Blockade of prostaglandin E₂ receptor 4 ameliorates nephrotoxic serum nephritis

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INTRODUCTION

Rapidly progressive glomerulonephritis (RPGN) often leads to end-stage renal disease with the need for renal replacement therapy. The disease itself and current standard therapy are still accompanied by a large reduction in life expectancy and quality (12). Experimental nephrotoxic serum (NTS) nephritis is a murine model of immune-complex glomerulonephritis (GN), closely resembling forms of human RPGN. Animals with NTS present gross proteinuria and proliferative and inflammatory glomerular changes, including crescent formation and kidney infiltrating leukocytes (9, 29, 36). The pathogenesis depends on T helper cells type 1 (Th1) and type 17 (Th17), which are counter regulated by regulatory T cells (30, 32, 36), but resident renal cells also play an important role in disease activity (8). Whereas Th1 cells mainly recruit macrophages to the kidney (16), Th17 cells have been proven to mediate recruitment of neutrophil granulocytes to the interstitium (7).

The lipid molecule and cyclooxygenase (COX) product prostaglandin E₂ (PGE₂) acts on four different G protein-coupled receptors, i.e., E-type prostanoid receptor (EP) 1–4. Activation of the Gαs-coupled EP4 leads to an intracellular increase in cyclic adenosine monophosphate levels and ensues activation of protein kinase A, although other signaling pathways, such as phosphoinositide 3-kinase, may also be involved (37). EP4 is expressed not only on a variety of immune cells; resident kidney cells (such as glomerular epithelial cells); mesangial cells; cells of theafferent arteriole; and collecting, proximal, and distal duct cells but also endothelial cells, which are all known to be involved in the pathogenesis of RPGN (15, 22).

PGE₂ has been found to modulate immune cells via EP4, mainly in an anti-inflammatory manner. Via EP4 signaling, PGE₂ limits proinflammatory cytokine production and activation of macrophages and neutrophil granulocytes. Although reports of EP4 activation are mainly associated with anti-inflammatory events, EP4 agonism also mediates the activation of Th17 cells and the increase of the expression of C-c motif chemokine receptor 7 and thereby facilitates the recruitment of T cells (6, 10, 15). Furthermore, EP4 agonism influences renal hemodynamics not only by exerting vasodilatory effects on the
affection to covering and increasing renin production in the juxtaglomerular apparatus (26).

So far, the targets of EP4 have been evaluated in different kidney disease models, and the EP4 receptor agonist was found to have both beneficial (20, 33) and harmful effects (18). Nagamatsu et al. (20) found an improved phenotype of NTS when mice were treated with an EP4 agonist, but the clear mechanism of protection remains elusive so far. Data on EP4 receptor antagonism are scarce. Recently, Thieme and coworkers (31) found an EP4 receptor antagonism to protect mice from the development of diabetic nephropathy.

In the presented study, we aimed to evaluate the effects of selective EP4 antagonism in NTS.

**MATERIALS AND METHODS**

**Animals and study design.** All experiments were performed with 8–10-wk-old male C57Bl/6j mice (Charles River Laboratories, Sulzfeld, Germany). The NTS model was induced as described earlier (9, 29). Briefly, C57Bl/6 mice were immunized subcutaneously with 2 mg/ml rabbit IgG (Jackson ImmunoResearch Laboratories, West Grove, PA) dissolved in incomplete Freund’s adjuvant (Sigma-Aldrich, St. Louis, MO) with desiccated, nonviable Mycobacterium tuberculosis H37a (Difco Laboratories, Detroit, MI). Three days later, NTS mice were injected intravenously via the tail vein with heat-inactivated rabbit anti-mouse glomerular basement membrane serum.

Starting on the day of NTS induction, mice were treated with an EP4 antagonist ONO AE3-208 (10 mg/kg body wt ·day⁻¹; kind gift from ONO Pharmaceutical, Osaka, Japan) or vehicle (3% DMSO) subcutaneously twice daily. One group was treated with the EP4-receptor antagonist ONO AE3-208 (10 mg/kg body wt ·day⁻¹) 30 min before injecting an EP4 agonist ONO AE1-329 (280 μg·kg body wt ·day⁻¹). In a separate experiment, mice were treated with the EP4-receptor antagonist ONO AE3-208 (10 mg/kg body wt ·day⁻¹) starting at 4 days after NTS induction. In one group of mice, we evaluated urinary output within 12 h using metabolic cages. Mice were euthanized after 14 days of NTS. As a control, healthy mice were treated with the EP4 antagonist ONO AE3-208 (10 mg·kg body wt ·day⁻¹) or vehicle for 7 days. We noted no dropouts in respective groups. All mice survived the follow-up period.

**Study approval.** All animal care and experimental procedures used in this study complied with national and international guidelines and were approved by the Austrian Federal Ministry of Science, Research, and Economy (BMWFZ GZ:66.010/042-WF/V/3b/2015) and conform with the Directive 2010/63/EU. Studies are reported in compliance with the ARRIVE guidelines (17).

**Biochemical analyses.** On day 7 and 14 after NTS induction, urinary albumin/creatinine ratio was determined in spot urine. Urinary albumin excretion was measured by enzyme linked immunosorbent assay (ELISA) (Abcam, Cambridge, MA) according to the manufacturer’s instructions. Urinary creatinine was evaluated photometrically with a picric acid-based kit (Sigma-Aldrich). Lipocalin-2/neutrophil elastase (NE) activity was determined using a commercially available mouse ELISA DuoSet kit (R&D Systems, Minneapolis, MN). Lipocalin-2 activity was determined using a commercially available mouse ELISA DuoSet kit (R&D Systems, Minneapolis, MN).

**Histological staining.** Kidneys were fixed in 10% neutral-buffered formalin for 24 h and embedded in paraffin. Kidneys were cut in 4-µm sections, and a periodic acid Schiff staining was performed (Merck KGaA, Darmstadt, Germany). 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 because of 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). Extra-capillary hypercellularity/crescents defined as cell proliferation of more than two cell layers was 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).

For performing immunohistochemistry, kidneys were shock-frozen with Tissue-Tek OCT Compound (Sakura Finetek Europe B.V., Alphen aan den Rijn, the Netherlands). The frozen 4-µm kidney sections were stained using rat-derived primary antibodies for CD4 (Serotec, Oxford, UK), CD8 (Serotec), CD68 (Serotec), and anti-Ly6G (Gr-1) (Abcam). The tissue was incubated for 1 h in a humidified chamber with the primary antibody followed by incubation with a biotin-conjugated goat anti-rat IgG (Jackson ImmmunoResearch 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 = 0–4 cells stained positive, 1+ = 5–10 cells, 2+ = 11–50 cells, 3+ = 51–200 cells, and 4+ = >200 cells stained positive per low-power field.

Sections were visually examined with an Olympus BX41 microscope (Olympus) and an Olympus U Plan Apo ×40/0.8 lens. Photographs were taken with an Olympus DP50 camera.

**Immunofluorescence staining.** Frozen 4-µm kidney sections were evaluated for autologous and heterologous IgG, as well as C3 deposition in kidneys. Serial dilutions of FITC-conjugated goat anti-mouse IgG (Jackson ImmmunoResearch Laboratories) as well as serial dilutions of FITC-conjugated goat anti-rabbit IgG (Jackson ImmmunoResearch Laboratories) were dispensed on kidney sections to evaluate autologous IgG and heterologous IgG deposition in the kidney using direct immunofluorescence titer determination. 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).

**Assessment of autologous antibody responses.** Rabbit IgG (100 μg/ml; Jackson ImmmunoResearch Laboratories) was incubated overnight on a 96-well plate. Mouse serum was incubated with serial doubling dilutions for the detection of circulating mouse anti-rabbit immunoglobulin using horseradish peroxidase-conjugated mouse anti-rabbit IgG (Jackson ImmmunoResearch Laboratories).

**Isolation of tubular cells from kidneys.** Mice were subjected to NTS. Tubules were isolated using two sieves (mesh size 150 and 90 µm), as has been described previously (13). Briefly, minced murine kidneys were passed through the top 150-µm sieve (Newark Wire Cloth, Clifton, NJ). From the top of the 90-µm sieve (Newark Wire Cloth), tubuli were collected and subjected to RNA isolation.

**Reverse transcription and quantification of mRNA by real-time PCR.** mRNA was isolated using TRI Reagent (Sigma-Aldrich). Complementary DNA transcripts from RNA was synthesized using the Superscript III Transcription Kit (Invitrogen) 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).

For quantification of respective genes, TaqMan gene expression assays (Applied Biosystems, Foster City, CA) were used for the following: Il6: Mm00446190_m1; Cxcl-1: Mm00433859_m1; Cxcl-5: Mm00446190_m1; Cxcl-5: Mm00433859_m1; Mm00446190_m1; Mm00433859_m1; Mm00446190_m1;
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Mm00436451_g1; Rory: Mm01261022_m1; and Renin: Mm02-342889_g1. SYBR Green Mastermix (BioRad) was used for the detection of hprt or β-actin using the following primers, respectively: forward: 5’GCT TCC TCA GAC CGC TTT TTG C 3’; reverse 5’ATC GAT AAT CAC GAC GCT GGG ACT G 3’; forward 5’GAA GTG TGA CGT CTA GCA ACC TCT CTC TCT GCT GGA 3’. Both housekeeping genes served as reference genes for kidney samples. Results were calculated with the 2–ΔΔCT method.

Evaluation of angiotensin in peripheral blood. A small number of mice was evaluated for their angiotensin levels in peripheral blood by mass spectrometry (liquid chromatography tandem-mass spectrometry-based angiotensin quantification) by Attotquant Diagnostics (Vienna, Austria), as described previously (25).

Blood pressure measurement. The noninvasive tail cuff measurement of blood pressure was performed daily for 14 days in the evening with a Kent Scientific Corporation CODA Non-Invasive Blood Pressure System (Kent Scientific CODA, Torrington, CT) in an observer-blinded manner. Blood pressure of each mouse was recorded by performing 5 acclimations and 10 experimental measurements. 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. The mean arterial pressure was calculated with accepted values as follows: mean arterial pressure = [2 × diastolic pressure] + systolic pressure]/3.

Cell culture. The murine distal convoluted tubules (DCT) cell line was used as described previously (8). Cells were serum- and glutamine-starved for 72 h and treated twice daily with either PGE2 (300 nmol/l; Cayman, MI), EP4 antagonist ONO AE3-208 (1 μmol/l), or the specific EP4 agonist ONO AE1-329 (300 nmol/l) for 72 h. Cells were subjected to RNA isolation and quantitative PCR as described above. Cxcl-5 protein was measured in the supernatant with a commercially available kit (Mouse LIX Quantikine ELISA Kit, R&D Systems, Minneapolis, MN).

Statistical analysis. Data are shown either as mean ± standard error of the mean or mean with raw data. Statistical comparisons were carried out using 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. When comparing only two-groups, either the Student’s t-test or Mann-Whitney U test was performed, depending on the distribution of the data. Normal distribution was assumed of all variables. 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 3.4.4 (R Foundation).

RESULTS

EP4 antagonism improves the phenotype of NTS. To evaluate the role EP4 receptors, mice were treated with the EP4 antagonist and compared with 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 that the EP4 receptor was completely blocked by our EP4 antagonist.

Fourteen days after induction of NTS, antagonism of EP4, as well as simultaneous application of EP4 agonist and antagonist, resulted in an improved histological appearance (Fig. 1A) compared with vehicle-treated mice, which was also reflected by decreased periodic acid Schiff-positive deposits and glomerular intraluminal thrombi, as shown by the chromotrope aniline blue-stained section. Whereas no difference in crescent formation was detected between the groups (Fig. 1B), the kidneys of mice treated with the EP4 antagonist, as well as with both agonist and antagonist, showed significantly decreased glomerular injury in terms of mesangial (Fig. 1C) and endocapillary proliferation (Fig. 1D) and intraluminal thrombi (Fig. 1E) and segmental capillary necrosis (Fig. 1F). Furthermore, decreased tubular injury (Fig. 1, G and H) was detected in the two treatment arms compared with vehicle controls. In line with these histological findings, serum NGAL (Fig. 1J) and BUN levels (Fig. 1J) were significantly lower 14 days after NTS induction in mice treated with EP4 antagonist and EP4 agonist plus antagonist as compared with vehicle-treated mice. Albuminuria was not different between the groups on day 7 and 14 (Fig. 1, K and L).

EP4 antagonism reduces renal infiltration of immune cells. Fourteen days after induction of NTS, a significant decrease in renal infiltration of CD4+ T cells was observed in EP4 antagonist-treated mice as compared with vehicle (Fig. 2, A and F).

No difference was detected for the infiltration of CD8+ T cells (Fig. 2, B and F) and CD68+ macrophages (Fig. 2, C and F) in immunohistochemistry. Strikingly, infiltration of interstitial Ly6G+ cells on day 14 was significantly decreased in mice treated with the EP4 antagonist and with agonist in combination with antagonist (Fig. 2, D and F), whereas there was no difference in Ly6G+ cell counts in glomeruli (Fig. 2, E and F).

To analyze whether the mice produced equal anti-rabbit IgG titers as a response to immunization, which is essential for the NTS phenotype, we performed a respective ELISA of mouse serum. Mouse anti-rabbit IgG titers did not differ between the groups 14 days after NTS induction (Fig. 3A). Furthermore, we did not detect a difference in deposited mouse and rabbit as well as mouse anti-rabbit IgG on glomerular basal membrane (Fig. 3, B and C). Moreover, we saw no difference in glomerular C3 deposition between the different groups (Fig. 4).

EP4 antagonism does not influence blood pressure levels, urinary output, and renin mRNA levels. EP4 antagonist treatment did not change blood pressure levels as compared with vehicle-treated healthy (data not shown) and nephritic mice (Fig. 5A). In contrast, EP4 agonism resulted in critical drop in blood pressure. Blood pressure levels were not detectable immediately after injection of the EP4 agonist within the first 2 to 4 days (data not shown). During the whole observation period, decreased blood pressure levels were detected in mice 30 min after treatment with the EP4 agonist (Fig. 5A). Pretreatment with the EP4 antagonist protected mice from EP4 agonist-induced hypotension (Fig. 5A).

Renin mRNA levels were evaluated in the kidney in nephritic mice treated with vehicle, the EP4 antagonist, or a combination of the EP4 antagonist and agonist. No difference in the gene expression of Renin was detected between the three groups (Fig. 5B). Furthermore, urinary output in healthy or nephritic mice treated with either the EP4 agonist or vehicle was quantified within a time period of 12 h using metabolic cages. EP4 antagonism did not influence urinary output after 6 days of treatment in healthy mice (Fig. 5C). No differences were observed in nephritic mice after 6 and 12 days of treatment with the EP4 antagonist as compared with vehicle-treated mice (Fig. 5, D and E). Finally, angiotensin levels were evaluated in a small number of mice in the peripheral blood by performing mass spectrometry. No differences in angiotensin 1 and 2 as well as angiotensin 1–5 were detected in the peripheral blood of
Fig. 1. E-type prostanoid 4 (EP4) receptor antagonism improves the phenotype of nephrotoxic serum nephritis (NTS). After NTS induction (14 days), kidneys of mice treated with vehicle (n = 8), EP4 antagonist/agonist (n = 4), or EP4 antagonist (n = 8) were harvested and processed for periodic acid Schiff staining. Representative pictures are shown (A). Magnification ×400/×600. Stained kidney sections were quantified for glomerular crescent formation (B), mesangial (C) and endocapillary proliferation (D), intraluminal thrombi (E) and segmental capillary necrosis (F), and acute tubular injury score (G). Tubular cast formation per 6 high power field (HPF) was evaluated (H) by an observer in a blinded manner. Serum neutrophil gelatinase-associated lipocalin (NGAL) levels (I) as well as serum blood urea nitrogen (BUN) levels (J) were evaluated on day 14. Furthermore, urine samples collected on days 7 (K) and 14 (L) were analyzed for albumin and creatinine. Medians are indicated by a horizontal line. Statistical significances are provided compared with vehicle-treated mice (**P ≤ 0.01 and ***P ≤ 0.001).
14-day nephritic mice treated with either vehicle or EP4 antagonist (Fig. 5).

Blocking the EP4 receptor 4 days after NTS induction improves disease severity. In further experiments, we addressed the potential therapeutic effect of the EP4 antagonist and treated mice with the EP4 antagonist or vehicle starting 4 days after NTS induction until day 14. Here, the EP4 antagonist also improved the phenotype of NTS. The ameliorated renal histology was reflected by a trend toward a decreased extent of crescents, mesangial and endocapillary hypercellularity, and tubular injury as compared with vehicle-treated controls (Fig. 6, A–F). Tubular cast formation and serum NGAL levels were significantly reduced after treatment with the EP4 antagonist (Fig. 6, G and H). BUN levels and albuminuria on day 14 did not differ between the two groups (Fig. 6, I and J).

EP4 antagonism reduces IL6 levels in NTS. We detected significantly decreased IL-6 mRNA in the kidneys of EP4 antagonist- and simultaneous EP4 agonist/antagonist-treated mice 14 days after NTS induction as compared with vehicle-treated controls (Fig. 7A). Since immune regulations in the lymph node are crucial for the development of NTS (2), we evaluated IL-6 mRNA expression also in the lymph node but found no differences between the groups (Fig. 7B). Interestingly, IL-6 was reduced on the protein level in the serum of EP4 antagonist-treated mice (Fig. 7C). Rorγt mRNA levels in the kidney remained unchanged between vehicle and EP4 antagonist-treated mice 14 days after NTS induction. In the EP4
agonist group, Rorγt were increasingly detected (Fig. 7D). Il-17 transcripts in the lymph node did not differ between the groups (Fig. 7E). Of note, no differences in renal mRNA levels of Tnfα, Tbet, Il10, Interferon-γ, and FoxP3, the key transcription factor controlling regulatory T cells, were found between the different groups (data not shown).

**EP4 antagonist treatment suppresses tubular Cxcl-1 and Cxcl-5 expression.** To further evaluate whether the decreased interstitial neutrophil cell infiltration in the EP4 antagonist-treated mice is due to alterations in Cxcl-1 and Cxcl-5 production, we performed quantitative PCR to assess Cxcl-1 and Cxcl-5 mRNA expression levels in the kidney 14 days after NTS induction (Fig. 8, A and B). Strikingly, Cxcl-1 and Cxcl-5 were both significantly decreased in the kidney of mice after treatment with not only the EP4 antagonist but also in the EP4 agonist plus antagonist group, as compared with the vehicle controls. Since Cxcl-1 and Cxcl-5 have been proven to be mainly produced by tubular cells in NTS (7), we evaluated Cxcl-1 and Cxcl-5 transcription in sieved tubular cells from mice treated with vehicle or EP4 antagonist from day 4 until day 14 after NTS induction (Fig. 8, C and D). Here, we could also show that treatment with the EP4 antagonist reduces tubular Cxcl-1 and Cxcl-5 mRNA expression (Fig. 8, C and D).

In vitro, murine DCT cells were starved for 72 h and treated with vehicle, EP4 antagonist, PGE2, or EP4 agonist and combinations of EP4 antagonist and either PGE2 or EP4 agonist. Cxcl-1 expression was not differentially regulated between the groups, even though DCT cells treated with either PGE2 or the EP4 agonist tended to show increased Cxcl-1 mRNA expression (Fig. 8E). PGE2 and EP4 agonist treatment increased Cxcl-5 transcripts significantly in DCT cells, whereas the EP4 antagonist blunted this increase to the level of vehicle-treated mice. EP4 antagonist treatment alone showed comparable Cxcl-5 mRNA levels in DCT cells as compared with vehicle controls (Fig. 8F). Cxcl-5 protein expression was regulated in a comparable manner (Fig. 8G).

**DISCUSSION**

Prostaglandins are important homeostatic regulators of kidney function. PGE2 is the major product of COX-2 and micro-
Fig. 5. Blocking the E-type prostanoid 4 (EP4) receptor does not influence blood pressure. Blood pressure was measured daily (A) in vehicle- \((n=7)\), EP4 antagonist- \((n=7)\), EP4 agonist- \((n=7)\), and the combination of EP4 antagonist/agonist- \((n=3)\) treated mice after nephrotoxic serum (NTS) induction. Blood pressure measurements were performed 30 min after injection in all 4 groups by employing the tail cuff method. Mean arterial pressure (MAP) is shown as mean ± SE. Statistical significances are provided comparing all 4 groups (*\(P<0.05\), **\(P<0.01\), and ***\(P<0.001\)). After NTS induction (14 days), quantitative PCR was performed to evaluate Renin mRNA expression in the kidney in healthy mice \((n=3)\), animals treated with vehicle \((n=8)\), EP4 antagonist/agonist \((n=4)\), or EP4 antagonist \((n=8)\) (B). Data are provided as mean of the fold change compared with healthy kidneys. Healthy (C) and nephritic mice (D and E) were treated with vehicle or EP4 antagonist \((n=3\) per group) and analyzed for their urinary output within 12 h at indicated time points. Plasma of mice subjected to 14 days of NTS and treated with either vehicle or EP4 antagonism \((n=2\) per group) were analyzed for angiotensin (Ang) levels by liquid chromatography tandem-mass spectrometry-based angiotensin quantification (F). Data are provided as mean ± SE.
Fig. 6. Blocking E-type prostanoid 4 (EP4) receptors 4 days after nephrotoxic serum (NTS) induction improves the phenotype of NTS. After 14 days of NTS, tissues from vehicle- \((n = 9–10)\) or EP4 antagonist- \((n = 10)\) treated mice were harvested and processed for further analysis. Stained kidney sections were quantified for glomerular crescent formation (A), mesangial (B) and endocapillary proliferation (C), intraluminal thrombi (D) and segmental capillary necrosis (E), and tubular cast formation per 6 high power field (HPF) (F and G) by an observer unaware of the treatments. Serum neutrophil gelatinase-associated lipocalin (NGAL) levels (H), blood urea nitrogen (BUN) levels (I), and urinary albumin/creatinine ratio (J) were evaluated on day 14. Means are indicated by a horizontal line; in the case of scores, the median is indicated by the horizontal line. Statistical significances are provided compared with vehicle treated mice (*\(P \leq 0.05\) and **\(P \leq 0.01\)).
somal PGE synthase 1 (mPGES1), and both of these enzymes have been shown to be elevated in renal diseases (23, 27). Nevertheless, studies to block PGE2 using COX-2 or mPGES1 inhibitors have failed (23), mainly because PGE2 binds to four different EP receptors (EP1–4), which are frequently coexpressed in various cells and usually have tissue-specific and often opposing effects (15, 22). Thus, targeting specific EP receptors, such as EP4, 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.

In the present study, we provide evidence that EP4 antagonism has beneficial effects in an experimental model of RPGN. This is somehow surprising because it has been shown that EP4 agonist treatment also improved the phenotype of NTS, even though the mechanism of protection has remained elusive so far (20). We speculate that this discrepancy might be explained by the fact that EP4 is expressed on different immune cells as well as resident kidney cells and vascular epithelial cells resulting in very different effects when targeting EP4 (22, 26, 27). Thus, targeting specific EP receptors, such as EP4, 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.

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Fig. 8. E-type prostanoid 4 (EP4) antagonist treatment suppresses tubular chemokine (C-X-C motif) ligand (Cxcl)-5 and Cxcl-1 expression in vivo and in vitro. After nephrotoxic serum (NTS) induction (14 days), quantitative PCR was performed to evaluate Cxcl-1 (A) and Cxcl-5 (B) mRNA expression in the kidney of vehicle- (n = 6), EP4 antagonist/agonist- (n = 4), or EP4 antagonist- (n = 8) treated mice. Cxcl-1 (C) and Cxcl-5 (D) mRNA expression were evaluated in isolated tubular cells from mice treated with vehicle (n = 8) or EP4 antagonist (n = 8) from day 4 until day 14 after NTS induction. Data are provided as mean ± SE of fold change compared with healthy kidneys or sieved tubular cells isolated from healthy mice, respectively. Statistical significances are provided compared with vehicle-treated mice (*P < 0.05). Murine distal convoluted tubules cells were starved and treated with vehicle, EP4 antagonist, prostaglandin E2 (PGE2), or EP4 agonist and a combination of PGE2/EP4 antagonist and EP4 agonist/EP4 antagonist. Cxcl-1 (E) and Cxcl-5 (F) mRNA expression was evaluated and provided as fold increase compared with vehicle control (vehicle, n = 10; antagonist, n = 6; agonist/PGE2, n = 5; antagonist/agonist, n = 4; PGE2, n = 6; agonist, n = 6). Cxcl-5 protein levels (G) were evaluated in supernatants (n = 4 per group). Statistical significances are provided compared with vehicle-treated mice (**P < 0.01).
has been proven that the production of Cxcl-1 and Cxcl-5 in tubular epithelial cells in NTS is mediated by the IL-17/Th17 axis (7, 28). Since we detected decreased renal infiltration of CD4⁺ T cells in EP4 antagonist-treated mice, we cannot completely exclude that this effect was mediated via the IL-17/Th17 axis rather than a direct effect of EP4 antagonism on tubular epithelial cells. Two facts point toward a direct EP4 mediated effect on tubular epithelial cells. First, the master gene regulator of Th17 cells, RORγt, was not regulated in NTS kidneys treated with the EP4 antagonist. Second, PGE2 and EP4 agonist treatment increased Cxcl-5 in an in vitro approach using murine DCT cells, which was blunted by EP4 antagonist treatment.

Furthermore, we detected significantly decreased IL-6 mRNA levels in the kidney and IL-6 protein in the serum of the EP4 antagonist-treated mice. PGE2-induced IL-6 production via EP4 has been demonstrated before in various cells (15, 37). IL-6 is mostly secreted by leukocytes but also by renal cells, including tubular epithelial cells (3). The observed decrease of IL-6 in the EP4 antagonist-treated mice could be explained by a direct effect of the EP4 antagonist on IL-6-producing cells but could also reflect the significantly decreased neutrophil numbers in the kidney, which also represent an important source of IL-6 (38). The role of IL-6 in the pathogenesis of NTS is still discussed controversially (4). Thus, we can only speculate whether the decreased in IL-6 induced by EP4 antagonism also added to the improved NTS phenotype.

EP4 receptor signaling plays an important role in the activation of thrombocytes. EP4 agonism has been shown to potently inhibit platelet aggregation and thereby thrombus formation, which was reversed by EP4 antagonism (24). In our hands, EP4 antagonism even improved intraglomerular capillary thrombi formation, as shown by histological evaluation. Obviously, the beneficial effects of EP4 signaling on tubular epithelial cells are dominant in our in vivo situation.

The renin-angiotensin-aldosterone system has been shown to be regulated by EP4 signaling (19, 26). Recently, prorenin receptor (PRR) signaling in the collecting duct has been shown to be mediated via EP4. EP4 antagonism was able to partly prevent the decrease in urine volume and the increase in urine osmolality and aquaporin 2 expression in a setting of water deprivation (35). In our experimental setup, we have indirect evidence that the renin-angiotensin-aldosterone system and also PRR signaling in the collecting duct are not critically influenced by EP4 antagonism since blood pressure levels, renin mRNA levels, and urinary output remain unchanged compared with vehicle controls in nephritic mice. In line, angiotensin levels were not regulated in nephritic mice treated with either vehicle or the EP4 antagonist, which was shown only in a few mice. PRR signaling is essential for the development of T cells (11). This signaling pathway serves as an explanation for the decreased renal CD4⁺ T cell infiltration in the EP4 antagonist-treated mice, but further studies are needed to outline this signaling pathway in the NTS model.

Although one might argue that pharmacological inhibition lack specificity, the agonist and antagonists we used have been characterized in great detail (10, 14, 18, 21). We further provide evidence that EP4 antagonism effectively blocked EP4 signaling since it completely abolished hypotension induced by EP4 agonism and showed an NTS phenotype comparable to mono-treatment with the EP4 antagonist. Furthermore, EP4 knockout mice show a drastic reduction in viability at birth, and they have an altered kidney development, which further limits their applicability in our model (1).

In summary, EP4 antagonism shows great potential in the treatment of GN. It seems to protect mice from NTS because of decreased tubular Cxcl-1 and Cxcl-5 production, leading to reduced infiltration of interstitial neutrophil granulocytes.

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DISCLOSURES
No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

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