardly be found in the glomeruli. Only 5% of the tubulointerstitial DCs belong to the CD8-like subset and have exclusive DC functionality. DCs located in the renal medulla are particularly potent in neutrophil recruitment (155).
In contrast to the DCs, macrophages can be found in the subcapsular renal area, in periarterial connective tissues and also in small amounts within the glomeruli. In the healthy kidney DCs are responsible to monitor their environment and to detect glomerular and tubular self-antigens

(171). By expressing the suppressive molecule programmed cell death ligand 1 (PDL1), DCs induce apoptosis in T cells specific for such antigens (172).

In the ischemia reperfusion injury (IRI) mouse model, a model to investigate acute kidney failure, kidney-resident DCs might have a proinflammatory role as they are the first to produce proinflammatory chemokines and cytokines like TNF. In contrast to this finding DCs might as well have a protective function in models of drug induced tubulotoxicity and acute crescentic GN. DCs are suggested to prevent excessive ischemic tissue damage by anti-inflammatory signalling mechanisms (155).

Circulatory monocytes are attracted to the inflammatory kidney microenvironment through chemokines and differentiate within the kidney into DCs and macrophages with a proinflammatory phenotype. This proinflammatory milieu pushes CKD progression by DCs stimulating T helper cells, while T cells in turn produce cytokines like IFN- γ to activate renal macrophages. The amount of tubulointerstitial mononuclear infiltration correlates inversely with kidney function. Thus, it is assumed that tubulointerstitial immune cell cross-talk promotes CKD progression (155). To slow disease progression, depletion of renal DCs and macrophages could be a new therapeutic approach. Nevertheless clodronate liposomes, used so far in mice for DC and macrophage depletion, is not exclusively harming DCs and macrophages but also damages other cell types. Therefore, new techniques and experimental settings are needed (155).

2.5. THE ROLE OF THE B CELL IN THE KIDNEY

B cells can be divided into $CD5^+$ B1 cells and $CD5^-$ B2 cells. When they turn into memory B cells, they switch their antigen receptors to IgG, IgA, or IgE. This immunoglobulin class switch is done in the germinal centres within the spleen and lymph nodes. In a last step, before B2 cells turn into antibody producing plasma cells, they differentiate outside of the germinal centres within the secondary lymphoid tissues (67,163).

B-cells link the innate, like the DCs and adaptive parts (T-cells) of the immune system by their ability to respond rapidly to DAMPs. Disruption of B-cell tolerance and impaired regulatory function of B-cell contribute to pathogenesis of autoimmune disorders. Depleting B-cells is therefore a therapeutic option in autoimmune renal disease and could potentially re-establish B-cell tolerance by destruction of autoreactive clones and pathogenic antibody producing B-cells. With the monoclonal (mAb) anti-CD20 antibody rituximab, autoreactive and pathogenic B-cells are destroyed and activation of pathogenic T cells is interrupted. However, rituximab is

not only depleting the pathogenic antibody producing B-cells but also the beneficial regulatory B-cells (Bregs). Future studies are needed to understand how pathogenic B-cells arise and to develop more selective autoreactive B-cell depleting therapies (155).

2.6. THE ROLE OF THE T CELL IN THE KIDNEY

Antigens are presented by the antigen presenting cells (APC) to the naive T-cells in the T-cell zones in the secondary lymphoid tissues and cause clonal expansion. These antigen presenting cells are DCs, expressing the co-stimulatory molecules B7.1 and B7.2 on their cell surface, which took up the pathogenic antigen on the spot of infection and subsequently became activated by other innate immune cells and migrated to local lymphoid tissue. The receptor for B7 molecules on the T cell is $CD28^+$ (67). Like B-cell receptors also T-cell receptors have variable regions (173,174). The various receptor regions are the V, D and J part. It is essential, that the T-cells express a functional antigen receptor and autoimmunity is excluded. Therefore, a positive and negative selection takes place. The positive selection is testing the functionality of the antigen receptor. The negative selections filters self-reactive cells (67). For T-cell activation, an antigen presenting cell is needed to present an antigen to the T-cell receptor. Moreover, B7 molecules are presented to $CD28^+$ T-cells. This is a regulatory step to prevent naïve T-cells from responding to self-antigens. If the T-cell is activated, the cell starts producing IL-2, which leads to proliferation and differentiation. Depending on the various cytokines released, T-cell phenotype is ultimately determined (163). In glomerulonephritis, especially antibodies are considered to be the main players in disease development and progression. However, there is more and more evidence that also T cells contribute directly to glomerular injury. In NTS, infiltrating T helper 1 (Th1) and T helper 17 (Th17) cells contribute to renal injury and mediate the pathogenesis (19). Th1 cells provide the cytokines to promote development of complement fixing antibodies, such as IgG1 and IgG3. Kidney inflammation and pathological changes are perpetuated by antigen presenting DCs, which recruit Th1 cells. Th1 cells themselves can recruit more pro-inflammatory cells (155,175). Counterparts in this pro-inflammatory milieu are the systemic and local regulatory immune cells (Tregs), which have suppressive effects on both adaptive and innate immune cells and protect the kidney from injury. In the early NTS stage Tregs suppress TH17 cell infiltration and in the later phase Tregs suppress not only Th17 cells but also Th1 activation (19,176). Tregs, as major mediators of tolerance, have multiple potential sites of action. Nevertheless, whether Tregs are a new treatment target is still controversially discussed and under investigation (176).

3. PROSTANOIDS IN THE KIDNEY

The lipid molecule prostaglandin (PG)E2 mediates pro- and anti-inflammatory effects, depending on its local concentration and on the cell type and receptors involved (177,178). Due to its short half-life, PGE2 is produced locally at the site of action. Prostaglandins originate from arachidonic acid (AA), a 20 carbon poly- unsaturated fatty acid, which occurs exclusively in membrane-bound phospholipids. It is liberated from its ester bond by phospholipases, especially cytosolic phospholipase A2 (PLA2). Since AA is lipophilic and thus it is difficult to store, it may only be released in times of need. Activity of PLA2 is regulated by various hormones, e.g. bradykinin, angiotensin II (AT II), endothelial growth factors (EGF) and corticosteoroids. Vice versa prostanoids can mediate or modulate the actions of these mentioned hormones. Also pro-inflammatory cytokines such as IL-1β increase the activity and the neosynthesis of PLA2. AA is converted to the endoperoxide prostaglandin G2 (PGG2) and further reduced to prostaglandin H2 (PGH2) by the cyclooxygenases 1 (COX1) or 2 (COX2). Whether this happens through COX1 or COX2 depends on the location and tissue condition. COX1 is thought to be a constitutive enzyme found in most body tissues, while COX2 is an inducible enzyme found mainly in inflamed tissue. Cytokines or cell wall components of gram-negative bacteria can stimulate induction of COX2. The different PG subtypes originate from the intermediate PGH2. These subtypes include the prostanoids: prostaglandin E2 (PGE2), prostacyclin (PGI2), prostaglandin F2α (PGF2α), prostaglandin D2 (PGD2) and thromboxane A2 (TXA2). PGE2 can also be transformed to prostaglandin A2 (PGA2). TXA2 occurs mainly in platelets where it is responsible for stimulating platelet aggregation (177,179).

NSAIDs

NSAIDs inhibit COX1 and 2 and therefore have analgesic, anti-pyretic and anti-inflammatory effects. Despite many clinical benefits, the use of NSAIDs is linked to the occurrence of many side effects ranging from gastrointestinal ulcers, and AKI to cardiovascular complications. It was thought that selectively blocking COX2 can reduce adverse reactions. But the expectations have not been fulfilled due to increased cardiovascular risk linked to the use of selective COX2 inhibitors (180). Apart from gastrointestinal bleeds, side effects of NSAID mostly concern the kidney leading to diminished renal blood flow and reducted GFR. Moreover NSAID's can cause an exacerbation of hypertension. Nevertheless, due to vasoconstriction of afferent arterioles it can decrease proteinuria in patients with glomerular diseases (181).

NSAIDs and interstitial nephritis

This disease is characterized by an inflammatory infiltrate in the kidney interstitial area which is in most cases drug associated, but may also be part of a systemic disease like SLE, Sjögren's syndrome, sarcoidosis, infections, or tubulointerstitial nephritis with uveitis (TINU) syndrome. Among the most common drugs causing an interstitial nephritis are NSAIDs as well as selective COX2 inhibitors. It is important to notice that the drug-induced interstitial nephritis is not dose dependent. Patients with an acute or chronic renal disease should avoid NSAIDs to exclude additional drug related nephrotoxicity (20,182).

3.1. Prostaglandin E2 and E prostanoid receptors 1-4 (EP1-4)

The ubiquitously occurring PGE2 is linked to mediation of pain, fever, inflammation, regulation of blood pressure, renal perfusion, angiogenesis and tumor growth. Various functions of PGE2, which are mediated by the four different G-coupled receptors namely EP1-4. The different EP receptors show differential patterns of tissue distribution and signaling pathways (177).

E PROSTANOID RECEPTOR 1 (EP1)

The Gq coupled EP1 receptor, can be found in humans on mast cells, in pulmonary veins, uterus, kidney and in the gastrointestinal tract (183–185). EP1 causes Ca^{2+} release with phospholipase C, which converts phosphatidylinositol bisphosphate (PIP2) to diacylglycerol (DAG) and inositol 1,4,5-triphosphate (IP3). IP3 opens Ca^{2+} channels in the endoplasmic reticulum, which increases its concentration in the cytoplasm (177,183,186). EP1 receptor mediates neuroendocrine stress responses and behavior by facilitate release of corticotropic-releasing hormone (CRH) which consequently stimulates adrenocorticotropic hormone (ACTH) secretion (187).

E PROSTANOID RECEPTORS 2 (EP2)

The Gαs coupled EP2 receptor is mainly expressed in the airways, ovary, bone marrow and olfactory epithelium (183–185). EP2 and EP4 cause a stimulation of the adenylate cyclase (AC). AC synthesizes cyclic adenosine monophosphate (cAMP) (177,183,186). EP2 and EP4 have redundant and similar functions in some processes. For example, both receptors mediate via PGE2 in osteoclasts induction of RANKL through cAMP. Nevertheless there are some functions where the EP2 receptor play a distinct role like in ovulation and fertilization due to selective expression of EP2 in ovaries (187).

E PROSTANOID RECEPTORS 3 (EP3)

The Gi coupled EP3 receptor can be detected mainly in adipose tissue, respiratory system, pancreas, kidney and the vena cava. Nevertheless it is assumed that there are splice isoforms of the EP3 receptor, also coupling to different signalling pathways (183–185). EP3 causing an inhibition of AC (177,183,186). EP3 is present in the airway epithelium and by activation of the receptor expression of allergy-related genes and therefore progression of allergic inflammation are suppressed (187).

E PROSTANOID RECEPTOR 4 (EP4)

The Gαs coupled EP4 receptor is found in the gastrointestinal tract, uterus, hematopoietic tissues and skin, but can also be detected in the kidney. As already mentioned it is thought that EP2 and EP4 have similar and redundant roles (183–185). EP4 but not EP2 can additionally activate the phosphoinositide-3-kinase (PI3K) signaling pathway, probably via Giin addition to activation of adenylate cyclase (177,183,186). The EP4 receptor is one of the most widely expressed PGE2 receptors in the body. Activation of the Gαs coupled EP4 receptor leads to an intracellular increase of cyclic adenosine monophosphate (cAMP) levels. EP4 receptors are expressed on different immune cells, resident kidney cells (epithelial cells of the glomerulus, mesangial cells, afferent arteriole) and endothelial cells (177,188). These cells play a crucial role in the pathophysiology of GN (189). Studies have shown that PGE2 mediated its anti-inflammatory effects via the EP4 receptor. On one hand, EP4 is responsible for the limitation of pro-inflammatory cytokine production, activation of macrophages and neutrophils. On the other hand, the EP4 receptor can mediate the activation of TH17 cells and the increase of the expression of C-C chemokin receptor 7 (CCR7) thereby facilitating the recruitment of T cells (177,190).

EP4 receptor signals via cAMP, protein kinase A (PKA) and endothelial nitric oxide synthase (eNOS) mainly mediate relaxant effects. Moreover previous studies described vasodilatory as well as bronchodilatatory effects mediated via the EP4 receptor (191,192). On the other hand EP4 receptors expressed on mouse podocytes signal via mitogen-activated protein (MAPK) and COX2 through a cAMP/ AMP-activated protein kinase (AMPK) dependent, but PKA-independent signalling cascade (193). In other words PGE2 via EP4 can activate different cAMP-dependent pathways including PKA and exchange protein directly activated by cAMP (Epac) and AMPK (194).

3.2. The immune system and PGE2

EP1 and EP2 receptors are expressed on T and B lymphocytes as well as macrophages. The EP3 receptor was detected only on B cells and macrophages, but not on T cells (195,196). The EP4 receptor is expressed on almost all immune cells (B- and T-lymphocytes, NK cells, DCs, eosinophils, monocytes and macrophages) (195,197). PGE2 impairs production of TNFα and IL-12 by macrophages and inhibits MHC class II expression, and may therefore can suppress antigen presentation (178,198). The anti-inflammatory actions of PGE2 has been mainly attributed to EP4 receptor-mediated signalling (199).

Especially in TH17 driven disease like NTS, the EP4 receptor mediated signalling can promote inflammation. Boniface et al demonstrated that mainly through EP2 Th17 cytokine expression is enhanced, whereas PGE2 inhibits via EP4 the production of the antiinfl ammatory cytokine IL-10 in Th17 cells (200). TH1 and TH17 cells are pro-inflammatory and mediate tissue damage in GN (200). It was shown that the EP4 receptor mediates the activation of Th17 cells and increases the expression of C-C chemokine receptor 7 (CCR7). CCR7 is expressed on DCs, Tregs and other subpopulations of $CD4^+$ T-cells. CCR7 facilitates cell migration and egress from peripheral tissue to the secondary lymphoid organs (21,177,195). It has been shown that CCR7 plays an important role in the suppression of GN, which is mediated by Tregs. All these cell populations play an important role in the progression of NTS (201–203).

Yao et al found promotion of TH1 differentiation and TH17 expansion and stabilization through EP4 stimulation via the PI3K signalling pathway (204). PGE2 acting via the EP4 receptor is crucial for DCs to attain a migratory phenotype for their entry into the lymph nodes and subsequent antigen presentation. Notably, expression of MMP-9 on DCs, which is also necessary for their migration into lymph nodes, is mediated via the EP2/EP4 receptor (201,205,206). *In vivo* experiments showed that DCs exposed to PGE2 drive the immune response via the EP4 receptor towards a TH2 phenotype. This observation suggests that EP4 may play an important role especially in allergic disease (207,208). This is supported by Fedyk et al. describing B-cell class switch towards IgE mediated via PGE2 and the EP2/EP4 receptor (209).

Macrophages express the EP4 receptor. PGE2 stimulates macrophage motility and inhibits chemokine release (210). This effect is not mediated by the cyclic adenosine monophosphate (cAMP) / Protein kinase A (PKA) / cAMP response element-binding protein (CREB) pathway but rather acts via the signaling pathway EP4 receptor-associated protein (211).

Eosinophils are poor phagocytes and they are mainly responsible for the defense against helminths and parasites. Besides releasing oxygen metabolites, cationic proteins, cytokines,

eosinophils are also secreting leukotriene and prostaglandin. Therefore eosinophils play an important role in allergic disease like asthma (212,213).

PGE2 and EP4 receptors, in particular the EP2/EP4-mediated expression of matrix metalloprotease-9 (MMP-9) are critical for dendritic cells to migrate to lymph nodes and activate lymphocytes (201,205,206).

Activation of EP4 receptor expressed on T cells makes them upregulate expression of IL23 and IL1 receptors what promotes Th2 polarization and suppresses IL10 production (200,214). Okano et al described that *in vitro* PGE2 via the EP2 and EP4 receptor suppresses the TH1 and TH2 specific response (215). Concerning B-cells PGE2 is promoting the antibody class switch towards IgE by activating the EP2 and EP4 receptor (209).

3.3. The role of PGE2 in the kidney

Many studies have shown the great impact of PGE2 on kidney physiology. Regulation of parameters like renal perfusion, diuresis, sodium excretion and regulation of the blood pressure by activation of the renin-angiotensin-aldosterone system is strongly influenced by prostaglandins. Renin and erythropoietin release, urinary concentration and electrolyte reabsorption are also associated with prostaglandin levels (177,179,185,216–218). As already mentioned, PGE2 is synthesised and acting in all tissues in the human body, but especially the kidney is synthesising a huge amount of prostanoids. The greatest amount of PGE2 production is detected in the glomeruli and collecting ducts (188). Mesangial cells synthesise PGE2 induced by IL-1 and TNF. Resident kidney cells express, depending on the cell type all E prostanoid receptors, EP1-4. According to the PGE2 amount being synthesised and the renal cell type various several cellular responses are mediated. Therefore, PGE2 can be also measured in the urine. Besides PGE2, also the prostanoids PGF2 and PGI2 act on kidney cells and, therefore, play a role in renal physiology (188,219,220). The PGE2 synthases microsomal PGE synthase 1 and 2 (mPGES-1, mPGES-2) and cytosolic PGE synthase (cPGES) have been found in the kidney (188). For PGE2 production mPGES-1 is coupling to COX1 as well as to COX2, in contrast to cPGES which is acting via COX1 mainly in the collecting ducts (188,221). Both COX isoforms occur in the renal inner medulla and lower concentrations in the outer medulla and renal cortex (218). Beside the kidney mPGE-1 is also expressed in adipose tissue, the stomach and the spleen (222).

EP1 AND THE KIDNEY

EP1 is expressed in the collecting duct, proximal tubule cells and glomerular mesangial cells (223,224). In the collecting duct the greatest amounts of EP1 can be measured, being responsible for sodium reabsorption (225). Moreover EP1 is hindering proliferation via cyclin dependent kinase inhibitor p27 and is promoting fibrosis (188). Rahal et al found that EP1 KO mice were unable to modulate potassium transport along the distal nephron, thereby prompting severe hyperkalaemia. Furthermore, creatinine and urea levels were as well exacerbated in EP1 KO mice. Rahal et al. hypothesise that the observed hyperkalaemia is due to direct effects on the aldosterone synthesis (226).

EP2 AND THE KIDNEY

The EP2 receptor is expressed on endothelial cells and can also be detected in the interstitial compartments of the kidney. EP2 takes part in sodium balance in response to a high-salt diet (227). EP2 knockout mice develop salt-sensitive hypertension (228). EP2 might play a role in cyst formation in polycystic kidney disease (229).

EP3 AND THE KIDNEY

EP3 was detected in the distal tubular cells and in the collecting duct. In the kidney EP3 takes part in the blood pressure regulation and in the diuretic homeostasis by inhibiting water and sodium reabsorption (188). EP3 has so far mainly been associated with water homeostasis and seems to play a role in diabetes insipidus (230).

EP4 AND THE KIDNEY

Ling et al. found EP4 expression in glomeruli, the media of arteries as well as in the vasa recta. EP4 was in this study not detected in proximal tubules, thin limbs and thick ascending limbs of Henle's loop, distal convoluted tubules, or collecting ducts (185). The expression of EP4 in the glomerula is additionally emphasized by Breyer et al. who demonstrated the expression of EP4 receptor mRNA in glomerular cells (217). Other studies which performed EP4 staining in mouse kidneys could detect EP4 expression on endothelial cells, e.g. the afferent arteriole, but also on tubular cells and the juxtaglomerular apparatus and macula densa, being responsible for renin release (231–233).

Of interest, kidneys of EP4 knock out (KO) mice have a decreased number of nephrons suggesting that EP4 plays an important role in kidney development (234).

3.4. *EP4 and blood pressure regulation*

The renin-angiotensin-aldosterone system (RAAS) is essential for blood pressure regulation as well as regulation of extracellular volume. Renin is produced by the juxtaglomerular cells and is released due to certain stimuli (235). Moreover it is speculated that the kidney is also releasing prorenin via a constitutive pathway. Prorenin-releasing stimuli can be: i) Insufficient perfusion of the kidney, ii) changes in sodium levels, as well as iii) a negative feedback loop via Angiotensin II. All this stimuli have in common that due to low blood pressure and therefore decreased kidney perfusion, the kidney releases renin to increase blood pressure and consequently kidney perfusion. Nevertheless the kidney is not the only organ being responsible for renin production, also the brain, adrenal gland, ovary, and visceral adipose tissue, heart and vascular tissue are synthesizing renin (236).

It was shown that PGE2 increases diuresis, modulates vascular tone and renal perfusion, and affects sodium homeostasis and water transport along the nephron; therefore, PGE is considered as an important component of the renal prostanoid system (218,220,237).

Several studies could demonstrate that EP4 receptor is expressed in renin-secreting juxtaglomerular granular cells. The kidney-produced hormone renin plays an important role in blood pressure regulation (232). Furthermore, EP4 plays a role in regulation renal water homeostasis by upregulating the sodium-hydrogen exchanger 1/3 (NHE1/3) and sodium-potassium ATPase (NKA) and consequently leading to sodium reabsorption (188). *In vitro* studies have shown that PGE2 and PGI2 directly stimulate renin secretion in isolated juxtaglomerular cells through elevation of intracellular cAMP. It is claimed that the stimulation of renin secretion by PGE2 and PGI2 are mediated via EP2, EP4, and IP receptors (235).

It was shown by Frölich et al. that a low salt diet in mice causes increased renal PGE2 synthesis, which in turn stimulates the renin-angiotensin-aldosterone system by activation of the EP4 receptor (218). Poschke et al. examined the role of the EP4 receptor in the RAAS in a salt restriction *in vivo* mouse model. Salt restriction enhanced plasma renin and aldosterone concentrations through enhanced by PGE2 release and COX induction. This effect was mediated via the EP4 receptor. EP4 receptor blockade - due to a negative feedback mechanism affecting the COX system - leads to overstimulated COX2 expression and PGE2 synthesis. Moreover, renin expression and activity was suppressed. Consequently electrolyte alterations, such as increased diuresis and increased potassium output, was observed (218). Further studies could also demonstrate that salt deprivation in mice causes COX2 induction in

the macula densa and enhanced PGE2 synthesis. These are key steps leading to renin secretion followed by RAAS activation (238,239). This effect is ascribed to the EP4 receptor (231).

Moreover, PGE2 is a potent vasodilator and intravenous administration of PGE2 induces a rapid (up to 20 seconds) dropping of the blood pressure. PGE2 acting via EP1/EP3 receptors cause vasoconstriction and via EP2/EP4 receptors vasodilatation (222). EP1 and EP3 cause vasoconstriction of the afferent arteriole caused by decreased renal perfusion, whereas EP2 and EP4 mediate vasodilatation of the vasa recta and therefore renal hyperfiltration (188).

To sum up, depending on the expression pattern of the EP receptor, counteracting hormones (endothelin, angiotensin II, nitric oxide) or PGE2 is mediating vasodilatation or vasoconstriction (188).

3.5. *EP4 and kidney disease*

It is thought that kidney damage leads to increased COX expression and consequently increased PGE2 production, which is acting via the EP4 receptor in podocytes, causing proteinuria and glomerular damage. Furthermore the EP4 receptor is described to promote cell survival and to increase proliferation (240–242). Via the EP4 receptor PGE2 promotes cell proliferation, but at the same time fibronectin and IL6 is elevated and connective tissue growth factor (CTGF) is inhibited, which promotes fibrosis (188). This is in contrast to Vucicevic et al., who showed in an AKI nephrotoxic mercury chloride rat model that EP4 receptor agonism leads to an improved kidney phenotype depicted by decreased fibrosis, less serum creatinine and increased survival (REF). They concluded that EP4 activation decreases the expression of intracellular adhesion molecule-1 is. Adhesion molecule-1 mediates the progression and intensity of renal necrosis and subsequently fibrosis (243). But this could not only be shown in an acute kidney injury model but also in an 5⁄6 nephrectomy rat model of chronic kidney failure (244).Yamamoto et al. demonstrated in a rat model of cisplatin-induced kidney injury, that rats treated with an EP4 receptor agonist had less kidney injury due to decreased apoptosis and increased proliferation (245). EP4 knockout mice subjected to unilateral ureteral obstruction causing an acute kidney failure had a worse phenotype compared to wildtype mice. This was accompanied by elevated inflammatory and fibrotic markers and increased renal fibrosis (243).

In rodent models of diabetic and non-diabetic CKD, EP4 blockade led to an improved kidney phenotype (246). Mohamed et al showed in a streptozotocin-induced diabetic mouse model

that treatment with an EP4 receptor agonist leads to worsening of the phenotype. This is due to IL-6-induced glomerulosclerosis and interstitial renal fibrosis (247). EP4 is expressed on mice podocytes (193) and via podocyte-selective depletion of EP4, proteinuria in diabetic mice was diminished. In contrast, Riyaz et al. showed that EP4 agonism promotes glomerulosclerosis and tubulointerstitial fibrosis in a diabetic mouse model through an increase in IL-6 expression (223).

Jian Hua et al. showed in a murine diabetes insipidus model that mice treated with an EP4 receptor agonist had less polyuria and polydipsia. They concluded that the ability to concentrate urine is mediated via the EP4 receptor expressed on collecting duct cells (249).

3.6. *EP4 and immune cells*

EP4 is also taking part in inflammatory responses in the kidney by inhibiting chemokine regulated on activation, normal T expressed and secreted (RANTES), also known as CCL5. RANTES is a chemotactic for T cells, eosinophils and basophils (188). Furthermore EP4 inhibits monocyte chemotactic protein-1 (MCP-1) or also known as CCL2. The cytokine CCL2 is responsible for recruiting monocytes, T cells and DCs to the spot of inflammation (188,250).

To sum up, the EP4 receptor has the potential to act on two axes, the immunological and the renin-angiotensin-aldosterone axis.

4. HYPOTHESIS AND AIMS

The research results of the past 20 years show a clear involvement of PGE2 and its receptors in the course of immune-mediated GN. Prostanoids are potent modulators of inflammatory cells and might thereby influence many different kidney diseases. Administration of PGE2 shows positive effects on the progress of NTS. It was hypothesized that PGE2 promotes recovery during NTS via the EP3 receptor (251). Nagamatsu et al. showed an improvement of NTS via EP4 receptor activation (252). Nevertheless, there are many contradictory findings regarding the nature of the influence of PGE2. To what extent this is done via which receptor subtypes, is still the subject of research. So far both pro- and anti-inflammatory effects of the EP4 receptor are documented, it is not clear which role a blockade or an activation of this receptor my play in NTS.

With this background we set out to elucidate the exact role of EP4-expressing immune cells in GN, using the mouse model of experimental Nephrotoxic Serum Nephritis (NTS). We further aimed to evaluate EP4 not only in mice *in vivo* but also EP4 expression and function on kidney cells *in vitro*. We evaluated whether PGE2 via EP4 receptor activation and blockade on distal convoluted tubular cells (DCT) influences cell viability.

We hypothesized that both activation and blockade of the EP4 receptor in NTS would alter the phenotype of the disease model, due to the multiple immunological effects of PGE2. To objectify the phenotype of the disease, parameters for renal function, glomerular damage, immune cell influx into the kidney and the expression of pro-inflammatory markers have been evaluated. The findings of these studies can be used to generate new hypotheses about potential therapeutic approaches in the therapy of immune-mediated renal diseases.

5. MATERIAL AND METHODS

Animals and study design

Male C57Bl/6J mice at the age of 8-10 week and a body weight of 22-25 g were used for the following experiments. The mice were obtained from Charles River Laboratories (Sulzfeld, Germany). To induce NTS, C57BL/6 mice were immunized s.c. with 2 mg/ml rabbit IgG (Jackson ImmunoResearch Laboratories, West Grove, PA, USA) dissolved in incomplete Freund´s adjuvant (Sigma-Aldrich, St. Louis, MO, USA) with desiccated, nonviable Mycobacterium tuberculosis H37a (Difco Laboratories, Detroit, MI, USA). Three days later, NTS mice were injected intravenously via the tail vein with heat-inactivated rabbit anti-mouse glomerular basal membrane (GBM) serum.

On the day of NTS induction, the mice were treated with high-dose [25 µg/mouse/day] or low-dose [7 µg/mouse/day] EP4 receptor agonist ONO AE2-329 (ONO Pharmaceutical), EP4 receptor antagonist ONO AE1-208 (ONO Pharmaceutical) [10mg/kg BW] or with the EP4 receptor antagonist ONO AE1-208 [10mg/kg BW] followed 30 minutes later by treatment with low-dose EP4-receptor agonist ONO AE2-329 [7 µg/mouse/day], or vehicle s.c. twice daily. Mice were sacrificed 14 days after NTS induction. To measure the influence of the EP4 receptor on diuresis, mice were placed in metabolic cages and urine was collected for 24 hours. Additionally, C57Bl/6J mice without induction of NTS were treated with an EP4-receptor antagonist ONO AE1-208 [10mg/kg BW] and vehicle s.c. twice daily for 7 days.

Study approval

The animal care and experimental procedures used in this study are in accordance with national and international guidelines and were approved by the Austrian Federal Ministry of Science, Research and Economy (BMWFW GZ:66.010/042- WF/ V/3b/2015), conform with the Directive 2010/63/EU. Studies are reported in compliance with the ARRIVE guidelines (253,254).

Urine albumin quantification by ELISA and urinary creatinine detection

7 and 14 after NTS induction, spotted urine was collected and the urinary albumin/creatinine ratio was determined using an albumin ELISA (Abcam, Cambridge, MA, USA). Urinary creatinine was evaluated photometrically using a picric acid-based kit (Sigma-Aldrich).

Serum lipocalin-2/NGAL ELISA

To evaluate the serum Lipocalin-2/ neutrophil gelatinase-associated lipocalin (NGAL) levels a commercially available Mouse ELISA DuoSet kit (R&D Systems, Park Abingdon, UK) was used.

Assessment of serum blood urea nitrogen (BUN)

For BUN evaluation in serum of the NTS mice after 14 days a commercially available BUN Colometric Detection Kit (Invitrogen, Carlsbad, CA) was used.

IL6 ELISA

Interleukin-6 levels in serum as well as in supernatant of DCT cells was determined using a commercially available mouse interleukin-6 ELISA kit (ImmunoTools, Friesoythe, Germany).

Assessment of angiotensin in plasma

To evaluate the angiotensin levels in mice treated with the EP4 receptor antagonist or agonist, plasma of mice was measured for angiotensin via mass spectrometry (liquid chromatography tandem-mass spectrometry-based angiotensin quantification) by Attoquant Diagnostics (Vienna, Austria) (255).

Immunohistochemistry staining

14 days after NTS induction, mice were sacrificed. Kidneys were harvest and fixed in 10% neutral-buffered formalin for 24 h and embedded in paraffin afterwards. Kidneys were cut in 4 µm sections following a periodic acid Schiff (PAS) staining (Merck KGaA, Darmstadt, Germany). For evaluation of tubular cast formations 6 low power fields have been evaluated in a blinded manner. Cell proliferations in the different glomerular compartments were assessed as follows: 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). Endocapillary hypercellularity, defined as hypercellularity due to an increased number of cells within glomerular capillary lumina, was subclassified as mild (score 1; present in a single glomerula), moderate (score 2; <50% affected glomerula), and severe (score 3; >50% affected glomerula). Extracapillary hypercellularity/crescents defined as cell proliferation of more than two cell layers were subclassified as mild (score 1; present in single glomerula), moderate (score 2; <50% affected glomerula), and severe (score 3; >50% affected glomerula). The presence of capillary necrosis and intraluminal capillary thrombi was assessed in chromotrope aniline blue-stained serial sections and subclassified as mild (score 1; present

in single glomerula), moderate (score 2; <50% affected glomerula), and severe (score 3; >50% affected glomerula).

Proliferating-cell nuclear antigen (PCNA) was stained on 4 µm paraffin sections using the three-layer immunoperoxidase staining protocol subsequent to standardized heat-mediated antigen retrieval with an automated de-cloaking chamber for 30 min at 120°C and 20 second at 80°C (Biocare Medical, Concord, CA, USA). Tissue sections were then stained with a mouse anti-mouse PCNA antibody (BioLegend, San Diego, CA, USA) using the M.O.M. staining kit (Vector Labs, Burlingame, CA, USA). Positive-stained cells were counted per 6 high-power fields.

Kidneys were snap-frozen with Tissue-Tek® OCT™ Compound (Sakura Finetek Europe B.V., Alphen an den Rijn, Netherlands). Frozen 4µm kidney sections were additionally stained using rat-derived primary antibodies for $CD4^+$ (Serotec, Oxford, UK), $CD8^+$ (Serotec), $CD68^+$ (Serotec) and Ly6 (Gr-1) (Abcam). The tissue was incubated for one hour in a humidified chamber with the primary antibody followed by incubation with a biotin-conjugated goat anti-rat IgG (Jackson ImmunoResearch Laboratories) as a secondary antibody. Positive $CD4^+$ and $CD8^+$ cells were counted per 6 high-power fields. A semi-quantitative scoring system for $CD68^+$ positive cells was performed as follows: 0 = 0 to 4 cells stained positive, 1+= 5 to 10 cells, 2+= 11 to 50 cells, 3+= 51 to 200 cells, and 4+= >200 cells stained positive per low-power field. Ly6 (Gr-1) positive stained cells were counted in 50 glomeruli per mouse.

Immunofluorescence staining

To quantify and evaluate renal deposition of autologous and heterologous IgG, as well as C3, kidneys were harvest 14 days after NTS and snap frozen followed by cutting them into 4 µm sections. Serial dilutions of FITC-conjugated goat anti-mouse IgG (Jackson ImmunoResearch Laboratories) as well as serial dilutions of fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit IgG (Jackson Immuno-Research Laboratories) were prepared and dispensed on kidney sections followed by histological evaluation of autologous IgG and heterologous IgG deposition in the kidney. For this purpose direct immunofluorescence titter determination was used. To evaluate C3 deposition in the kidneys, serial dilutions of FITC-conjugated goat anti mouse Complement C3 (MP Biomedicals, Eschwege, Germany) were dispensed on kidney sections. C3-stained slides were mounted in mounting medium for fluorescence with DAPI (Vector Laboratories).

IgG ELISA

To assess the autologous antibody respons, rabbit IgG (100 µg/ml; Jackson ImmunoResearch Laboratories) was absorbed over-night on a 96-well plate, followed by incubation with the serum of our mice in serial-doubling dilutions. For the detection of circulating mouse anti-rabbit immunoglobulin horseradish peroxidase-conjugated rabbit anti-mouse IgG (Jackson Immuno Research Laboratories) was used.

Blood pressure measurement

The non-invasive tail cuff blood pressure measurement was performed daily for 14 days in the evening with a Kent Scientific Corporation CODA Non-Invasive Blood Pressure System (Torrington, CT, USA). Mice were accustomed to the procedure three times before the recording started. The measurement was performed by the same person in a quiet room; animals were placed on a heating unit and measurement was only started when the tail skin temperature reached 35°C. Each measurement consisted of 5 acclimations and 10 experimental measurements. The session was repeated two times. The mean arterial pressure (MAP) was calculated with accepted values as followed MAP= ((2xdiastolic pressure) + systolic pressure) /3.

Flow cytometry

Lymph node and blood single cells suspensions were stained for $CD45^+$, $CD4^+$,$CD8^+$,$B220^+$,$CD3^+$,$CD69^+$, $FoxP3^+$, $CD11b^+$, $CD11c^+$, $Ly6G^+$ and a live/dead cell stain using fluorescent-labelled antibodies (eBioscience, San Diego, CA, USA). APC-conjugated rat anti-mouse $CD4^+$ (Clone RM4-5; BD Biosciences, San Jose, CA, USA), FITC-conjugated anti-mouse $CD8a^+$ (clone 53–6.7; BioLegend, San Diego, CA, USA), PE-conjugated anti-mouse $CD69^+$ (clone H1.2F3; BioLegend) PE-conjugated anti-mouse $B220^+$ (clone RA3-6B2; BioLegend), eFluor450-conjugated anti-mouse $CD11b^+$ (clone M1/70; eBioscience), PerCP-Cy5.5 conjugated anti-mouse $CD45^+$ (clone 30-F11; BioLegend), Alexa Fluor 700 conjugated anti-mouse $CD3^+$ (clone 17A2, BioLegend), Alexa Fluor anti-mouse $FoxP3^+$ (clone 647:150D, BioLegend), APC-conjugated anti-mouse $CD11c^+$ (clone HL3, BioLegend), PE-Cy5 conjugated anti-mouse $Ly6G^+$ (clone RB6-8C5, BioLegend) and LIVE/DEAD Fixable Aqua Dead Cell Stain Kit ((Applied Biosystems, Thermofisher) . Samples were analysed on a LSRII cytometer (BD Biosciences).

RNA isolation, reverse transcription (RT) real-time polymerase chain reaction (PCR)

RNA was isolated using TRI Reagent® (Sigma-Aldrich). Complementary DNA (cDNA) transcripts from RNA were synthesized using Superscript III Transcription Kit (Invitrogen, Carlsbad, CA, USA) and random primers (Roche, Basel, Switzerland) for reverse transcription of 0.5 to 2 µg of total RNA. Real-time PCR was performed in duplicates on a CFX96 Real-Time System (BioRad, Hercules, CA, USA). For quantification of respective genes, TaqMan gene expression assays (Applied Biosystems, Foster City, DA, USA) for *FoxP3*: Mm00475162_m1, *Tnf-alpha*: Mm00443258_m1, *Il10*: Mm00439616_m1, *Il6*: Mm00446190_m1, *Tbx*: Mm00450960_m1, *Ifn-gamma*: Mm00801778_m1, *Il17a*: Mm00439619_m1, *Gata-3*:
Mm00484683_m1, *Cxcl1*: Mm00433859_m1, *Cxcl5*: Mm00436451_g1 and *Renin*: Mm02-342889_g1 were used. Real-time PCR for EP receptor expression was performed using Advanced™ Universal SYBR® Green Supermix with PrimePCR™ SYBR® Green Assay primers for PTGER1-4 or GAPDH, human (all Biorad) according to the manufacturer's instructions. SYBR Green Mastermix (BioRad) was used for the detection of *hprt* or *β-Actin* using the following primers, 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´GAA GTG TGA CGT TGA CAT CCG 3´; reverse 5´TGC TGA TCC ACA TCT GCT GGA 3´. Both housekeeping genes served as reference genes for kidney samples. Results were calculated with the 2-ΔΔCT method. Seeded tubular cell mRNA from mice treated with vehicle or EP4 receptor antagonist from day 4 until day 14 after NTS induction were normalized to *hprt* or *β-Actin.*

Cxcl-5 ELISA

To measure the amount of Cxcl-5 in supernatant a commercially available ELISA kit (Mouse LIX Quantikine ELISA kit, R&D Systems, Minneapolis, MN) was used.

Isolation of kidney tubular cells

Fourteen days after NTS induction in the mice, kidneys were harvest and minced. Tubules were isolated using two sieves (mesh size 150 and 90 µm) Newark Wire Cloth, Clifton, NJ) (256). From the top of the 90-µm sieve (Newark Wire Cloth), tubuli were collected and subjected to RNA isolation.

Cell culture experiments

The Distal convoluted tubular cell (DCT) line was kindly provided by Miriam Banas, (University of Regensburg, Germany). DCT cells were grown in DMEM/Ham's F-12 media (Gibco, life technology) supplemented with 5% heat-inactivated FCS (Gibco) and an antibiotic mixture of 1% Penicillin/ streptomycin, and 100 mg neomycin/100 ml (Gibco, life technology) in a humidified atmosphere of 95% air and 5% CO_2. $4x10^3$ or $1x\ 10^3$ DCT cells were seeded per well on a 12 well or 96 well plate respectively and were grown confluent. Cells were serum and glutamine starved for 72h and treated twice daily with either PGE2 (30 – 300 nmol/L) (Cayman), EP4 antagonist, ONO AE3-208 (250-1000 nmol/L) or the selective EP4 agonist, ONO AE1-329 (30-300 nmol/L) for 72 hours. Experiments were performed in duplicates 5 times in total. The supernatant was collected and stored at -70°C for further analysis.

Cell cycle determination

Cells were fixed and permeabilized and incubated with propidium iodide (Sigma-Aldrich), Triton X-100 (0.1%) (Fluka analytical) and Ribonuclease I (Sigma-Aldrich), followed by incubation at 37°C for 40 min. Stained cells were analysed by flow cytometry on a FACSCalibur flow cytometer (BD Biosciences). Percentage of cells in each cell cycle phase was analyzed with the analyzing software FLOW JO ®.

Proliferation assay

DCT cells were seeded, grown to confluence and treated as described before and incubated in a 96 well plate. Culture medium from the 96-well plates was removed and exchanged with 100µl Opti-MEM® Medium (Gibco, life technology) and 10µl per well of MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium))
(Promega, Madison, WI, USA) was added. Plates were incubated at 37°C for 4-5 hours and absorbance was measured on a plate reader (Bio-Rad Laboratories, Vienna, Austria) at 490-500 nm.

cAMP enzyme immunoassay

cAMP secretion of DCTs was measured using a competitive enzyme immunoassay system (GE Healthcare Europe, Vienna, Austria). DCTs were grown to confluence on 96-well plates and starved for 2 hours. Afterwards cells were treated with either PGE2 (30 – 300 nmol/L) (Cayman), EP4 antagonist, ONO AE3-208 (250-1000 nmol/L) or the specific EP4 agonist,

ONO AE1-329 (30-300 nmol/L). Cells were lysed, followed by a 5–10 minute incubation before assay.

Radioimmunoassay (RIA)

A radioimmunoassay for the detection of PGE2 in DCT cells was performed as previously described (257). IC50 of PGE2 was 93.1 ± 12.43 pg/ml, detection limit was defined as 10% inhibition of binding and was 9.7 ± 1.38 pg/ml.

Statistical analysis

Data are shown either as mean ± standard error of the mean (SEM) or mean with raw data. Statistical comparisons were carried out using one-way ANOVA followed by Dunnett's test against vehicle as the control group if not otherwise stated. Mean arterial pressure was compared for each day using one-way ANOVA for repeated measurements. When comparing only two-groups either Student's t-test or Mann- Whitney U test was performed depending on the distribution of the data. Normal distribution was tested by Kolmogorov-Smirnov test. When comparing scores, Fisher's exact test was used to assess for independence due to the fact that the comparing groups are independent and not correlated. P-values of less than 0.05 were considered statistically significant. Statistical analyses and graphs were done with GraphPad Prism version 5.05 (GraphPad Software, La Jolla, CA) or with the statistical software R version 3.3.1. (R Foundation).

6. RESULTS – FINDINGS

Parts of this results have been published in the Am J Physiol Ren Physiol 315. 2018;315:1869–80 (1).

3.1. HIGH-DOSE EP4 AGONISM AND ANTAGONISM IMPROVE THE PHENOTYPE OF NTS

To evaluate the therapeutic effects of targeting EP4 in NTS, mice were treated with two different doses of EP4 agonist or the EP4 antagonist and compared to vehicle-treated controls starting from the day of NTS induction. Furthermore, one group was treated with both the EP4 antagonist and low-dose EP4 agonist to prove the specificity of targeting EP4. Fourteen days after induction of NTS, vehicle-treated mice showed histological features of NTS, such as PAS positive deposits in the glomeruli, occasional crescent formations and interstitial immune cell infiltrates (Figure 1, A). While low doses of EP4 agonist histologically resembled vehicle-treated mice, high doses of EP4 agonist resulted in an improved histological phenotype. Interestingly, antagonism of EP4, as well as simultaneous application of low-dose EP4 agonist and antagonist, also resulted in an improved histological appearance (Figure 1, A). Crescent score (Figure 1, B), tubular casts (Figure 1, H) and serum NGAL and BUN level (Figure 1, I-J), as markers for NTS activity, were significantly decreased in mice treated with high-dose EP4 agonist, the low-dose EP4 agonist plus antagonist as well as EP4 compared to vehicle-treated mice 14 days after NTS induction (Figure 1, B-J). The low-dose EP4 agonist group had a comparable crescent-score and NGAL, but decreased tubular casts as well as BUN serum levels compared to the vehicle controls (Figure1, B-J).

Albuminuria was not different between the groups on day 7 (Figure 1, K) and 14 (Figure 1, L) except for significantly increased albuminuria in high-dose EP4 agonist-treated mice on day 14 (Figure 1, L).

3.2 EP4 ANTAGONISM DECREASE INFILTRATION OF INTERSTITIAL LY6G$^+$ CELLS

Fourteen days after induction of NTS, a significant decrease in renal infiltration of CD4$^+$ T cells was observed in mice treated with high and low dose EP4 agonist, and in EP4 antagonist-treated mice compared to vehicle (Figure 2, B). No difference was detected for the infiltration of CD8$^+$ T cells and CD68$^+$ macrophages in immunohistochemistry (Figure 2, D-E). Strikingly, infiltration of interstitial Ly6G$^+$ cells on day 14 was significantly decreased in mice treated with the high dose of the EP4 agonist, with low-dose agonist in combination with antagonist and

also in mice treated with the EP4 antagonist only (Figure 2, B), whereas there was no difference in $Ly6G^+$ cell counts in glomeruli (Figure 2, C).

3.3. RESPECTIVE CYTOKINES ARE NOT REGULATED BY TARGETING THE EP4 RECEPTOR IN KIDNEYS AND LYMPH NODES

Next, we evaluated whether this striking difference in cell recruitment after administration of the EP4 agonist or antagonist was caused by differences in kidney cytokine profiles. No differences in renal mRNA levels of Tnfα, Tbet, Il10, Interferon-γ, and FoxP3, the key transcription factor controlling Tregs, were found between the different groups (Figure 3, A-F). The immune response in the lymph node was not altered by targeting EP4. Since the phenotype of the NTS model is critically influenced by immune regulations in the lymph node, we evaluated cytokines and immune cell populations in the draining lymph node 14 days after NTS. The Th17 marker Il17a showed decreased expression levels in high-dose EP4 agonist-treated mice in the draining lymph node (Figure 3, J). No differences were detected in mRNA levels of Tnfα, FoxP3 and Il-10 in the lymph node (Figure 3, G-I).

3.4. EP4 RECEPTOR ANTAGONISM REDUCES IL-6 LEVELS IN NTS

It has been shown previously that targeting EP4 influences IL-6 production. The role of IL-6 in NTS has been discussed controversially (177,188,247). *In vivo*, we detected significantly decreased *Il-6* mRNA levels in the kidneys of EP4 antagonist-treated mice 14 days after NTS induction as compared to vehicle-treated controls (Figure 4, A). Since leukocytes are transported to the draining lymph nodes and T cell activation and regulation in NTS takes place in the lymph node (19), we evaluated *Il-6* mRNA expression also in the lymph node, but found no differences between the groups (Figure 4, B). Interestingly, Il-6 was reduced on protein level in the serum of EP4 antagonist-treated mice (Figure 4, C). Since IL-6 is not only produced by immune cells, but also tubular cells (258) which are key players in the pathogenesis of NTS, we now aimed to evaluate the impact of IL-6 production and its regulation by EP4 using a tubular cell line (DCT cells) *in vitro*. DCT cells were treated with the EP4 agonist, antagonist or PGE2 under starving conditions followed by *Il-6* mRNA quantification by RT-qPCR. Low concentrations of the *Il-6* transcript were found in vehicle treated cells, but concentrations increased upon treatment with either the EP4 agonist or PGE2. Pre-treatment with the EP4 antagonist inhibited the increased *Il-6* expression by PGE2 (Figure 4, D).

3.5. TARGETING THE EP4 RECEPTOR DOES NOT INFLUENCE LYMPH NODE AND BLOOD T CELL POPULATIONS AND B CELLS IN NTS

Pathogenic changes in NTS depend on Th1 and Th17 cells as well as Tregs, accompanied by an important role of resident renal cells in disease activity. We therefore evaluated cytokines and immune cell populations in the draining lymph node and in the blood 14 days after NTS. In line with our findings, respective cytokines are not regulated by targeting EP4 receptor, and we further did not find differences in T cell populations and B cells evaluated by flow cytometry in the lymph node and peripheral blood 14 days after NTS induction (Figure 5, A-G).

3.6. EP4 ANTAGONIST TREATMENT SUPPRESSES TUBULAR CXCL-5 AND CXCL-1 EXPRESSION

The Th17/IL-17 axis contributes to tubular cell expression of Cxcl-1 and -5, thereby leading to neutrophil infiltration into the kidney and causing glomerular damage (258). To investigate whether the diminished renal interstitial neutrophil cell influx in the EP4 receptor antagonist-treated mice is caused by alterations in Cxcl-1 and -5 production, we performed qPCR to assess *Cxcl-1* and *-5* expression levels in the kidney 14 days after NTS induction (Figure 6, A-B). Strikingly, Cxcl-1 as well as Cxcl-5 were both significantly decreased in kidneys after EP4 antagonist treatment, but also in the EP4 antagonist in combination with low-dose agonist group as compared to the vehicle controls. Due to another research work (258) describing that *CXCL-1* and *-5* are mainly produced by tubular cells in NTS, we evaluated *Cxcl-1* and *-5* mRNA levels in sieved tubular cells from mice treated with vehicle or EP4 antagonist from day 4 until day 14 after NTS induction (Figure 6, C-D). Here, we could also show that treatment with the EP4 antagonist reduces tubular Cxcl-1 and -5 mRNA expression (Figure 6, C-D).

3.7. TREATMENT OF NTS MICE WITH EP4 ANTAGONIST OR AGONIST DOES NOT AFFECT IGG OR C3 DEPOSITION IN THE KIDNEY

To analyze whether the mice produced equal anti-rabbit IgG titers in response to immunization, which is essential for the NTS phenotype, we performed an IgG ELISA of mouse serum. Mouse anti-rabbit IgG titers did not differ between the groups 14 days after NTS induction (Figure 7, A). Furthermore, we did not detect a difference in deposition of mouse anti-rabbit IgG and rabbit anti-mouse IgG on glomerular basal membrane (Figure 7, B), as well as glomerular C3 deposition between the different group (Figure 7, C).

3.8. THERAPEUTIC TREATMENT WITH EP4 RECEPTOR ANTAGONIST 4 DAYS AFTER NTS INDUCTION IMPROVES THE PHENOTYPE OF NTS

Blocking EP4 receptors four days after NTS induction improves the phenotype. Since EP4 agonism resulted in hypotensive episodes (see Figure 9), we decided to treat mice only with the EP4 antagonist compared to vehicle starting 4 days after NTS induction until day 14 (Figure 8) in order to mirror the typical clinical situation. Here, the EP4 antagonist also significantly improved the phenotype of NTS as shown by decreased PAS score (Figure 8, A), tubular casts (Figure 8, B), and serum NGAL levels (Figure 8, C). Albuminuria was also decreased 14 days after NTS induction in EP4 antagonist-treated mice, but statistical significance was not reached (Figure 8, D). There was no difference concerning BUN serum levels (Figure 8, E).

3.9. EP4 AGONISM CAUSES HYPOTENSIVE EPISODES IN NTS

It is already described that EP4 is expressed on the vasculature and their activation leads to vasodilatation (177), therefore we performed arterial blood pressure measurements before the start of treatment with the EP4 receptor agonist and antagonist, immediately and 30 minutes after injection via a tail cuff method. Mean arterial pressure (MAP) was comparable in all groups before we started the treatment. In the high-dose EP4 agonist group MAP dropped below the lower threshold of detection after the fourth day of treatment (Figure 9, see Table 4). Thirty minutes after injection, MAP was again detectable in the high-dose EP4 agonist group, but was significantly lower compared to vehicle-treated mice (Figure 9, see Table 4). A significant decrease in MAP was observed in low-dose agonist-treated mice (Figure 9, see Table 4). EP4 antagonist-treated mice showed a stable MAP similar to vehicle-treated mice (Figure 9, see Table 4).

3.10. BLOCKING THE EP4 RECEPTOR BEFORE EP4 RECEPTOR AGONIST TREATMENT ABOLISHES HYPOTENSION

As already described in Figure 9 blood pressure was significantly decreased immediately after treatment with low-doses of the EP4 receptor agonist (Figure 10, B), but the mice adapted 30 minutes after treatment (Figure 10, A) to a normotensive blood pressure. In contrast to those vehicle treated mice where no blood pressure changes after treatment could be detected. Blood pressure was always measurable and stable in vehicle treated mice throughout the 14 days (Figure 10, A-B). Treatment with the EP4 receptor antagonist did not influence blood pressure (Figure 10, B) neither directly after the treatment, nor 30 minutes later (Figure 10, A). Pre-treatment with the EP4 antagonist before the EP4 receptor agonist treatment in the same mouse protected mice from EP4 agonist-induced hypotension (Figure 10, A-B), showing that

EP4 receptors were entirely blocked and that the hypotensive effect can be attributed solely to the EP4 receptor. Moreover angiotensin plasma levels, as well as *renin* mRNA expression and diuresis was measured. Neither angiotensin levels, nor renin levels, diuresis, nor blood pressure showed any difference in EP4 receptor antagonist treated NTS mice compared to vehicle treated NTS mice (Figure 10, C-G).

3.11. TREATMENT OF HEALTHY MICE WITH AN EP4 RECEPTOR ANTAGONIST DOES NOT INFLUENCE BLOOD PRESSURE

To evaluate effects of EP4 receptor antagonism on the healthy kidney, mice were treated with an EP4 receptor antagonist or vehicle for 7 days. After 7 days of treatment, no effects on albuminuria, BUN serum levels or blood pressure between the two groups were detected (Figure 11**,** A-C).

3.12. EP4 AGONISM INCREASES TUBULAR CELL VIABILITY AND PROLIFERATION IN NTS

EP4 activation has been described to increase cell proliferation and viability (177). We hypothesized that increased tubular cell proliferation via improvement of tubular regeneration might be an explanation for the improved NTS phenotype in EP4 agonist-treated mice. In vivo, we observed significantly increased numbers of PCNA positive, proliferating tubular cells in the high-dose EP4 agonist group 14 days after induction of NTS as compared to the vehicle group (Figure 12).

3.13. EP4 AGONIST TREATMENT LEADS TO SIGNIFICANTLY DECREASED CELLS IN THE SUB G1 PHASE AND INCREASED CELLS IN THE G1 PHASE OF DCTS

Cell cycle analysis by propidium iodide staining was performed in DCT cells incubated for 72h in complete medium (Figure 13, A) or under starving conditions (Figure 13, B-G). Starved cells were treated with vehicle (Figure 13, B), 300nM EP4 receptor agonist (Figure 13, C), 300nM PGE_2 (Figure 13, D), 1µM EP4 receptor antagonist (Figure 13, E), 300nM PGE_2 and 1µM EP4 receptor antagonist (Figure 13, F) or 300nM EP4 receptor agonist and 1µM EP4 antagonist (Figure 13, G). Representative histogram plots are shown. At least 5 independent experiments were performed.

In vitro, DCT cells were treated for 72 hours with EP4 agonist, antagonist or PGE2 under starving conditions. We detected increased, concentration-dependent cell viability when cells were treated with PGE2 or the EP4 agonist (Figure 13, A-B), whereas incubation with EP4 antagonist had no effect (Figure 13, C). Coincubation of PGE2 or the EP4 agonist with the EP4 antagonist abolished the increased viability of cells, which returned to control levels (Figure 13, D-E). Cell cycle analysis by propidium iodide (PI) staining (Figure 13) revealed that PGE2 as well as the EP4 agonist reduced the proportion of late apoptotic cells seen in the sub G1 phase (Figure 13, A-B), whereas the EP4 antagonist had no effect (Figure 13, C). PGE2, but not the EP4 agonist, significantly increased the S phase (Figure 13, B).

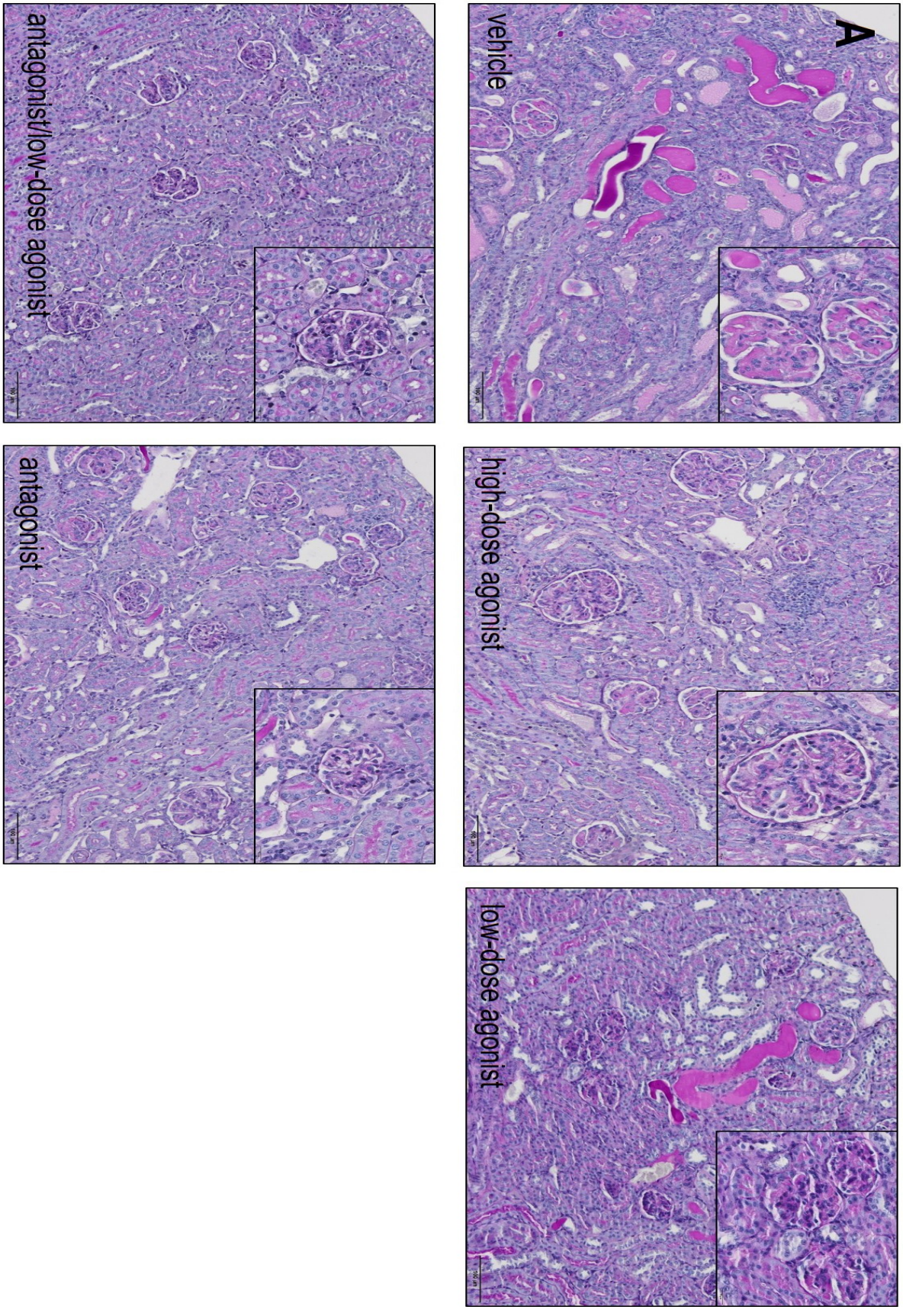
3.14. EP4 RECEPTOR AGONISM INCREASES TUBULAR PROLIFERATION IN VITRO

In vitro, DCT cells were treated for 72 hours with EP4 receptor agonist ONO AE1-329 [30nM-300nM], antagonist ONO AE3-208 [250nM-1000nM] or PGE2 [30nM-300nM] under starving conditions. Cell proliferation was evaluated by MTS test, which revealed increased, concentration-dependent cell viability when cells were treated with PGE2 or the EP4 receptor agonist (Figure 15, A-B), whereas incubation with the EP4 receptor antagonist had no effect (Figure 15, C) on proliferation. Co-incubation of PGE2 or the EP4 receptor agonist with the EP4 receptor antagonist abolished the increased viability of cells to control levels (Figure 15, D-E).

3.15. EP4 STIMULATION OF DCTS WITH ONOAE1-329 (300 NMOL/L) SIGNIFICANTLY INCREASED CAMP PRODUCTION

It is known that activation of the Gαs coupled EP4 receptor leads to an intracellular increase in cyclic adenosine monophosphate (cAMP) levels (177). The Gαs protein–coupled receptor EP4 leads to time and concentration dependent increase in intracellular cAMP levels when DCT cells are stimulated (Figure 16, A). *DCTs* were first investigated for the expression of the EP receptors by evaluating mRNA levels of the different EP receptors via qPCR (Figure 16, B). DCT cells are equally express EP1, EP3 and EP4 receptors. The EP2 receptor was not detectable on DCT cells. To determine the concentration of PGE2 endogenously produced by the DCT cells we performed a radioimmunoassay (RIA) (Figure 16, C). Although we could detect endogenous PGE2 production, we found only low amounts of PGE2 (0.05 ng/ml) in the supernatants of DCT cells after 24 hours.

Figure 1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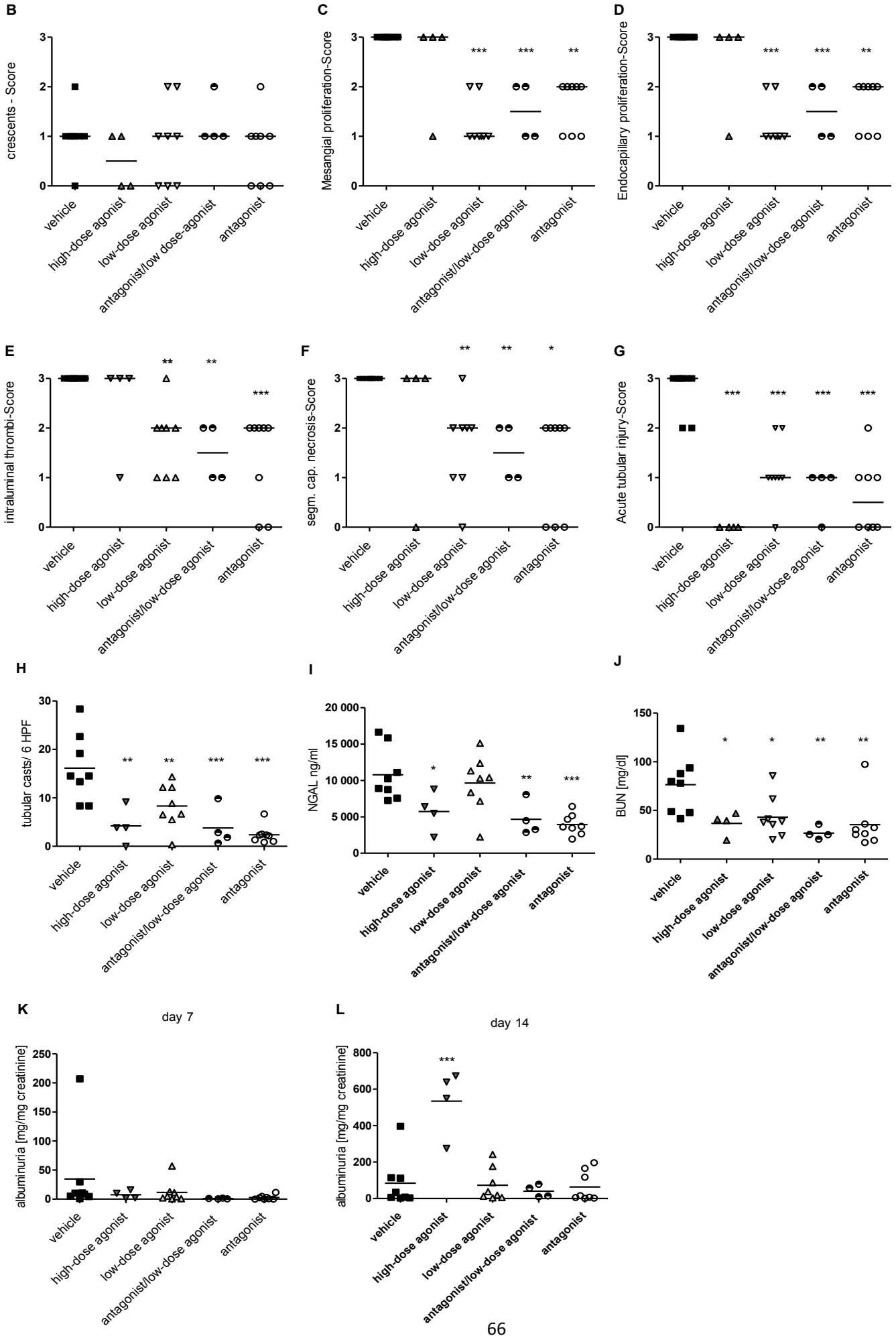
antagonist/low-dose agonist
high-dose agonist
antagonist
low-dose agonist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Acute tubular injury-Score
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tubular casts/ 6 HPF
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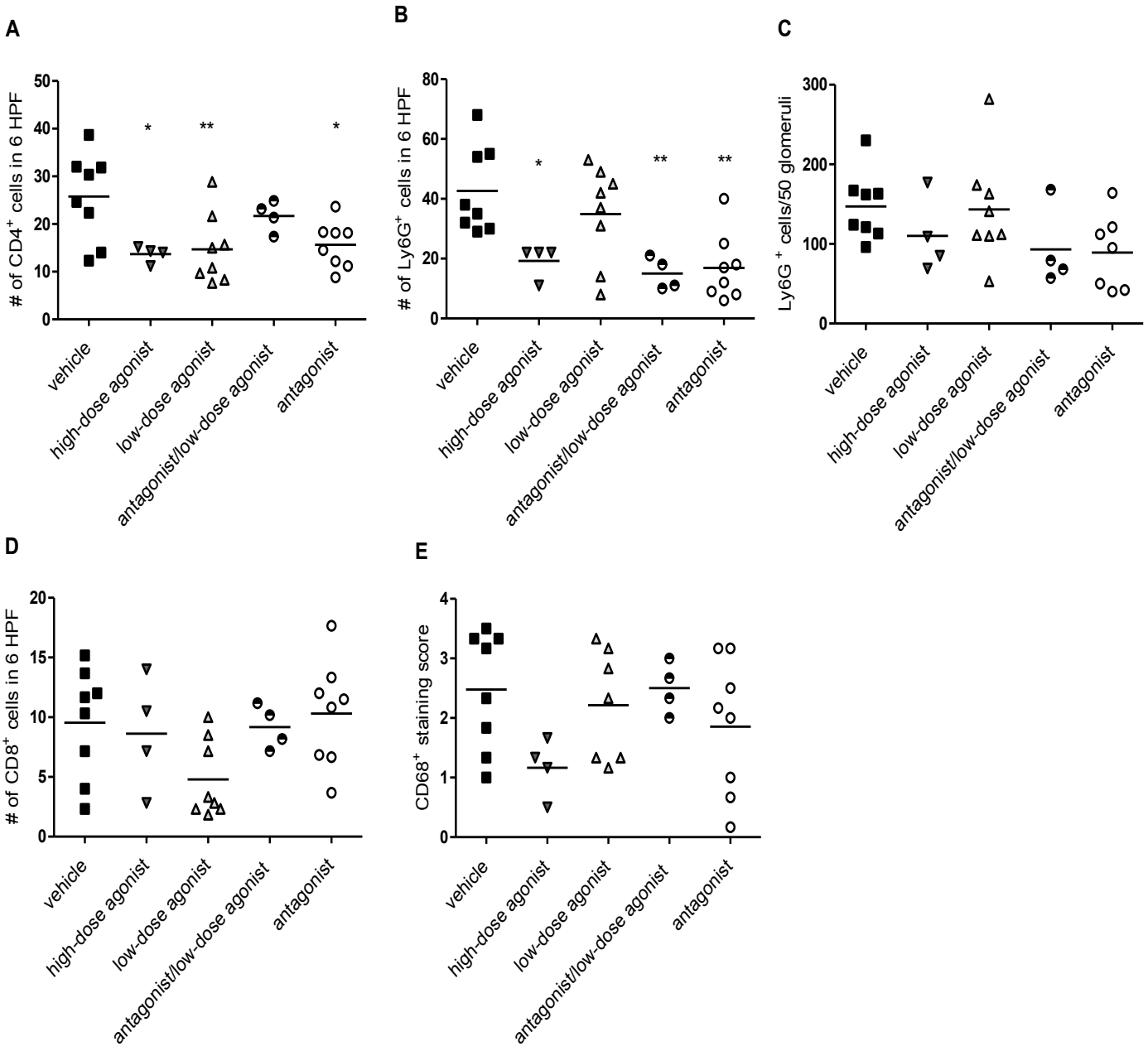
NGAL ng/ml
J
BUN [mg/dl]
K
day 7
albuminuria [mg/mg creatinine]
L
day 14
albuminuria [mg/mg creatinine]
vehicle
high-dose agonist
low-dose agonist
antagonist/low dose-agonist
antagonist/low-dose agonist
antagonist

Figure 1 - High-dose EP4 receptor agonism and antagonism improves the phenotype of NTS

Fourteen days after NTS induction kidneys of mice treated with vehicle, high-dose EP4 receptor agonist, low-dose EP4 receptor agonist, EP4 receptor antagonist/low-dose agonist or EP4 receptor antagonist were harvested and processed for PAS-staining (**A**). Representative pictures are shown. Magnification x400/x600. Stained kidney sections were quantified for glomerular and kidney damage (**B-G**) and tubular cast formation per 6 high power field (HPF) (**H**) by a blinded observer. Serum NGAL levels (**I**) as well as BUN levels were evaluated on day 14 (**J**). Furthermore, urine samples collected on day 7 (**K**) and 14 (**L**) were analyzed for albumin and creatinine (1).

Figure 2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# of CD4+ cells in 6 HPF
50
40
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antagonist
B
# of Ly6G+ cells in 6 HPF
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high-dose agonist
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antagonist/low-dose agonist
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high-dose agonist
low-dose agonist
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antagonist
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antagonist

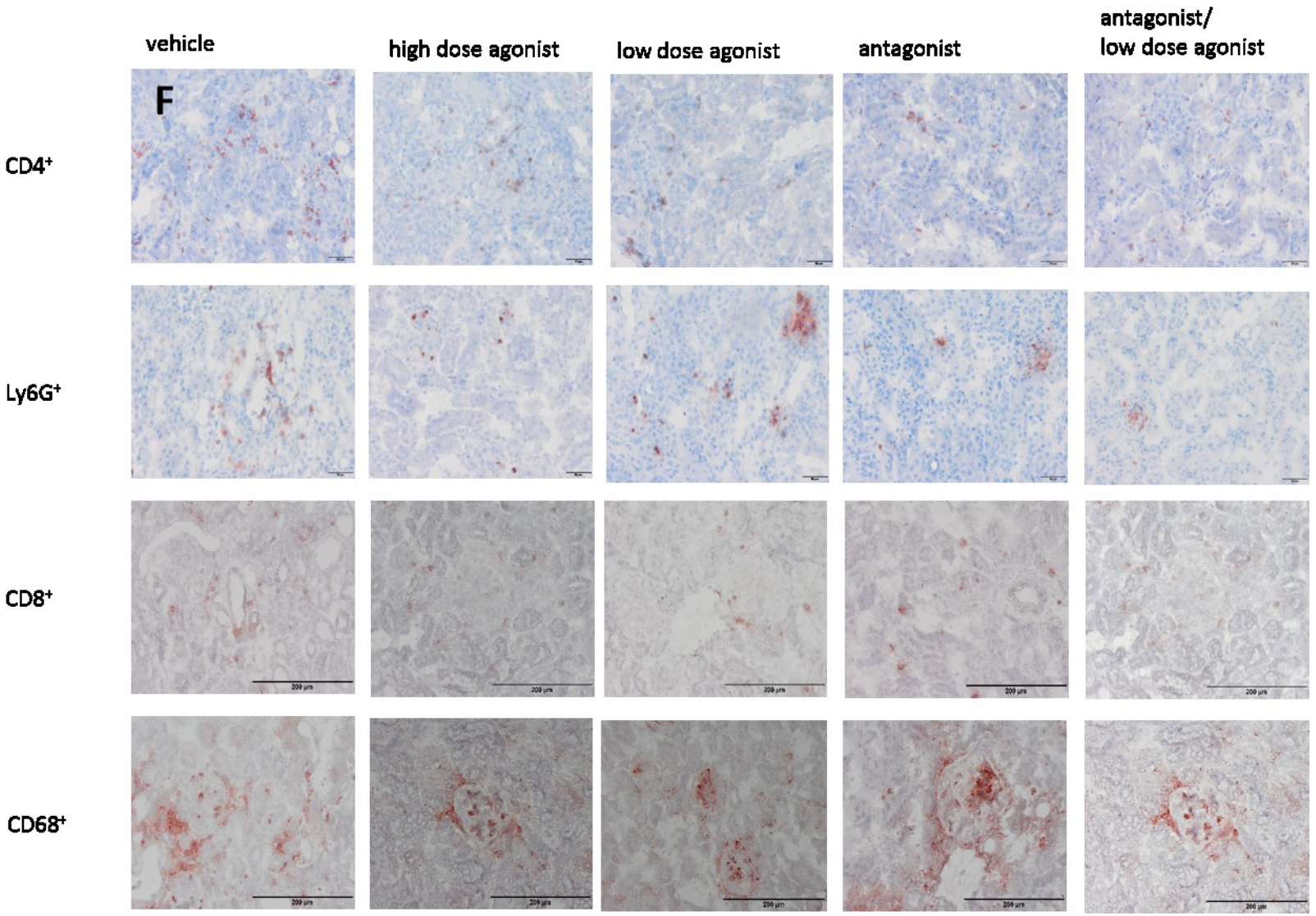

F
vehicle
high dose agonist
low dose agonist
antagonist
antagonist/
low dose agonist
CD4+
Ly6G+
CD8+
CD68+

Figure 2 - EP4 antagonism decreases infiltration of interstitial Ly6G+ cells
Fourteen days after induction of NTS, a significant decrease in renal infiltration of $CD4^+$ T cells was observed in mice treated with high and low dose EP4 receptor agonist, and in EP4 antagonist-treated mice compared to vehicle (**A**). No statistical significant difference was detected for the infiltration of $CD8^+$ T cells and $CD68^+$ macrophages in immunohistochemistry (**D-E**). Strikingly, infiltration of interstitial $Ly6G^+$ cells on day 14 was significantly decreased in mice treated with the high dose of the EP4 receptor agonist, with low-dose agonist in combination with antagonist and also in mice treated with the EP4 antagonist only (**B**), whereas there was no statistical significant difference in $Ly6G^+$ cell counts in glomeruli (**C**). Immunohistochemical staining was performed to evaluate the infiltration of $CD4^+$, $CD8^+$ T cells, $CD68^+$ macrophages and $Ly6G^+$ neutrophil granulocytes (**F**) in the kidney after 14 days of NTS. Means are indicated by a horizontal line. Statistical significances are provided compared to vehicle treated mice (*$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$) (1).

Figure 3

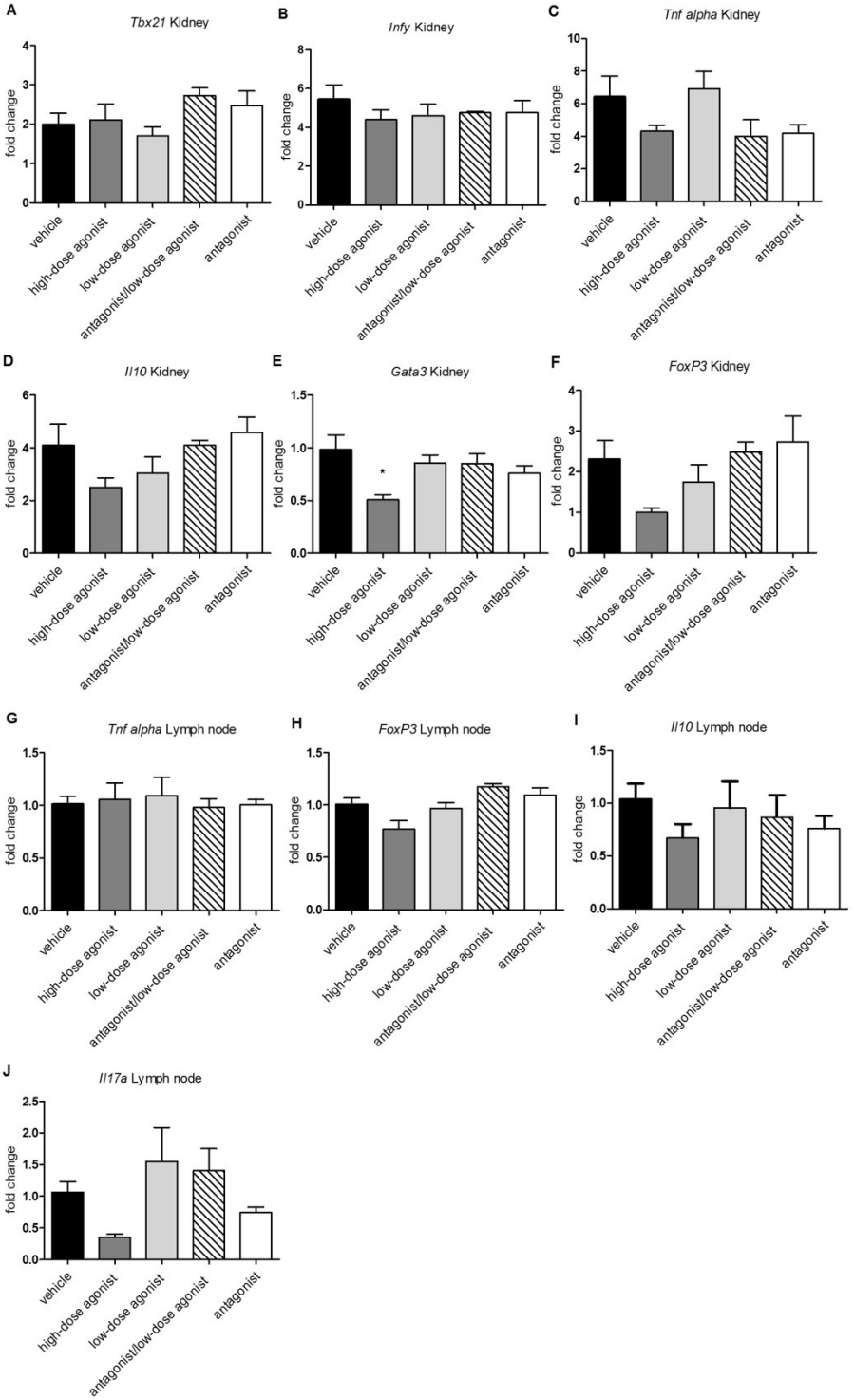

A
Tbx21 Kidney
B
Infy Kidney
C
Tnf alpha Kidney
D
Il10 Kidney
E
Gata3 Kidney
F
FoxP3 Kidney
G
Tnf alpha Lymph node
H
FoxP3 Lymph node
I
Il10 Lymph node
J
Il17a Lymph node
fold change
vehicle
high-dose agonist
low-dose agonist
antagonist/low-dose agonist
antagonist
*

Figure 3 - Respective cytokines are not regulated by targeting the EP4 receptor in kidneys and lymph nodes

Fourteen days after NTS induction RT PCR was performed to evaluate cytokine expression in the kidney (**A-F**) and in the draining lymph node (**G-J**) in healthy mice (n=3), and in mice reated with vehicle (n=6), high-dose EP4 receptor agonist (n=3-4), low-dose EP4 receptor agonist (n=7-8), EP4 receptor antagonist /low-dose agonist (n=4) and EP4 receptor antagonist (n=7-8). Data are provided (mean ± SEM) as fold increase compared to healthy kidneys or vehicle-treated lymph nodes. Significances are provided compared to vehicle-treated mice (*p≤ 0.05) (1).

Figure 4

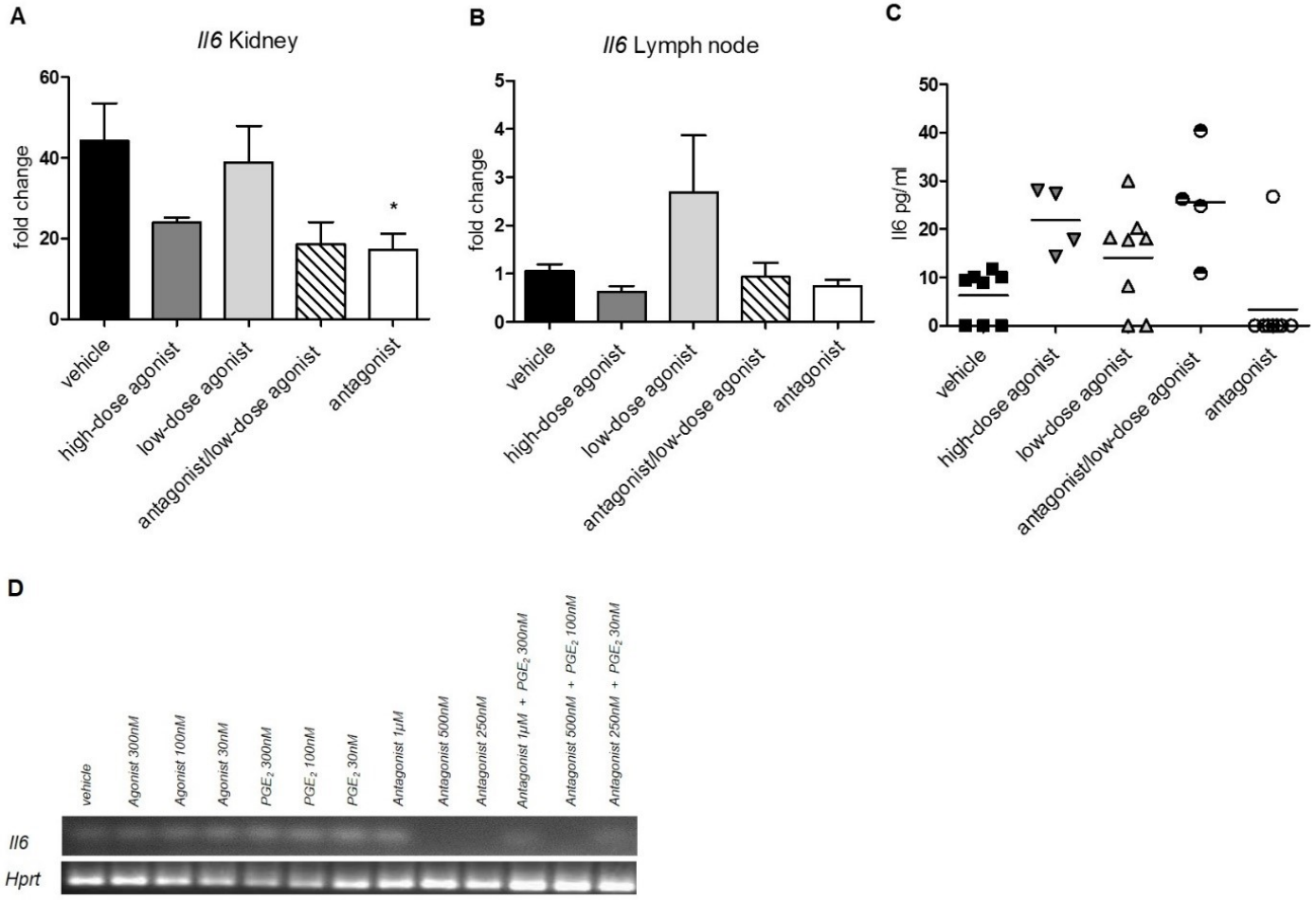

A
Il6 Kidney
fold change
60
40
20
0
*
vehicle
high-dose agonist
low-dose agonist
antagonist/low-dose agonist
antagonist
B
Il6 Lymph node
fold change
5
4
3
2
1
0
vehicle
high-dose agonist
low-dose agonist
antagonist/low-dose agonist
antagonist
C
Il6 pg/ml
50
40
30
20
10
0
vehicle
high-dose agonist
low-dose agonist
antagonist/low-dose agonist
antagonist
D
vehicle
Agonist 300nM
Agonist 100nM
Agonist 30nM
PGE2 300nM
PGE2 100nM
PGE2 30nM
Antagonist 1µM
Antagonist 500nM
Antagonist 250nM
Antagonist 1µM + PGE2 300nM
Antagonist 500nM + PGE2 100nM
Antagonist 250nM + PGE2 30nM
Il6
Hprt

Figure 4 - EP4 receptor antagonism reduces Il6 levels in NTS

Fourteen days after NTS induction RT PCR was performed to evaluate *Il6* expression in the kidney (**A**) and in the draining lymph node (**B**) in healthy mice (n=3), and animals treated with vehicle (n=6), high-dose EP4 receptor agonist (n=3-4), low-dose EP4 receptor agonist (n=7-8), EP4 receptor antagonist/low-dose agonist (n=4) or EP4 receptor antagonist (n=7-8). Furthermore, *Il6* levels were evaluated in the serum of respective mice 14 days after NTS induction (**C**). Finally, DCT cells were treated with vehicle, EP4 receptor agonist, PGE_2, EP4 receptor antagonist and PGE_2/EP4 receptor antagonist for 72h under starving conditions. RT PCR was performed for *Il6* and analysed on a 2.5% agarose gel. A representative experiment out of at least 3 independent experiments is shown (**D**). Data are provided as mean of the fold change compared to healthy kidneys or vehicle-treated lymph nodes ± standard error of the mean (SEM). Means are indicated by a horizontal line. Statistical significances are provided compared to vehicle treated mice (*$p \leq 0.05$) (1).

Figure 5

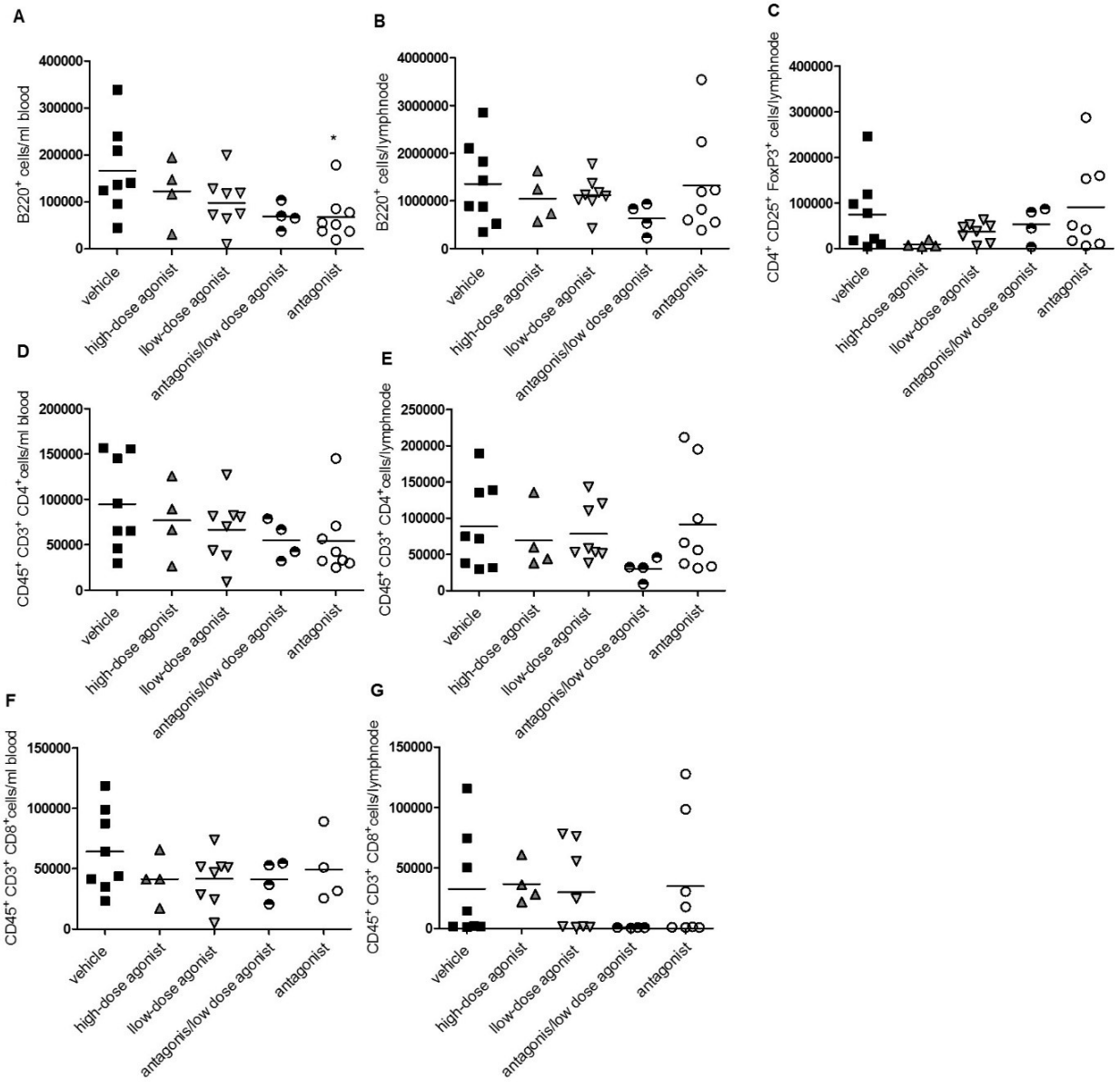

A
B220+ cells/ml blood
400000
300000
200000
100000
0
*
vehicle
high-dose agonist
llow-dose agonist
antagonis/low dose agonist
antagonist
B
B220+ cells/lymphnode
4000000
3000000
2000000
1000000
0
C
CD4+ CD25+ FoxP3+ cells/lymphnode
400000
300000
200000
100000
0
D
CD45+ CD3+ CD4+cells/ml blood
200000
150000
100000
50000
0
E
CD45+ CD3+ CD4+cells/lymphnode
250000
200000
150000
100000
50000
0
F
CD45+ CD3+ CD8+cells/ml blood
150000
100000
50000
0
G
CD45+ CD3+ CD8+cells/lymphnode
150000
100000
50000
0

Figure 5 - Targeting the EP4 receptor does not influence lymph node and blood T cell populations and B cells in NTS

We did not find statistical significant differences in different T cell populations and B cells evaluated by flow cytometry in the lymph node and peripheral blood 14 days after NTS induction: $B220^+$ B-Cells in the peripheral blood (**A**), $B220^+$ B cells in the lymph node (**B**), $CD4^+CD25^+FoxP3^+$ Treg cells in the lymph node (**C**), $CD45^+CD3^+CD4^+$ T-cells in the blood (**D**), $CD45^+CD3^+CD4^+$ T-cells in the lymph node (**E**), $CD45^+CD3^+CD8^+$ T-cells in the blood (**F**) and $CD45^+CD3^+CD8^+$ T cells in the lymph node (**G**) were evaluated quantitatively (**A-G**). Means are indicated by a horizontal line. Statistical significances are provided compared to vehicle treated mice (*$p \leq 0.05$) (1).

Figure 6

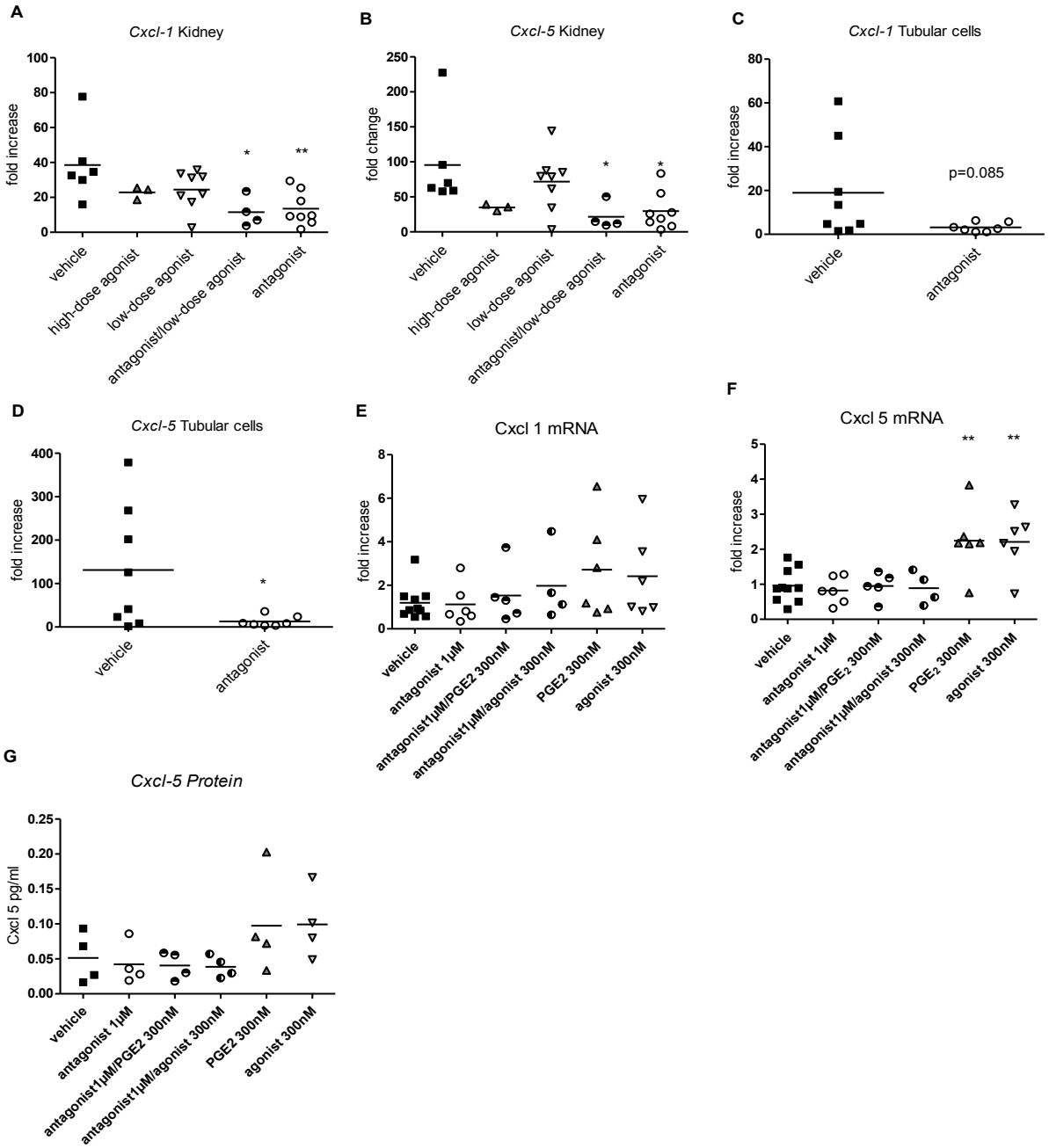
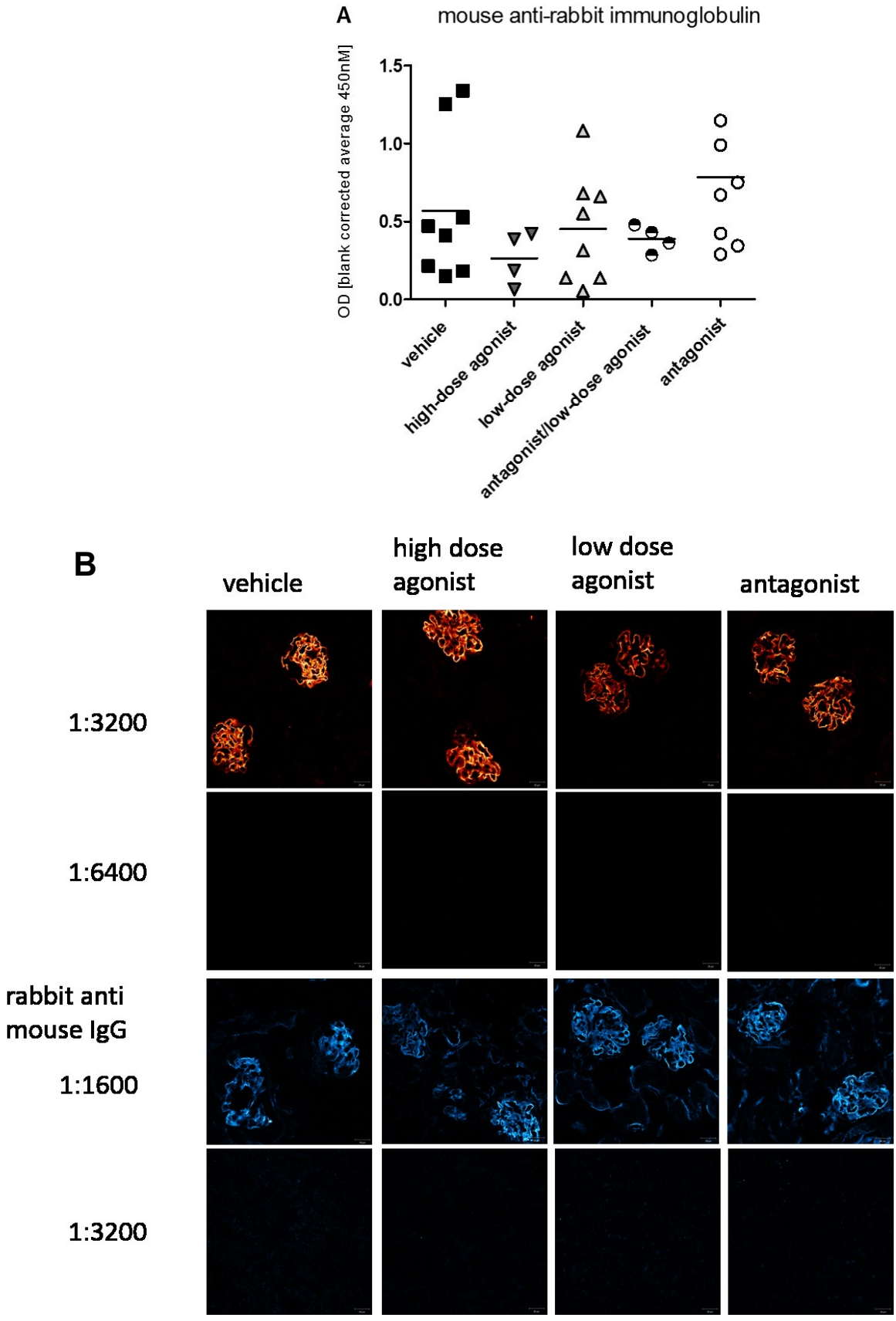

A
Cxcl-1 Kidney
fold increase
vehicle
high-dose agonist
low-dose agonist
antagonist/low-dose agonist
antagonist
B
Cxcl-5 Kidney
fold change
C
Cxcl-1 Tubular cells
fold increase
p=0.085
D
Cxcl-5 Tubular cells
E
Cxcl 1 mRNA
F
Cxcl 5 mRNA
G
Cxcl-5 Protein
Cxcl 5 pg/ml
antagonist 1μM
antagonist1μM/PGE2 300nM
antagonist1μM/agonist 300nM
PGE2 300nM
agonist 300nM

Figure 6 - EP4 antagonist treatment suppresses tubular Cxcl-5 and Cxcl-1 expression

Fourteen days after NTS induction qPCR was performed to evaluate Cxcl-1 (**A**) and Cxcl-5 (**B**) mRNA expression in the kidney of mice treated with vehicle, high-dose EP4 receptor agonist, low-dose EP4 receptor agonist, EP4 receptor antagonist/low dose agonist or EP4 receptor antagonist. Cxcl-1 (**C**) and -5 (**D**) mRNA expression was evaluated in isolated tubular cells from mice treated with vehicle or EP4 receptor antagonist from day 4 until day 14 after NTS induction. Data are provided as fold change compared to healthy kidneys or sieved tubular cells isolated from healthy mice, respectively. Data are provided as mean ± SEM. Statistical significances are provided compared to vehicle-treated mice (*p≤ 0.05, **p≤ 0.01) (1).

Figure 7


A
mouse anti-rabbit immunoglobulin
OD [blank corrected average 450nM]
1.5
1.0
0.5
0.0
vehicle
high-dose agonist
low-dose agonist
antagonist/low-dose agonist
antagonist
B
vehicle
high dose agonist
low dose agonist
antagonist
1:3200
1:6400
rabbit anti mouse IgG
1:1600
1:3200

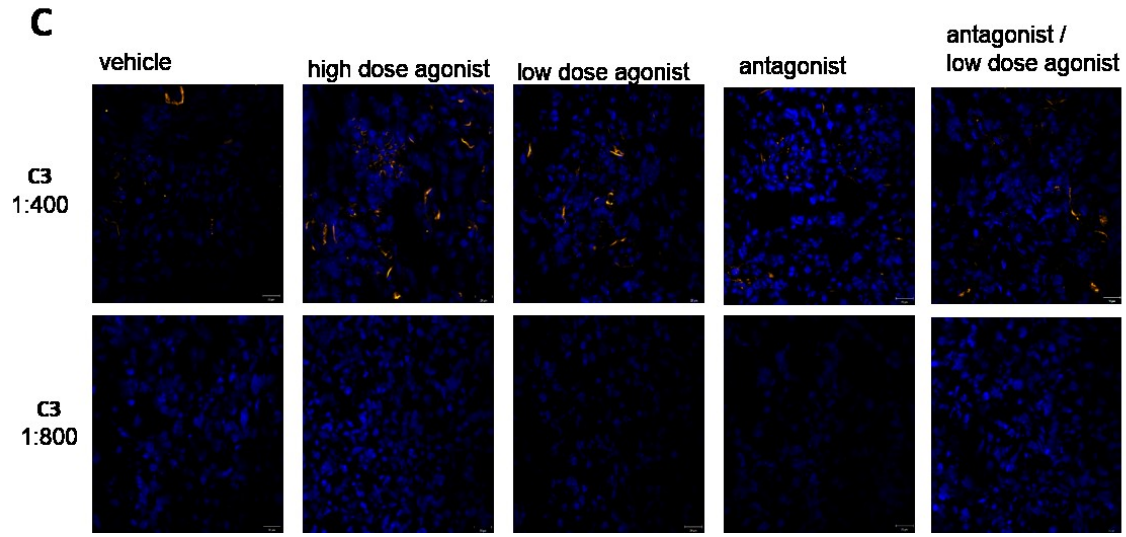


Figure 7 - Treatment of NTS mice with EP4 antagonist or agonist does not affect IgG or C3 deposition in the kidney

Mouse anti-rabbit IgG titters did not differ between the different treatment arms (**A**). Moreover, we did not detect a difference in deposition of mouse anti-rabbit IgG and rabbit anti-mouse IgG on glomerular basal membrane (**B**). Furthermore, no difference in glomerular C3 deposition between the different groups has been detected (**C**) (1).

Figure 8

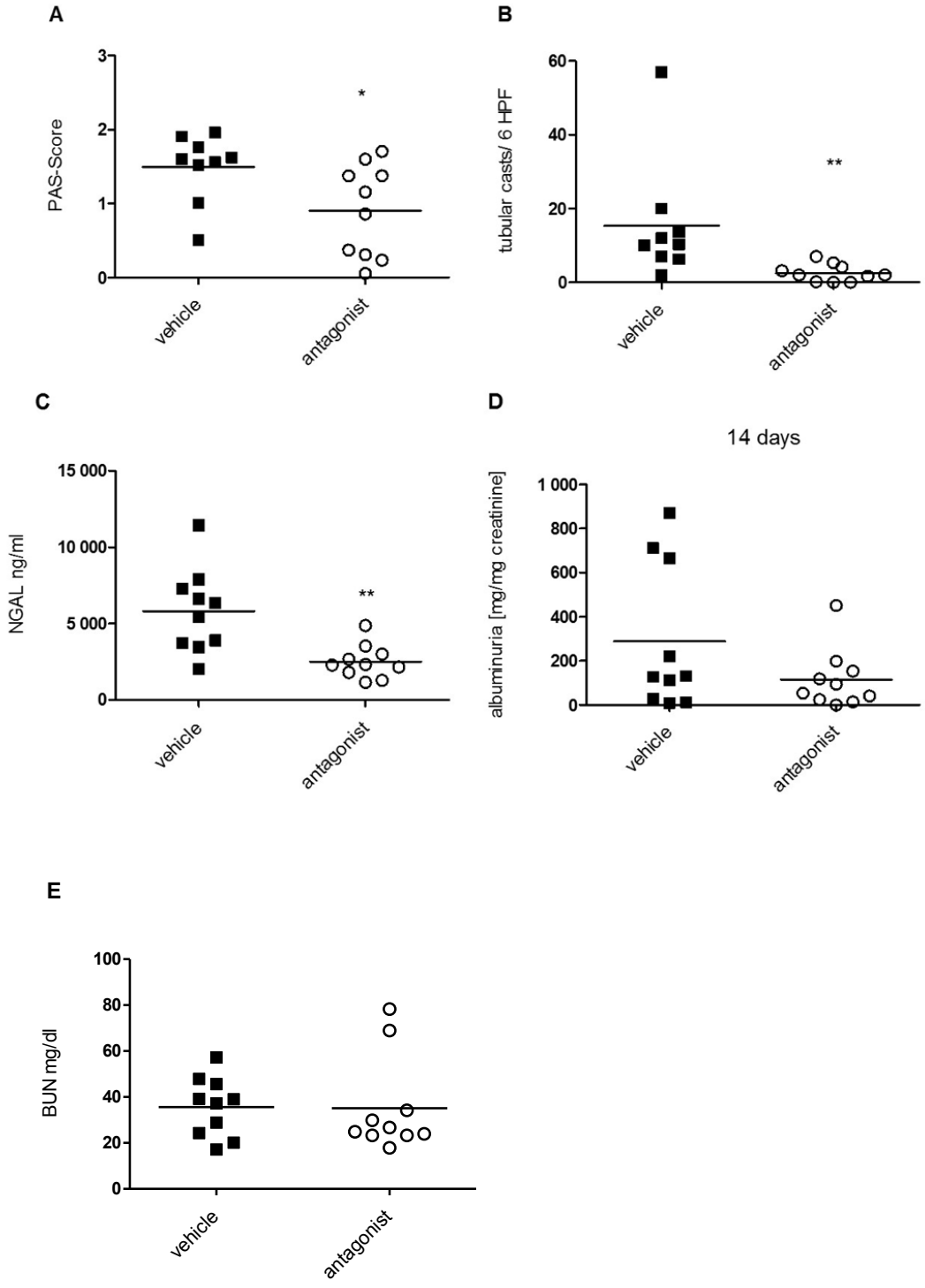

A
PAS-Score
3
2
1
0
*
vehicle
antagonist
B
tubular casts/ 6 HPF
60
40
20
0
**
vehicle
antagonist
C
NGAL ng/ml
15 000
10 000
5 000
0
**
vehicle
antagonist
D
14 days
albuminuria [mg/mg creatinine]
1 000
800
600
400
200
0
vehicle
antagonist
E
BUN mg/dl
100
80
60
40
20
0
vehicle
antagonist

Figure 8 - Starting EP4 receptor antagonism 4 days after NTS induction improves the phenotype of NTS

After 14 days of NTS, tissues from vehicle or EP4 receptor antagonist treated mice were harvested and processed for further analysis. Glomerular PAS score (**A**), tubular casts (**B**), and serum NGAL (**C**) were evaluated. Albuminuria was also decreased 14 days after NTS induction in EP4 antagonist-treated mice, but statistical significance was not reached (**D**). There was no difference concerning the BUN serum levels (**E**). Means are indicated by a horizontal line. Statistical differences were determined by Man-Whitney U-test. Statistical significances are provided compared to vehicle treated mice (*$p \leq 0.05$, **$p \leq 0.01$) (1).

Figure 9

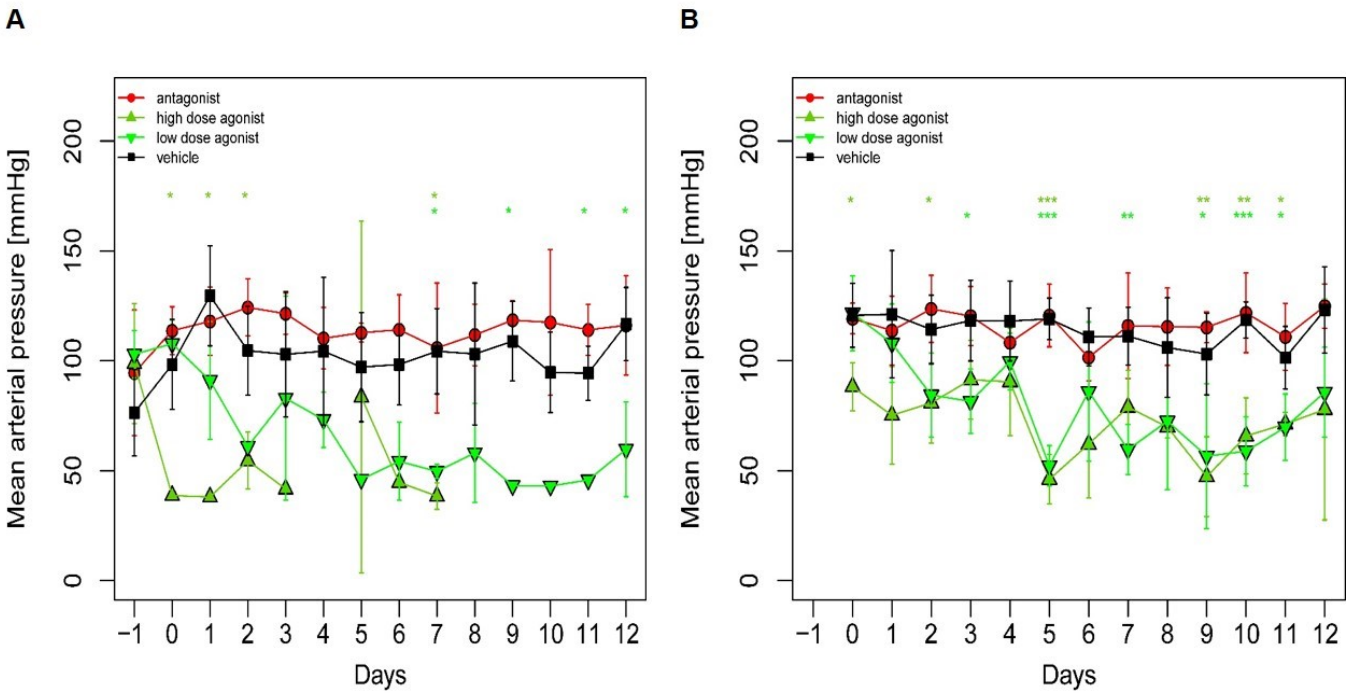


Figure 9 - Hypotensive episodes after EP4 receptor agonist administration
Blood pressure was measured daily (in total 12 days) in healthy mice as well as in vehicle (n=4), EP4 receptor high-dose agonist (n=4), EP4 receptor low-dose agonist (n=4) and in EP4 receptor antagonist (n=4) treated mice after NTS induction. Blood pressure measurements were performed immediately (**A**) and 30 minutes after injection (**B**) in all 4 groups by employing the tail cuff method. Mean arterial pressure (MAP) is shown as mean ± SEM using Dunnett's test with vehicle as the control group. Statistical significances are provided compared to vehicle treated mice (*p≤ 0.05, **p≤ 0.01, ***p≤ 0.001) (1).

Figure 10

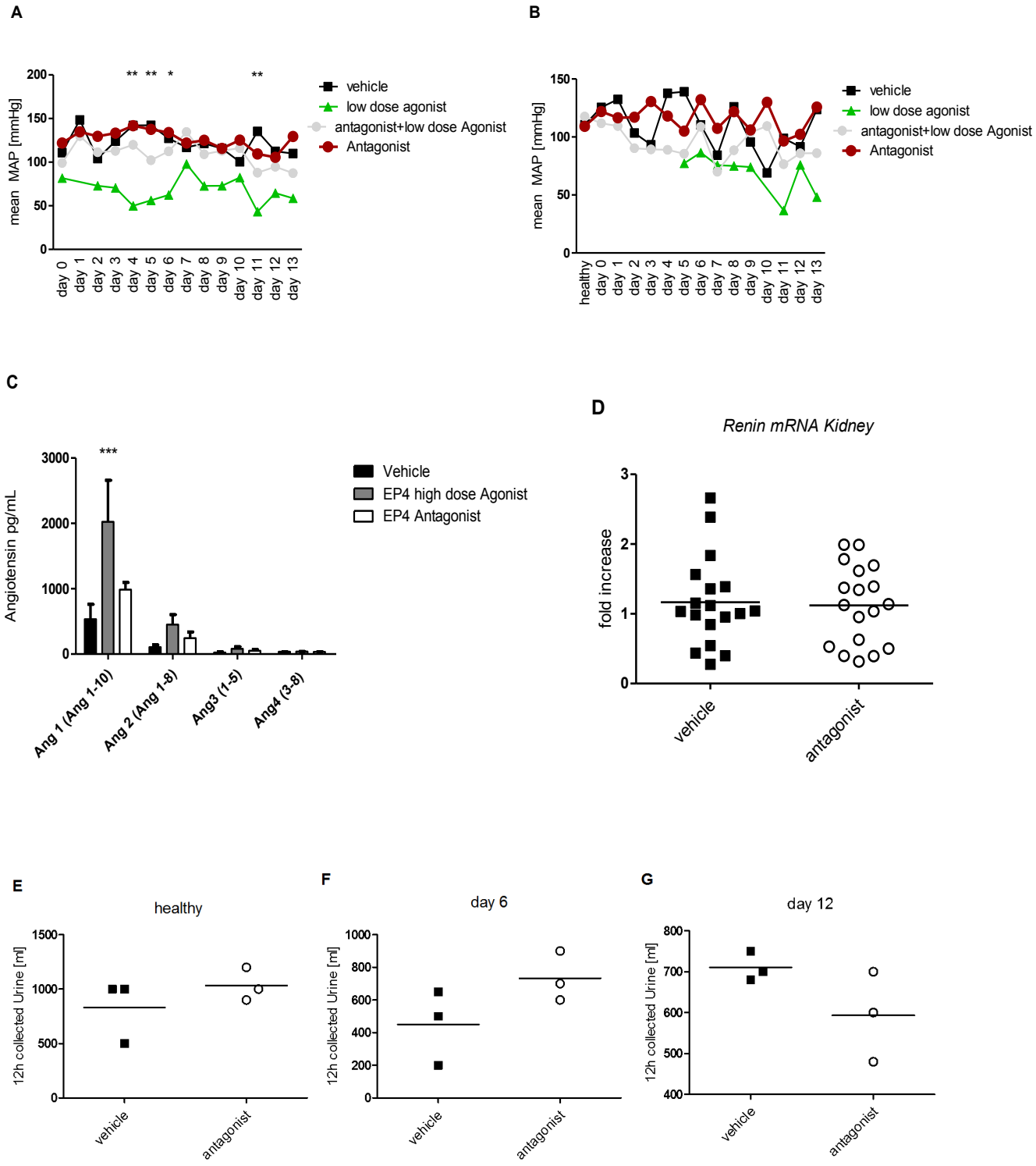

A
mean MAP [mmHg]
vehicle
low dose agonist
antagonist+low dose Agonist
Antagonist
B
mean MAP [mmHg]
vehicle
low dose agonist
antagonist+low dose Agonist
Antagonist
C
Angiotensin pg/mL
Vehicle
EP4 high dose Agonist
EP4 Antagonist
Ang 1 (Ang 1-10)
Ang 2 (Ang 1-8)
Ang3 (1-5)
Ang4 (3-8)
D
Renin mRNA Kidney
fold increase
vehicle
antagonist
E
healthy
12h collected Urine [ml]
vehicle
antagonist
F
day 6
12h collected Urine [ml]
vehicle
antagonist
G
day 12
12h collected Urine [ml]
vehicle
antagonist

Figure 10- Blocking the EP4 receptor before EP4 receptor agonist treatment abolishes hypotension

Blood pressure was measured daily in vehicle (n=7), EP4 antagonist (n=7), EP4 low-dose agonist (n=7) and combined EP4 antagonist/agonist (n=3) treated mice after NTS induction (**A**). Blood pressure measurements were performed 30 minutes after injection in all 4 groups by employing the tail cuff method (**B**). Mean arterial pressure (MAP) is shown as mean ± SEM. Fourteen days after NTS induction plasma of mice treated either with vehicle, high-dose EP4 receptor agonist or an EP4 receptor antagonist (n=2 per group) were harvested and angiotensin levels (**C**) were measured by liquid chromatography tandem-mass spectrometry. Moreover, qPCR was performed to evaluate renin mRNA expression in the kidney of vehicle (n=8) and EP4 receptor antagonist (n=8) treated mice (**D**). Data are provided as mean of the fold change compared to healthy kidneys. To detect the influence of the EP4 receptor on diuresis, healthy (**E**) and nephritic mice (**F-G**) were treated with vehicle or an EP4 receptor antagonist (n=3 per group) and analysed for their urinary output within 12 hours at indicated time points. Neither angiotensin levels, nor renin levels, diuresis, or blood pressure showed any difference in EP4 receptor antagonist treated NTS mice compared to vehicle treated NTS mice. Data are provided as mean ± SEM. Statistical significances are provided comparing all 4 groups (*p≤ 0.05, **p≤ 0.01, ***p≤ 0.001) (1).

Figure 11

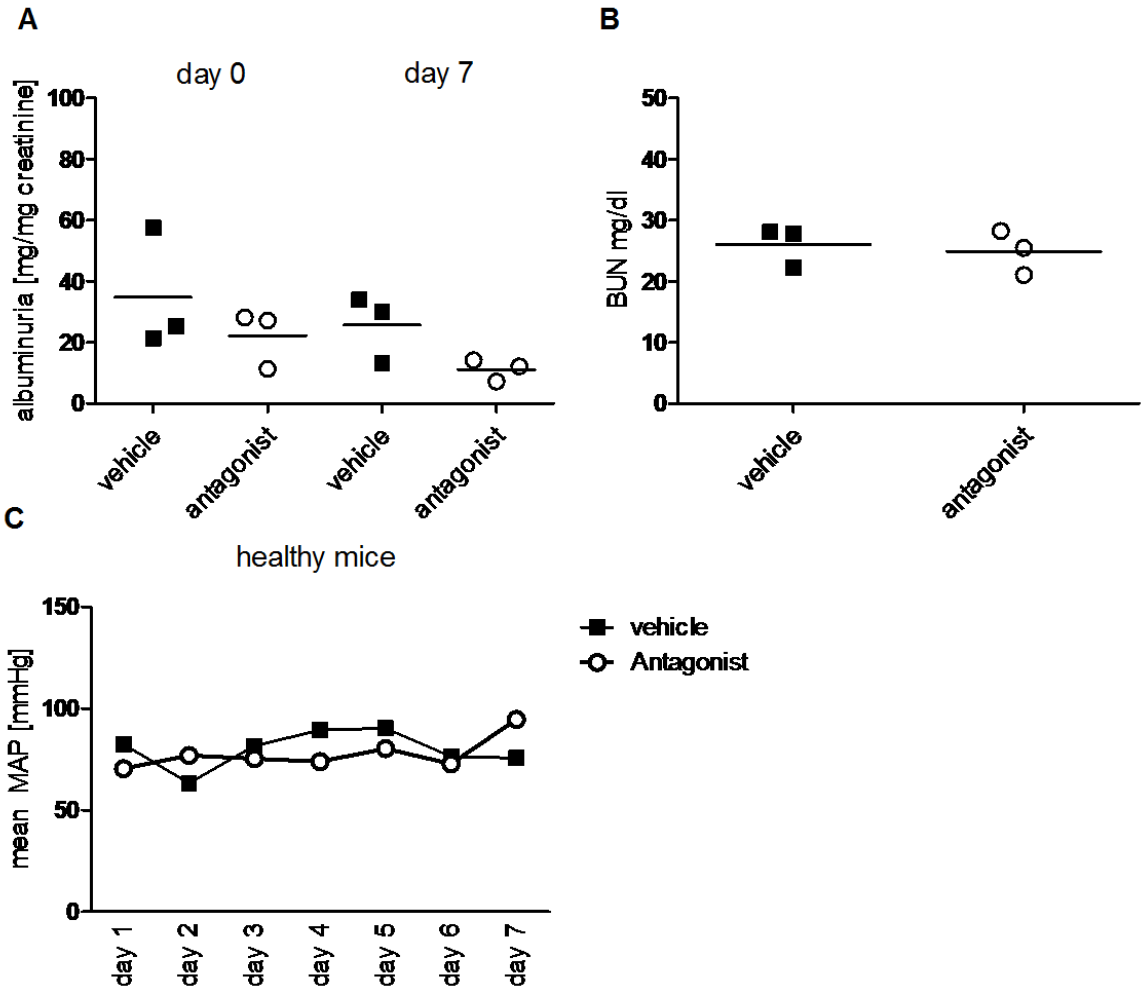


Figure 11 - Treatment of healthy mice with an EP4 receptor antagonist does not influence the blood pressure

Healthy mice were treated for7 days with an EP4 receptor antagonist or vehicle. After 7 days of treatment we evaluated albuminuria, BUN serum levels and blood pressure between the two groups (**A-C**) (1).

Figure 12

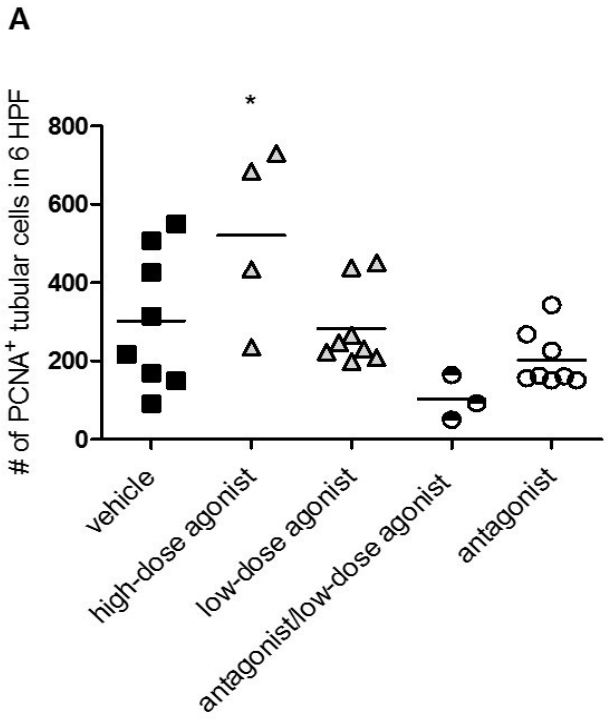


Figure 12 - Agonist-treated mice show increased proliferation of tubular cells in vivo

Fourteen days after NTS induction mice treated with vehicle, high-dose EP4 receptor agonist, low-dose EP4 receptor agonist, EP4 receptor antagonist/low-dose agonist and EP4 receptor antagonist were evaluated for $PCNA^+$ tubular cells per 6 high power fields (HPF). *In vivo*, we observed statistically significant increases in the numbers of PCNA positive, proliferating tubular cells in the high-dose EP4 agonist group 14 days after induction of NTS as compared to the vehicle-treated group (**A**). All other groups showed no statistically significant differences in the number of proliferating cells compared to vehicle-treated mice (**A**). Means are indicated by horizontal lines. Statistical significances are provided compared to vehicle-treated mice (*$p \leq 0.05$) (1).

Figure 13

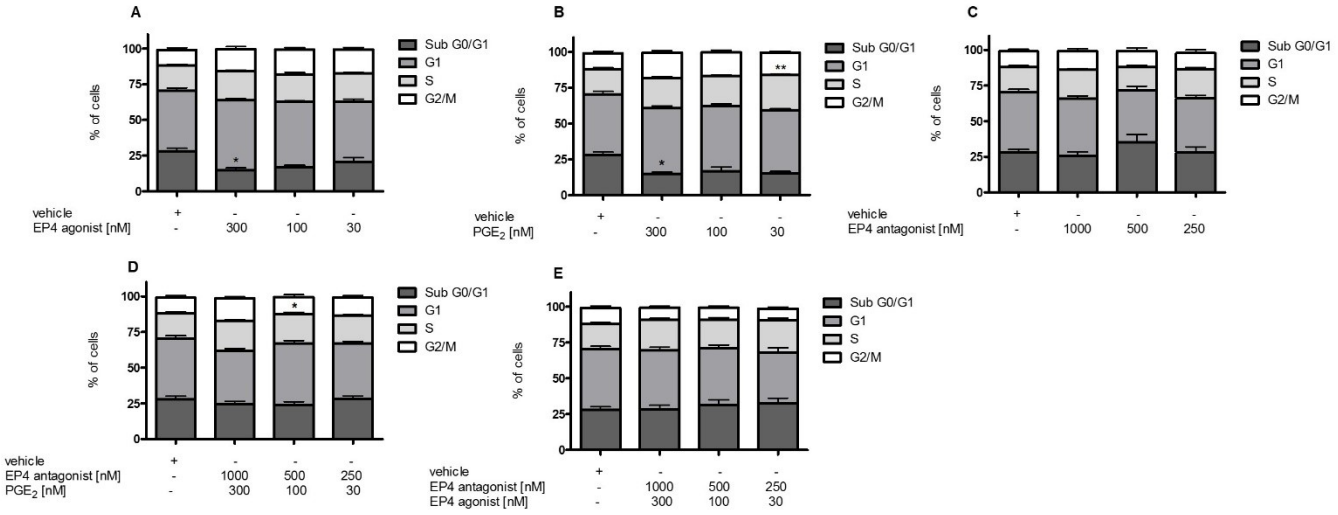


Figure 13 - EP4 agonist treatment leads to significantly decreased cells in the sub G1 phase and increased cells in the G1 phase of DCTs

To evaluate cell viability a cell cycle analysis was performed. DCT cells were treated with vehicle (n=7), EP4 receptor agonist (n=6-7) (Figure 13, **A**) PGE_2 (n=5-6) (Figure 13, **B**), EP4 receptor antagonist (n=5-6) (Figure 13, **C**) and the EP4 receptor antagonist together with PGE_2 (n=6-7) (Figure 13, **D**) or together with the EP4 receptor agonist (n=4) (Figure 13, **E**) under starving conditions for 72 hours. Cell cycle analysis by propidium iodide staining revealed that EP4 agonist (Figure 14, **A**) as well as PGE_2 (Figure 14, **B**) treatment reduced the proportion of dysfunctional cells seen in the sub G1 phase. Data are shown as mean ± SEM and cell cycle state using Dunnett's test with vehicle as the control group. Statistical significances are provided compared to vehicle (*p≤ 0.05, **p≤ 0.01) (1).

Figure 14

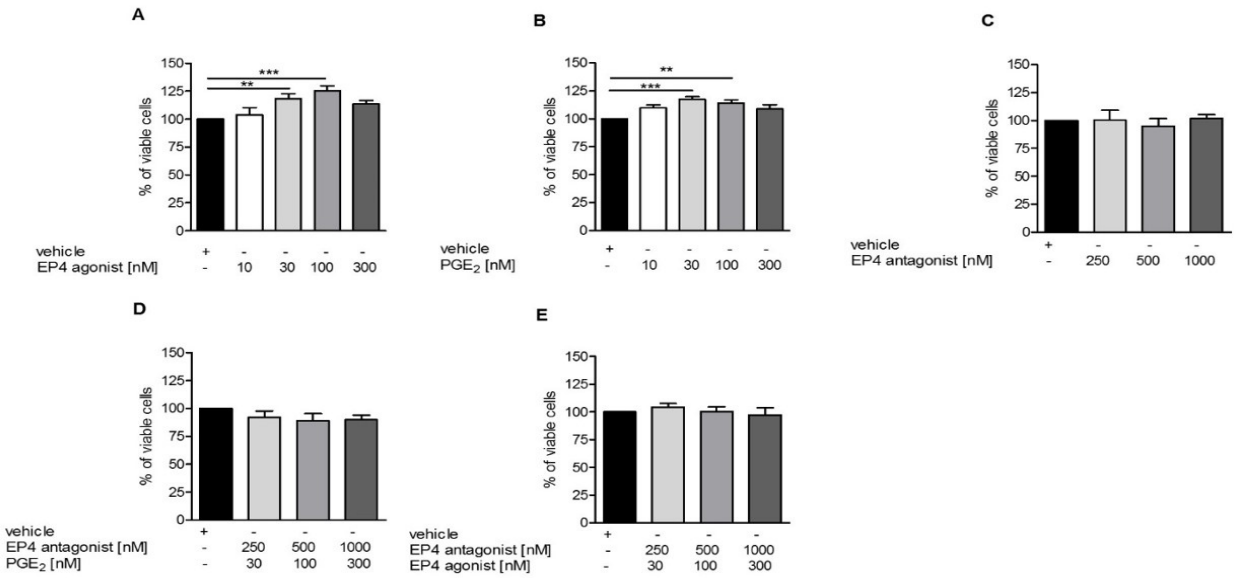


Figure 14 - EP4 receptor agonism increases tubular proliferation in vitro

To evaluate proliferative capacity of DCT cells, the MTS test was performed. DCT cells were treated with vehicle, EP4 receptor agonist (**A**), PGE2 (**B**), EP4 receptor antagonist (**C**) and the EP4 receptor antagonist together with PGE2 (**D**) as well as EP4 receptor antagonist together with EP4 receptor agonist (**E**) under starving conditions for 72 hours. Data are provided as mean ± SEM from at least 5 independent experiments. Statistical significances are provided compared to vehicle treated cells (**p≤ 0.1, ***p≤ 0.001) (1).

Figure 15

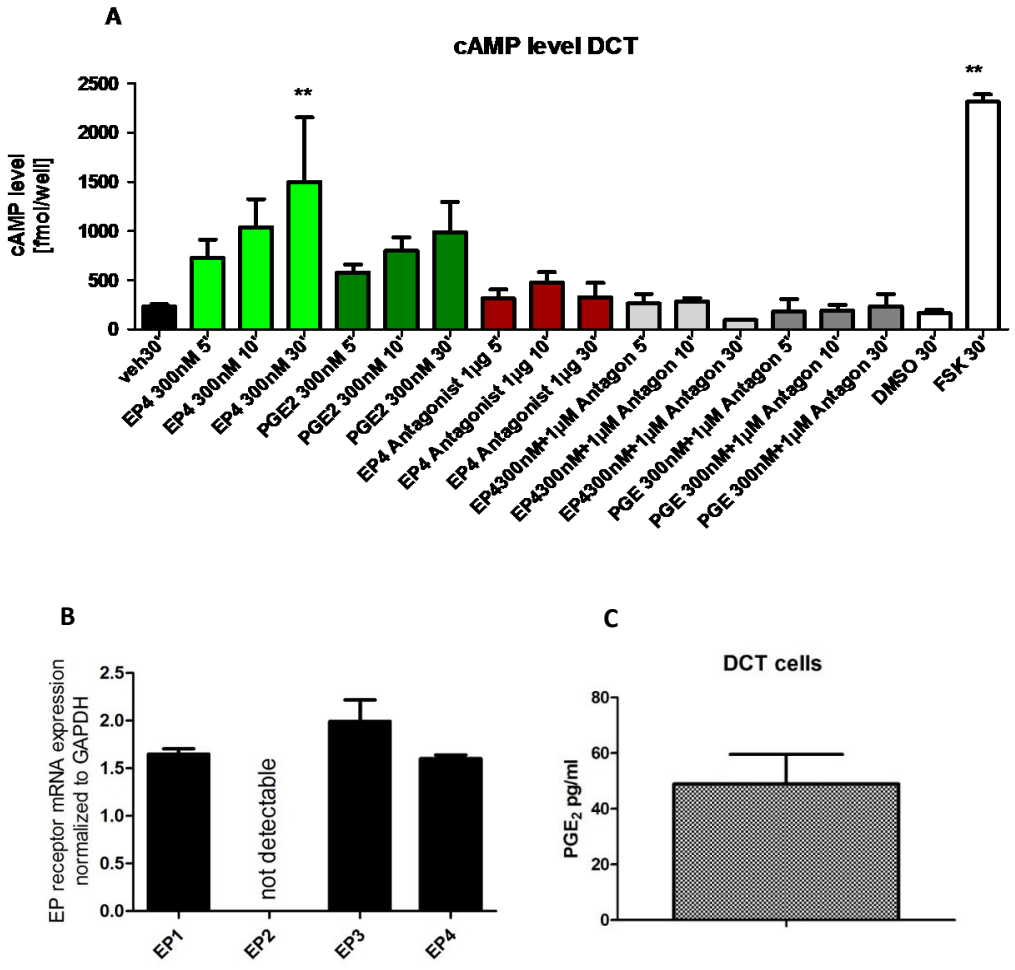


Figure 15 - EP4 stimulation of DCTs with ONOAE1-329 (300 nmol/L) significantly increased cAMP production

Pre-incubation with the EP4 antagonist ONOAE3-208 prevented EP4 agonist–induced increase in cAMP in DCT cells (**A**). EP1, 3 and 4 receptors were detected in equal proportion, while the EP2 receptor was not expressed on DCT cells (**B**). RIA revealed low PGE2 production of DCT cells after 72 hours of incubation in starving medium (48.9 ± 10.6 pg/ml) (**C**). Experiments were performed in duplicates and in total at least five different experiments were performed. (**p≤ 0.1, ***p≤ 0.001) (1).

Table 8 - Treatment Table

To evaluate therapeutic effects of targeting EP4 in NTS, mice were treated with two different doses of EP4 agonist, or the EP4 antagonist, as compared to vehicle treated controls starting from the day of NTS induction. Furthermore, one group was treated with both the EP4 antagonist and low-dose EP4 agonist to prove the specificity of targeting EP4. Fourteen or seven days after induction of NTS mice were sacrificed and organs were harvest. Additionally, healthy mice were treated with an EP4-receptor antagonist and vehicle for 7 days.

Time	Day -3	Day 0	Day 4	Day 7	Day 14
1.	Immunization	Start with the GN			Sacrifice mice
		Treatment every 12h until day 14: 1. ONO AE1-329 EP4 receptor agonist 25µg or 7µg/mouse/day 2. ONO AE3-208 EP4 receptor antagonist 250µg/mouse/day 3. ONO AE3-208 EP4 receptor antagonist 250µg/mouse/day + ONO AE1-329 EP4 receptor agonist 7µg/mouse/day 4. vehicle			
2.	Immunization	Start with the GN	Start with the treatment		Sacrifice mice
			Treatment every 12h until day 14: 1. ONO AE3-208 EP4 receptor antagonist 250µg/mouse/day 2. vehicle		
3.	Immunization	Start with the GN	Measure blood pressure and collectin urine		Sacrifice mice
		Treatment every 12h until day 14: 1. ONO AE1-329 EP4 receptor agonist 7µg/mouse/day 2. ONO AE3-208 EP4 receptor antagonist 250µg/mouse/day 3. ONO AE3-208 EP4 receptor antagonist 250µg/mouse/day + ONO AE1-329 EP4 receptor agonist 7µg/mouse/day 4. vehicle			
4.		Start with treatment of healthy mice		Sacrifice mice	
		Treatment every 12h until day 7: 1. ONO AE3-208 EP4 receptor antagonist 250µg/mouse/day 2. vehicle			

starting on day 0 with treatment		starting on day 4 with treatment	
vehicle	n=8	vehicle	n=10
Low dose Agonist	n=8	Antagonist	n=10
High dose Agonist	n=4		
Antagonist/low dose Agonist	n=4		
Antagonist	n=8		

starting on day 0 with treatment (date for Blood pressure measurement and urine volume collection)		Healthy mice treated	
vehicle	n=3	vehicle	n=3
Low dose Agonist	n=3	Antagonist	n=3
Antagonist/low dose Agonist	n=3		
Antagonist	n=3		

Table 9 - Blood pressure measurements

Blood pressure measurements throughout NTS until day 12 (d0-d12) are provided. The measurements were performed immediately after injection of vehicle, high dose EP4 receptor agonist, low dose EP4 agonist and EP4 receptor antagonist (max n=4 per group). Values are indicated in mmHg. NA is stated when measurement was not possible due to too low measured values.

High dose agonist (max n=4)

	healthy	d0	d1	d2	d3	d4	d5	d6	d7	d8	d9	d10	d11	d12
N	4	1	1	4	1	0	2	1	2	0	0	0	0	0
Miss	0	3	3	0	3	4	2	3	2	4	4	4	4	4
Min	65.2	38.7	38.0	39.3	41.7	NA	42.7	44.7	35.3	NA	NA	NA	NA	NA
1st Qu	81.3	38.7	38.0	45.6	41.7	NA	63.1	44.7	36.9	NA	NA	NA	NA	NA
Median	103.8	38.7	38.0	56.0	41.7	NA	83.5	44.7	38.4	NA	NA	NA	NA	NA
3rd Qu	121.3	38.7	38.0	65.0	41.7	NA	103.9	44.7	40.0	NA	NA	NA	NA	NA
Max	122.4	38.7	38.0	66.9	41.7	NA	124.3	44.7	41.5	NA	NA	NA	NA	NA
Mean	98.8	38.7	38.0	54.6	41.7	NA	83.5	44.7	38.4	NA	NA	NA	NA	NA
SD	27.8	NA	NA	13.3	NA	NA	57.7	NA	4.4	NA	NA	NA	NA	NA

Low dose agonist (max n=4)

	healthy	d0	d1	d2	d3	d4	d5	d6	d7	d8	d9	d10	d11	d12
N	4	4	4	1	3	4	1	2	3	2	1	1	1	2
Miss	0	0	0	3	1	0	3	2	1	2	3	3	3	2
Min	95.0	93.3	65.1	61.0	52.6	58.4	46.0	45.2	46.7	46.6	43.2	43.0	45.8	48.7
1st Qu	95.7	104.4	69.2	61.0	59.6	64.7	46.0	49.7	48.3	52.3	43.2	43.0	45.8	54.2
Median	99.0	109.1	89.4	61.0	66.7	74.2	46.0	54.2	49.9	58.1	43.2	43.0	45.8	59.8
3rd Qu	106.2	112.5	111.2	61.0	98.2	82.7	46.0	58.8	51.1	63.8	43.2	43.0	45.8	65.3
Max	119.0	119.6	120.1	61.0	129.7	85.8	46.0	63.3	52.3	69.6	43.2	43.0	45.8	70.8
Mean	103.0	107.8	91.0	61.0	83.0	73.2	46.0	54.2	49.6	58.1	43.2	43.0	45.8	59.8
SD	11.1	10.9	27.3	NA	41.1	12.8	NA	12.8	2.8	16.3	NA	NA	NA	15.6

Vehicle (max n=4)

	healthy	d0	d1	d2	d3	d4	d5	d6	d7	d8	d9	d10	d11	d12
N	4	4	4	4	4	4	4	4	4	4	4	4	4	4
Miss	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Min	59.0	76.0	104.5	84.8	67.7	66.1	70.5	75.7	85.9	53.9	82.1	67.5	83.7	95.2
1st Qu	59.8	82.8	118.2	88.3	88.3	84.5	80.9	91.6	88.2	100.5	105.9	89.8	84.5	109.0
Median	73.8	100.1	126.8	103.5	103.5	102.5	94.7	98.0	103.3	117.3	114.7	101.7	91.7	117.8
3rd Qu	90.3	115.6	138.2	119.8	118.2	122.4	110.9	104.7	119.5	119.9	117.6	106.6	101.6	125.6
Max	99.0	117.1	160.3	126.7	137.1	146.4	128.7	121.7	124.9	124.0	124.2	107.8	110.3	135.9
Mean	76.4	98.3	129.6	104.6	103.0	104.4	97.1	98.3	104.4	103.1	108.9	94.7	94.4	116.7

Antagonist (max =4)

	healthy	d0	d1	d2	d3	d4	d5	d6	d7	d8	d9	d10	d11	d12
N	4	4	4	4	4	4	4	4	4	4	4	4	4	4
Miss	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Min	66.9	100.4	99.6	107.1	110.7	89.3	108.8	97.1	63.8	95.0	111.4	78.5	102.7	82.8
1st Qu	74.7	108.4	109.1	117.3	114.9	108.6	109.2	105.5	96.8	106.8	113.5	97.2	106.7	109.5
Median	89.5	113.7	117.7	127.5	122.6	115.1	112.4	112.1	112.4	111.0	115.5	117.4	111.8	123.9
3rd Qu	109.4	119.0	126.5	134.5	129.2	116.7	116.0	120.8	121.4	116.0	120.5	137.7	119.3	130.6
Max	132.4	127.0	137.0	135.2	130.0	121.5	117.4	135.4	134.9	130.0	131.7	156.5	129.9	133.9
Mean	94.6	113.7	118.0	124.3	121.5	110.2	112.8	114.2	105.9	111.8	118.5	117.5	114.1	116.1
SD	29.1	11.1	15.9	13.3	9.5	14.3	4.4	16.1	30.2	14.3	9.1	33.8	11.8	23.1

7. DISCUSSION

GN is a heterogeneous clinical picture that can lead to terminal renal insufficiency (21). Especially rapidly progressive GNs (ANCA vasculitis, Goodpasture's disease and nephritis in systemic lupus erythematosus) lead to terminal renal failure very frequently and rapidly (21). These three forms are autoimmune diseases, where autoantibodies initiate an inflammatory process and ultimately lead to destruction of the renal parenchyma.

The experimental mouse model of NTS is an optimal convenient method to further investigate these three rapidly progressive forms of GN (19,189,203,259,260). The murine NTS is a well-established mouse model that resembles forms of human RPGN (189,260). It is induced by the injection of a rabbit antibody against mouse GBM and accelerates by a preceding immunization of mice with anti-rabbit IgG. The NTS animals develop an immune-complex glomerulonephritis, with proteinuria, glomerular damage and kidney parenchyma damage and infiltration of pro- and anti-inflammatory innate and adaptive immune cells into the kidney (260). These pathophysiological changes depend on TH1 and TH17 as well as Treg cells (189,202,258,261), but also resident renal cells are playing an important role in disease activity (259) .

In the first NTS phase, γδ T cells infiltrate into the kidney. They are activated by interleukin 23 (IL-23) and release interleukin 17 (IL-17) and thereby mediate neutrophil recruitment to the kidney. Neutrophils cause glomerular damage (262). Meanwhile in the lymphatic tissue T-cells are primed, entering the kidney in a second wave being responsible for producing heterologous antibodies. Approximately 4 days after NTS induction CC-chemokine receptor 6 (CCR6) and receptor-related orphan receptor γt (RORγt) expressing T helper 17 (TH17) infiltrate the kidney (263). TH17 cells cause CXC- chemokine ligand 5 (CXCL-5) expression in tubular epithelial cells, what leads to further recruitment of neutrophils to the renal interstitium. This process is regulated by CXCL-5 rather than CXC- chemokine ligand 1 (CXCL-1) (258). Immature renal DCs influence invariant natural killer T (iNKT) cells, which in turn, regulate TH17 activity. Through their production of Interleukine-4 (IL4) and Interleukine-10 (IL10) iNKT can abolish TH17 cell infiltration to the kidney (264).

In case of chronic kidney inflammation, pathological changes are perpetuated by antigen presenting DCs, which produce CXC - chemokine ligand 9 (CXCL9) and recruit CXC-chemokine receptor 3 (CXCR3) expressing TH1 cells (175). TH1 cells themselves can recruit more pro-inflammatory cells like fibrocytes, monocytes and tumour necrosis factor α (TNFα) producing macrophages by the secretion of interferon-γ (IFNγ) (202,265,266). Counterparts in this pro-inflammatory milieu are the regulatory immune cells like $CCR6^{+}/CCR7^{+}$ Tregs which

in the early NTS stage suppresses TH17 cell infiltration and in the later phase suppress not only TH17 cells but also TH1 activation (19). This is attributed to the production of IL10 as well as the expression of the programmed cell death ligand 1 (PDL1) (12). Beside the kidney, also the secondary lymphoid organs play an important role in the immune response in NTS. Especially early immune regulation in NTS takes place in the lymph nodes (267). Moreover, inhibition of complement pathway improves the disease phenotype in mice, what highlights the role of this system in the NTS progression (268–270). Kidneys are frequent targets of systemic autoimmunity, therefore understanding disease pathophysiology and detecting disease causing autoantibodies, immune complexes and complement factors in kidney disease is crucial to develop for example autoantibody assays (12).

Velden et al suggest that an IL-17-specific antibody might be a therapeutic option in crescentic glomerulonephritis and postulate that neutrophils act as an important source of IL-17 and thereby initiate and promote ongoing renal inflammation (271). In our NTS mice we found significantly decreased infiltration of interstitial $Ly6G^+$ cells on day 14 in mice treated with the high dose of the EP4 agonist, with low-dose agonist in combination with antagonist and also in mice treated with the EP4 antagonist only. Moreover we detected a decreased expression of Il17a in high-dose EP4 agonist-treated mice in the draining lymph node. The other treatment groups showed no difference in Il17a expression levels (1).

TNF-specific antibodies could be a treatment in Lupus nephritis, anti-GBM and glomerulonephritis. Karkar et al investigated the role of TNF-α in an experimental model of crescentic glomerulonephritis in Wistar Kyoto rats by blocking endogenous TNF- α. Rats treated with a modified recombinant human soluble TNF receptor (sTNFr) p55 had an improved phenotype depicted by reduction in albuminuria, glomerular inflammation, decreased crescent formation and decreased renal fibrosis (272). We detected no differences in renal mRNA levels of Tnfα between the different groups.

Studies performed so far in NTS mouse models demonstrate that the member of the TNF-ligand and receptor superfamily, tumor necrosisfactor-like weak inducer of apoptosis (TWEAK) might be associated with NTS activity and renal lupus activity in human SLE patients. Stimulation of TWEAK in human as well as in murine podocytes induce a proinflammatory response mediated via the activation of the NFkB signalling pathway (273). TWEAK promotes development of glomerulonephritis by inducing multiple inflammatory mediators, like RANTES, MCP-1, IP-10, MIP-1a, ICAM-1, and VCAM-1 in human kidney cells and it thereby causes a

local inflammatory environment and proliferation of kidney cells (273). Clinical trials are ongoing in lupus nephritis testing a TWEAK-specific antibody (URL 10.07.2019: https://clinicaltrials.gov/ct2/show/results/NCT01499355?view=results) (12).

Urushihara et al demonstrated in an anti-GBM nephritis rat model that a combined therapy with inhibition of the MCP-1/CCR2 pathway and an ARB ameliorated renal injury depicted by decreased proteinuria and markedly reduced glomerular crescent formation. The treatment also suppressed macrophage infiltration and decreased expression of MCP-1, AGT, Ang II, and TGF-β1 (274). A CCR5 antagonist could be as well a possible target in immune complex glomerulonephritis. CCR5 is predominantly expressed on monocytes and Th1-polarized T cells and is mainly responsible in inflammatory diseases for T cell and monocyte recruitment. Turner et al induced NTS in CCR5 KO mice. CCL5 , CCL3 as well as CCL4 were upregulated in CCR5 KO NTS mice. Furthermore increased infiltration of monocytes and T cells and albuminuria was detected in CCR5 KO NTS mice compared to WT mice. Turner et al concluded that CCR5 deficiency aggravates glomerulonephritis via enhanced CCL3/CCL5-CCR1- driven renal T cell recruitment (275). Strikingly, we found significantly decreased Cxcl-5 and Cxcl-1 expression in kidneys after EP4 antagonist treatment, but also in the EP4 antagonist in combination with low-dose agonist group as compared to the vehicle controls (1). We neither evaluated CCL5, CCR1, nor CCR5 expression levels in our NTS mice.

PGE2 is synthesized from AA by COX. PGE2 plays an important role in many biological processes. Thus, PGE2 mediates fever, pain and angiogenesis. PGs are important homeostatic regulators of kidney function. PGE2 is not only the major product of COX2, but also mPGES1, and both of these enzymes have been shown to be elevated in renal diseases (248,276). Nevertheless, studies to block PGE2 using COX2 or mPGES1 inhibitors have failed (277), mainly because PGE2 binds on 4 different EP receptors (EP1–4). The EP receptors are often co-expressed in cells and usually have opposing effects (248). The EP4 receptor is expressed on various cells of the innate and adaptive immune system, as well as in the kidney (endothelial cells, glomerular and mesangial epithelial cells). Studies have shown that PGE2 mediates immunoregulatory effects mainly via the EP4 receptor (177). Thus, targeting specific EP receptors such as EP4 receptor may be advantageous in the treatment of kidney diseases to control the deleterious effects of COX-2/mPGES1/PGE2, while leaving the protective responses intact. Many studies have already shown the great impact of PGE2 on kidney physiology, bearing in mind the regulation of renal perfusion, diuresis, salt excretion and blood pressure (177). However, the role of the EP4 receptor, as well as its precise immunoregulatory

mechanisms, have still not been adequately elucidated, therefore in this study PGE2 and the role of EP4 in the NTS mouse model was examined in more detail.

Previous studies demonstrated the protective as well as pro-inflammatory effect of prostaglandin EP4 receptor agonists in mice with GN or other autoimmune disease (188,190,247,252,278). On the one hand, PGE2 limits the production of pro-inflammatory cytokines and the activation of macrophages and neutrophil granulocytes via the EP4 receptor. On the other hand administration of PGE2 has been shown to positively affect the course of experimental NTS. However, since this effect was attributed to the activation of the EP3 receptor, it should be noted that this was only assumed due to only one dose being used of the respective EP receptor agonists (251). In addition, it was shown that EP4 agonism reduces the development of GN (252). In a rat model of nephrotoxic, mercury chloride-induced acute kidney failure and in a 5/6-nephrectomy model, treatment with the EP4 receptor agonist improved the phenotype of both the acute and chronic kidney disease model by limiting apoptosis and increasing proliferation of tubular epithelial cells (244). In contrast, EP4 signaling also has harmful effects in kidney conditions such as polycystic kidney disease and diabetic nephropathy (247,279). In the latter, EP4 agonism worsened glomerulosclerosis and tubulointerstitial fibrosis (279).
In the present study, we provide evidence that high-dose EP4 receptor antagonism, but also EP4 receptor agonism have beneficial effects in an experimental model of RPGN due to different mechanistic roles of action. As highlighted in a recent review by Nasrallah et al. the published data on targeting EP4 in different experimental models of kidney diseases showed protective, but also harmful effects on the outcome (188,225,280). Nasrallah et al. as well as our data add to the observed discrepancies, since both EP4 receptor agonism and antagonism is protective in the NTS mouse model. We speculate that this is due to the fact that the EP4 receptor is expressed on different immune cells as well as resident kidney cells such as endothelial cells and tubular epithelial cells including cells of the juxtaglomerular apparatus, therefore EP4 receptor agonism as well as antagonism results in different effects depending on the cell type (177,218,225). Beneficial effects of high-dose EP4 receptor agonist treatment have previously been observed in this NTS model. So far, previous studies have used 25-300µg/kg/day of the EP4 receptor-selective agonists ONO AE1-329 and 10 mg/kg/day of the EP4 receptor-selective antagonists ONO AE3-208 (177). It must be noted that we have used 280 µg/kg/day or a higher dose of 1000 µg/kg/day of the EP4 receptor agonists ONO AE1-329 compared to all the studies performed so far.

In our setup, EP4 receptor agonism mainly acted on the vascular system leading to recurrent hypotensive episodes after treatment, which is a well-known effect of PGE2 leading to vasodilation via EP4 receptor signaling (281). Prostaglandins influence blood pressure on various levels: Via acting directly on endothelial cells, by renal hemodynamic effects via the RAAS axis and by regulating salt and water balance in the nephrons and therefore have a potent diuretic and natriuretic role in the kidney mediated by all four EP receptors. The role of renal PGE2/EP receptors in the development of hypertension is already described, although it is unclear whether PGs are increased primarily or secondarily(248). Additionally, PGE2 play as well a part in the endocrine functions of the kidney. Renin and erythropoietin release, urinary concentration and electrolyte reabsorption are also associated with prostaglandins. The juxtaglomerular apparatus composed of macula densa, juxtaglomerular cells and mesangial cells secrete the blood pressure regulating hormone renin. Interestingly, the EP4 receptor, which is expressed in the juxtaglomerular apparatus is suggested to take part in renin production (218). The EP4 receptor contributes to renal hemodynamic and leads to vasodilation in the glomerular ateriola afferens. The hormones of the RAAS are responsible for the regulation of blood pressure, electrolyte and water balance. Renin is mainly produced by the macula densa (distal tubular cells) in the kidney (188,219). It is suggested that in GN renin secretion is increased and the RAAS is activated (282). The RAAS and hypertension plays a crucial role in the pathophysiology of this chronic kidney disease and aggravates the progression of the disease not only inducing renal oxidative stress but also renal scarring and dysfunction (188,274).

Kinoshita et al treated nephritic rats with an angiotensin II (Ang II) type I receptor (AT1R) blocker (ARB). While vehicle-treated nephritic rats developed a crescentic GN, accompanied by enhanced expression of TGF-β, angiotensinogen (AGT), Ang II, AT1R, nephritic rats treated with the ARB showed an improved kidney phenotype and decreased proteinuria.

To further investigate the underlying mechanism, Kinoshita et al isolated rat glomeruli with or without ARB treatment and measured Ang II and TGF- β 1 in supernatants. In vehicle treated nephritic rats production of glomerular AGT protein was increased, whereas treatment with ARB attenuated the production of AGT and Ang II as well as the glomerular expression of Nox2, which is a major component of Nox (ROS-generating system) and TGF- β. Moreover, they demonstrated that Ang II activates MCP-1 and TGF-β1 (274) .

Neugarten et al examined the effects of an antihypertensive therapy in a rat model of nephrotoxic serum nephritis in which hypertension occurs. NTS was induced in uninephrectomized male Sprague Dawley rats and blood pressure was treated with reserpine, hydralazine and hydrochlorothiazide. In contrast to normotensive NTS rats, diffuse glomerular

endo- and extracapillary proliferation was observed in hypertensive NTS rats. Neugarten et al suggested that due to the fact that renal histopathological changes were limited primarily to focal segmental proliferation in less than 1/3 of glomeruli in normotensive NTS rats in contrast to hypertensive NTS rats, a hemodynamic mechanism may have been responsible for enhanced glomerular injury in the hypertensive nephritic animals (283). Anyway, blood pressure in our vehicle treated NTS mice was stable and normotensive (1).

When mice were either treated with a lower-dose of the EP4 receptor agonist or with the EP4 receptor antagonist ONO AE3-208, an improved phenotype of NTS nephritis was observed, depicted by significantly improved histological scores concerning kidney injury and no signs of acute renal failure since tubular casts were reduced to the levels of vehicle-treated mice. So far, it can only be speculated on the mechanisms of EP4-mediated renal protection.
Due to our results we hypothesize that the recurrent hypotensive episodes following the injection of the EP4 agonist in our NTS mice protected the kidney comparable to ischemic preconditioning known from ischemia-reperfusion models (284–286). Low-dose agonist treatment also moderately reduced the blood-pressure, which has been shown to be beneficial in a combined model of NTS and hypertension in rats (283). The effect of the EP4 receptor agonist in NTS was dose-dependent since low-dose agonist treatment caused less hypotensive episodes and a NTS phenotype comparable to vehicle controls. Burne-Taney and coworkers have already shown that ischemic preconditioning influences systemic immune cells, but did not provide evidence of the exact immune cell subtype responsible for the observed effect (286). Subsequently, Tregs migrating to the kidney due to ischemic preconditioning were found to protect mice from ischemia reperfusion injury (285). More recently, evidence was provided that limb ischemic preconditioning reduces systemic Il-17 and thereby prevents from cerebral ischemia (284). In line, we could show that recurrent hypotension in mice exposed to high-dose EP4 receptor agonist resulted in decreased infiltration of immune cells into the kidney as well as reduced *Il-17* mRNA in the draining lymph node. Systemic T and B cell numbers were not influenced by high-dose agonist treatment. Also Treg numbers in the lymph node and *FoxP3* mRNA expression in the kidney were not increased in high-dose EP4 agonist-treated mice. Nevertheless, we observed a decrease in CD4 cell infiltration in low-dose treated mice. It has previously been shown that EP4 receptor signaling is involved in the activation of TH1 and TH17 cells in a model of experimental autoimmune encephalitis (190). Furthermore, effector cells such as macrophages and neutrophil granulocytes, which are recruited by respective TH cell subtypes (12) did not show

a difference. Thus, we concluded that EP4-mediated immune mechanisms are only to a lesser extent responsible for the protective role of low-dose EP4 receptor agonist treatment.

Tubular epithelial cells play a crucial role in tubulointerstitial fibrosis and ultimately progression of chronic kidney disease by the production of several chemokines (e.g. MCP-1, RANTES) and attraction of macrophages and lymphocytes into the renal interstitial space. The recruited macrophages play a pivotal role in the progression of fibrosis by inducing resident fibroblast proliferation and their differentiation into myofibroblasts through the production of profibrotic growth factors, such as transforming growth factor-β1(TGFβ1) and connective tissue growth factor (CTGF) (243) .

Nakagawa et al demonstrated in a mouse model of unilateral ureteral obstruction (UUO) that endogenous PGE2 and the EP4 agonist ONO-4819 decreased tubulointerstitial fibrosis and therefore improved the kidney phenotype. Moreover they demonstrated in UUO EP4 knock out mice that tubulointerstitial fibrosis was increased. Nakagawa et al found elevated macrophage infiltration, myofibroblast proliferation and increased mRNA levels of MCP-1 and RANTES in the kidneys of UUO EP4 knock out mice. Therefore they hypothesized that tubulointerstitial fibrosis is mediated by the EP4 receptor. In line with this finding, EP4 and COX-2 mRNA levels in the kidney were elevated in WT UUO mice. Moreover, cultured murine renal tubular epithelial cells treated with the EP4 agonist ONO-AE1-329 had decreased expression of platelet-derived growth factor (PDGF) and less TGF-β1-induced epithelial–mesenchymal transition (EMT), a phenotypic conversion of tubular epithelial cells into mesenchymal fibroblasts causing interstitial fibrosis (243).

Yamamoto et al showed in a cis-diammine dichloroplatinum-induced rat model of renal failure (CCDP) that PGE2, produced by increased COX-1 and mPGES-1 expression, induced epithelial regeneration via the up-regulated EP4 receptor in renal tubular cells. Moreover, they speculate that endogenous PGE2 suppresses apoptosis and EMT in CCDP-induced rat renal failure. Yamamoto et al demonstrated that rat renal epithelial cells (NRK-52E) treated with cis-diammine dichloroplatinum and additionally treated with 11- deoxy-PGE1 inhibited cell death and increased cell proliferation. Furthermore, they saw an increased cell number in the G0/G1 phase, indicating a G0/G1 arrest and therefore protection from the renal toxic effect of chemotherapeutic agents like cis-diammine dichloroplatinum (245).

In line with the findings of Nakagawa et al and Yamamoto et al we observed an increase in proliferating tubular epithelial cells in the high-dose EP4 receptor agonist treated group *in vivo*, which prompted us to evaluate the influence of PGE2, selective EP4 receptor agonism and antagonism on a murine distal tubular epithelial cell line *in vitro*. In line with our *in vivo* data,

EP4 receptor agonism resulted in increased numbers of viable cells and decreased numbers of cells in the sub G1 phase reflecting late-stage apoptotic cells (1). The latter observation was also made when cells were incubated with PGE2. The importance of EP4 receptor signaling in this mechanism was underlined by the fact that co-incubation of tubular cells with both PGE2 and a selective EP4 receptor antagonist resulted in numbers of cells in the sub G1 phase comparable to vehicle-treated cells. Proliferation of tubular cells in vitro was likely due to a PGE2 and EP4 mediated increase in cAMP. Our data are in line with previous publications providing evidence that EP4 receptor signaling is crucially involved in the regeneration of tubular epithelial cells in different forms of kidney diseases (243,245).

Nevertheless, further studies are needed to unravel the exact mechanisms of EP4 receptor agonist-induced immune suppression in NTS. From a clinical point of view, these data somehow limit further therapy approaches targeting EP4 since the therapeutic range seems to be very small and the expression of EP4 on various cell populations results in opposing effects on kidney function. Another limitation of this study is that it could not prove the EP4 specificity of the used agonist *in vivo*. EP4 receptor knock out mice would be needed to provide this piece of evidence, but they are critical in breeding because most of the EP4 receptor knock-out mice are not viable, due to a patent ductus arteriosus. The surviving mice have an altered kidney development and reduced numbers of nephrons in these mice are further limits for feasibility and applicability in our model. Also a bone marrow transfer from EP4 KO mice to WT mice and vice versa could be useful to investigate the role of EP4 receptor expressed on bone marrow derived cells in more detail (234,287).
In order to investigate EP4 receptor functions individually, specific cell or tissue types can be genetically modified while leaving other tissues untouched. This technique is called tissue-specific gene cre/lox knockout(288). Further, constitutive induction as for example using tamoxifen receptor coupled promotors might be used.

More promising from a clinical perspective is the pharmacological exploitation of EP4 receptor antagonism since it was accompanied neither by changes in the blood pressure nor by any other obvious adverse effects. To our knowledge, there are no data available on the effects of EP4 receptor antagonist in the treatment of NTS so far. In this study, EP4 receptor antagonist treatment protected mice from NTS without affecting blood pressure, which was also shown in healthy mice treated with EP4 receptor antagonist. Even when EP4 receptor antagonist treatment was started 4 days after induction of NTS, the kidney phenotype was improved. Fourteen days after induction of NTS, EP4 receptor antagonist treated mice had an improved

histological renal phenotype compared with vehicle-treated mice, depicted by significantly decreased glomerular intraluminal thrombi. One can speculate that platelet activation and formation of thrombi also play a part in the pathophysiology and glomerular injury in GN. Donadio et al. demonstrated in a prospective, randomized, double blind placebo-controlled trial treating idiopathic MPGN patients with two platelet inhibitor agents, dipyridamole and aspirin, or placebo that platelet inhibitors resulted in a better maintained GFR. It must be mentioned that they saw no significant difference in serum creatinine between the two treatment arms (289).

Moreover, we found Cxcl-1 and -5 transcription in tubular cells to be significantly decreased in mice treated with the EP4 receptor antagonist. Moreover, PGE2 has been shown to increase CXCL-1 production in a model of colorectal cancer (290), but so far data on PGE2-dependent Cxcl-5 transcription are lacking. It was demonstrated by Disteldorf et al that the two chemokines CXCL-1 and -5 in the NTS mouse model are crucially involved in the recruitment and influx of neutrophil granulocytes into the kidney (258). Tubular cells stimulated with IL-17A and IL-17F secreted from γδ T cells and Th17 cells were found to produce CXCL-1 and -5 leading to the recruitment of neutrophil granulocytes to the kidney in NTS (291). In line with these date, EP4 receptor antagonist-treated NTS mice had significantly less neutrophil granulocytes in the renal interstitial area. Furthermore, we measured significantly decreased *Il-6* mRNA levels in the kidney and IL-6 protein in the serum of the EP4 receptor antagonist-treated NTS mice. PGE2-induced IL-6 production via EP4 has been demonstrated before in various cells such as macrophages, neutrophils, smooth muscle cells, and fibroblasts (292). Leukocytes are the main producers of Il-6, but resident renal cells like tubular epithelial cells are able to secrete IL-6 as well (293). We only detected marginal expression of *Il-6* mRNA in our DCT cells *in vitro*, but a strong signal when stimulated with PGE2 or the EP4 receptor agonist, which was blunted by adding the EP4 receptor antagonist. Thus, PGE2 via EP4 receptor signaling also induces *Il-6* transcription in DCT cells. *In vivo*, the observed decrease of IL-6 in the EP4 receptor antagonist-treated mice could be explained by a direct effect of the EP4 receptor antagonist on IL-6-producing cells such as leukocytes and renal cells, but we cannot exclude that this finding is directly related to the significantly decreased neutrophil numbers in the kidney, accounting to those cells capable of secreting IL-6 (294).

Also, an IL-6-specific antibody has been tested in a mouse and rat model of lupus nephritis, anti-GBM disease and immune complex glomerulonephritis. The role of IL-6 in autoimmune kidney disease is controversial, on one hand IL-6 acts proinflammatory in murine lupus nephritis, and on the other hand IL-6 has protective effects in a mouse model of nephrotoxic

serum nephritis (295,296). Luig et al tested an antibody blockade of the IL-6R in a murine model of nephrotoxic serum nephritis and found a disease aggravation. They hypothesised that impaired classic IL-6 signalling in macrophages is causative for aggravation of nephritis (296). This is in contrast to our findings. We found reduced IL-6 on protein level in the serum of EP4 antagonist-treated mice, which had an improved kidney phenotype (1).

Nevertheless, the role of IL-6 in the NTS mouse model is still not clear and is discussed controversially, and obviously IL-6 effects involve different pathways (297). Therefore, we can only hypothesize that reduced IL-6 release caused by the EP4 receptor antagonist can be attributed to the improved NTS phenotype.

Importantly, in this study we have shown *in vivo* and *in vitro* that treatment with the EP4 receptor antagonist reversed the phenotype of EP4 receptor agonist treated mice and tubular cells, proving the specificity of pharmacological EP4 receptor targeting. Although one might argue that pharmacological inhibition could lack specificity, the agonist and antagonists we used have been well-characterized (177). Each prostanoid receptor preferentially binds one of the natural prostaglandins and is named after the one with the highest affinity. Thus PGE2 preferentially activates the EP receptor. The naphthyl derivative ONO-AE3-208 developed by Ono Pharmaceuticals has an affinity of Ki = 1.3 nM and ONO-AE1-329 an affinity of Ki = 9.7 nM, EC_{50} = 3.1 nM. Several compounds highly selective for each EP receptor subtype have been investigated and developed using cultured cell lines stably expressing each EP receptor subtype. The well characterized structure of EP receptors enables a systematic analysis and development of the binding characteristics and the development of more chemically stable EP4 agonists and antagonists. A chemical library screening resulted in further chemical modifications and new potent and selective agonists and antagonists were designed (187,298,299). Following selective agonists stimulate EP4: 1-hydroxy-PGE_1, rivenprost (ONO-4819), OOG-308, ONO-AE1-329, AGN205203, ONO-4819, CP-734,432m AE1-329, SC-19220, SC-51089, and EP4RAG. Following selective antagonists block the EP4 receptor: grapiprant (CJ-023,423), ONO-AE3-208, GW627368X, AH23848, and ONO-AE2-227 (300).

In the present study we provide evidence that both EP4 receptor antagonist and agonist have indeed beneficial, but also harmful effects in an experimental model of glomerulonephritis depending, on the dose applied. Opposing effects of EP4 receptor agonism in the model of NTS depending on the dose applied has been observed. One can speculate, that the major cause of these effects is based on the fact that the EP4 receptor is expressed on many different

resident kidney cells such as endothelial cells, cells of the arterioles but also on tubular epithelial cells including cells of the juxtaglomerular apparatus (177,188,218,280). Furthermore, EP4 receptor targeting has been described to have effects on cells of the innate and adaptive immunity, such as neutrophils, macrophages and T helper cells, which are all crucially involved in the pathogenesis of NTS nephritis (12).

In summary, high-dose EP4 receptor agonism, but also antagonism improved the NTS phenotype (1). Whereas high-dose EP4 agonist treatment resulted in recurrent hypotensive episodes and reduced infiltration of immune cells into the kidney as well as increased proliferative capacity of tubular cells. EP4 antagonism protected mice from NTS due to decreased tubular Cxcl-1 and Cxcl-5 production leading to reduced infiltration of interstitial neutrophil granulocytes into the kidney. We thus provide further evidence that targeting the EP4 receptor shows great potential in the future treatment of glomerulonephritis.

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