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Podocytes Produce and Secrete Functional Complement C3 and Complement Factor H

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Podocytes are an important part of the glomerular filtration barrier and the key player in the development of proteinuria, which is an early feature of complement mediated renal diseases. Complement factors are mainly liver-born and present in circulation. Nevertheless, there is a growing body of evidence for additional sites of complement protein synthesis, including various cell types in the kidney. We hypothesized that podocytes are able to produce complement components and contribute to the local balance of complement activation and regulation. To investigate the relevant balance between inhibiting and activating sides, our studies focused on complement factor H (CFH), an important complement regulator, and on C3, the early key component for complement activation. We characterized human cultured podocytes for the expression and secretion of activating and regulating complement factors, and analyzed the secretion pathway and functional activity. We studied glomerular CFH and C3 expression in puromycin aminonucleoside (PAN)-treated rats, a model for proteinuria, and the physiological mRNA-expression of both factors in murine kidneys. We found, that C3 and CFH were expressed in cultured podocytes and expression levels differed from those in cultivated glomerular endothelial cells. The process of secretion in podocytes was stimulated with interferon gamma and located in the Golgi apparatus. Cultured podocytes could initiate the complement cascade by the splitting of C3, which can be shown by the generation of C3a, a functional C3 split product. C3 contributed to external complement activation. Podocyte-secreted CFH, in conjunction with factor I, was able to split C3b. Podocytes derived from a patient with a CFH mutation displayed impaired cell surface complement regulation. CFH and C3 were synthesized in podocytes of healthy C57Bl/6-mice and were upregulated in podocytes of PAN treated rats. These data show that podocytes produce functionally active complement components, and

could therefore influence the local glomerular complement activation and regulation. This modulating effect should therefore be considered in all diseases where glomerular complement activation occurs. Furthermore, our data indicate a potential novel role of podocytes in the innate immune system.

Keywords: complement secretion, proteinuria, glomerulus, kidney, local regulation

INTRODUCTION

Proteinuric kidney diseases are very common in adult and pediatric patients (1–3). In these diseases the glomeruli of the kidneys are damaged and patients then suffer from severe proteinuria. Podocytes (glomerular epithelial cells) are a critical part of the glomerular filtration barrier, which also consists of the glomerular basement membrane (GBM) and endothelial cells. They show a complex, highly developed architecture and one of their main tasks is to maintain the structure of the glomerular filter. Structurally, podocytes form major processes and smaller foot processes. The foot processes of neighboring podocytes interdigitate with each other and form the slit-diaphragm, which works as a semi-selective membrane and maintains the filtration of urine. Thus, larger plasma proteins, such as albumin, are held back in the serum. Podocytes also secrete proteins such as VEGF, which not only maintains their own phenotype but also maintains the fenestrated glomerular endothelium (4). When podocytes are damaged, their foot processes are effaced and the filter becomes leaky and patients then experience proteinuria. Proteinuria also occurs in complement-associated renal diseases (5, 6).

The complement system is an important player in the innate immune system. It consists of more than 30 proteins (7). C3 activation is a central step in the initiation and amplification of the system. All pathways result in the formation of a C3 convertase, which hydrolyses C3 to generate C3a and C3b. C3b, together with factor B form another C3 convertase (C3bBb) and together with another C3b fragment a C5 convertase (C3b2Bb), which leads to the formation of the terminal complement complex (TCC/C5b-9) (7). The C5b-9 complex can lead to destruction of invading pathogens, foreign or apoptotic cells or, in sublytic concentrations, to an activation of several cell types. Furthermore, the occurring split products (e.g., C3a and C5a) act as anaphylatoxins and recruit additional immune cells. Beyond the long-known function of complement components in the initiation of the complement cascade—especially C3—seem to play a role in differentiation, proliferation and immune response in T-cells and therefore link the innate to the adaptive immunity (8).

Serum-based and membrane-bound complement inhibitors regulate the activation of the complement system, to prevent inappropriate activation. Complement factor H (CFH) is one of the most important circulating regulators of the alternative pathway. It serves as an essential cofactor for complement factor I (CFI)-mediated C3b cleavage and has important tasks in the decay of C3 convertase (9, 10). Therefore, C3 and CFH can be

hypothesized to be important protagonists in the initiation and regulation of the complement cascade.

There is uncontrolled complement activation in a number of renal diseases, notably atypical hemolytic uremic syndrome (aHUS) (11), C3 glomerulopathy (10, 12, 13), IgA nephropathy (14), and membranous nephropathy glomerulopathy (10, 12, 13), IgA nephropathy (14), and membranous nephropathy (6). The activation of the complement system is thought to cause the system or an uncontrolled regulation. Besides these diseases, which are directly triggered by the systemic complement cascade, the complement system can also be activated locally in non-primary immune-mediated diseases, for example in Shiga toxin-associated hemolytic uremic syndrome or kidney transplantation (15). This secondary toxin-associated hemolytic uremic syndrome or kidney transplantation (15). This aggravate severe renal damage. The reason the glomerulus is particularly vulnerable to unregulated systemic or local complement activation is not fully clarified, yet.

Most complement proteins are produced in the liver and circulate in the blood. In recent years, several extrahepatic sources have been reported, including the kidney, brain, lungs, and intestines. Several studies have shown these organs can also produce small amounts of complement proteins, which could contribute to local complement activation and regulation (16–20). The kidney is one of the most important extrahepatic sources. It has been shown that kidney-derived C3 contributes up to 10% of the circulating C3 (21). The local production of complement proteins in the human kidney has already been described in healthy individuals (22) and in those with glomerular diseases (23–27). Primary renal cell culture studies confirmed that mesangial, endothelial, and epithelial cells are capable of synthesizing the activating circulating complement proteins C3, C4, and factor B (28–32). CFH mRNA was also detected in human glomeruli B (28–32). CFH mRNA was also detected in human glomeruli (33). Sheerin and co-workers demonstrated that local complement production is and that the loss of local complement synthesis in this proteinuria model prevents severe renal damage (34). Based on these findings, a recently published study demonstrates that podocytes, key players in the development of proteinuria, may themselves be one important source of complement C3 in proteinuric diseases (35). Furthermore, Zoshima and colleagues suggested a potential role of podocyte derived CFH in clearing immune complexes in a murine model of immune-complex glomerulonephritis (36). However, the production of complement proteins by podocytes remains controversial.

Podocytes do not have any direct contact to serum-based complement activation, which is in most cases the reason for complement-mediated renal damage. Nevertheless, in special circumstances the glomerular filtration barrier is leaky and allows components of the blood to pass through, thus permitting contact with podocytes. Additionally, local complement activation could contribute to podocyte damage. Furthermore, there is a growing body of evidence that podocytes may act as immune modulating cells (37, 38). It has been shown that podocytes express several toll-like receptors (TLR) (39–42), which are regularly expressed in macrophages, T- and B-cells and B7-1, which is expressed by B-cells or antigen-presenting cells. Even though the role of TLR and B7-1 in podocytes is highly debated, these data indicate that podocytes may have additional roles beyond the stabilization of the glomerular filtration barrier.

Therefore, we hypothesized that podocytes produce functionally active complement proteins. While most studies have focused either on the activating complement perspective or the expression of regulating factors, we were interested in both aspects to give a more complete overview. We investigated the expression, functionality and secretion mechanisms of the activating central complement factor C3 and the regulating CFH in human podocyte cells and in a rat model of proteinuria.

MATERIALS AND METHODS

Antibodies, Proteins, and Serum

For Western blot antibodies against human C3 (goat, Pierce, Thermo Scientific, Waltham, MA, USA, catalog number: PA1-29715), CFH (goat, Calbiochem, Merck, Darmstadt, Germany, catalog number: 341276), CD 46 (LS Bio, Seattle, WA, USA, catalog number: 6754), C5b-9 (abcam, Cambridge, UK, rabbit catalog number: ab55811), which seems to recognize a neo-epitope on C9 and was used in the assay with rat-podocytes, C5b-9 (mouse, Pierce, catalog number: MA5-28502) were used in dilution 1:500–1,000. Mitogen-activated protein kinases (MAPK) and β -actin were evaluated with rabbit antibodies from Cell Signaling (1:100–500, Cell Signaling Technology, Danvers, MA, USA, catalog numbers: 4668, 9101, 4631, 9252, 9102, 9212). For immunofluorescence antibodies against C3 (goat, Pierce, 1:100), CFH (goat, Calbiochem, 1:100), giantin (rabbit, 1:100, abcam, catalog number: ab24586), tubulin and laminin (both rabbit, 1:100, Sigma, St. Louis, MO, USA, catalog numbers: T3526, L9393) were used. Nuclei were stained with 4, 6-Diamidine-2-phenylindole dihydrochloride (DAPI) (1:10,000, Sigma, St. Louis, MO, USA). IRDye conjugated secondary antibodies for Western blot were obtained from Licor (1:5,000, Lincoln, NE, USA). Appropriate secondary antibodies for immunofluorescence were used from Thermo Fisher (1:200–400, Waltham, MA, USA): Alexa Fluor, goat anti-rabbit A488 and donkey anti-goat A594. Normal human serum (NHS) was obtained from healthy laboratory staff (10 persons, pooled and stored in aliquots at -20°C). Recombinant CFH, CFI, C3 and C3b were purchased from Merck (Darmstadt, Germany).

Animal Experiments

Animals were housed in a controlled animal facility with free access to water and standard animal chow. Experiments were performed according to national and institutional animal care and ethical guidelines and have been approved by the local ethical committees at the Medical University of Hamburg-Eppendorf and the Ministry of Health and Consumer Protection. Puromycin aminonucleoside-induced nephropathy (PAN) was induced in male Sprague–Dawley rats by intraperitoneal injection of puromycin aminonucleoside in phosphate buffered saline (PBS) (Sigma) as described (43). Urine was collected in metabolic cages and proteinuria was measured in semi-quantitative conventional dip sticks (Siemens, Berlin, Germany). Rats were euthanized on day 28 and glomeruli were isolated: kidneys were encapsulated, cortex was minced and sieved through a $90\ \mu\text{m}$ filter (BD, Franklin Lakes, NJ, USA). The filtrate was sieved through a $53\ \mu\text{m}$ filter (BD) and glomeruli were removed from this filter with cold PBS. Glomeruli were centrifuged at 1,000 rpm and washed three times with 0.5% BSA in PBS (Sigma). Glomeruli were frozen and kept at -80°C until mRNA isolation. Small parts of the kidney cortex were fixed in formaldehyde and paraffin embedded for light microscopy. Sections 1–2 μm were stained with periodic acid-Schiff reagent (Sigma) according to the manufacturer's instructions.

Cell Culture

Primary human podocytes were transfected with temperature sensitive SV40 and cultured as previously described (44). These cells were used for all experiments with human podocytes. The phenotype-differentiation of the cells was routinely controlled in the microscope, and differentiation markers were analyzed repeatedly as cited in the original manuscript (44). Cells were used below passage 25. RPMI-media with glutamine was supplemented with 10% fetal bovine serum (FBS) (both Gibco, Life Technologies, Carlsbad, CA, USA) 100 U/ml penicillin, 0.1 mg/ml streptomycin (Gibco), and insulin, transferrin, and sodium selenite mixture (ITS) (Sigma). Conditionally immortalized human glomerular endothelial cells (CiGenC) were produced from primary glomerular endothelial cells in a similar way (45). Cells were used till passage 30, and provided with the microvascular endothelial cell growth media EGM2TM-MV (Lonza, Basel, Switzerland). Podocytes from an aHUS patient with a known Arg1182Ser (G3546T) CFH mutation were isolated from a nephrectomized kidney with informed consent. This mutation affects the ability of CFH to bind to C3b and heparin (46). Cells were isolated as described in (44) and transfected with SV40.

To test protein secretion, differentiated podocytes were washed with PBS, incubated in serum-free RPMI-media (SFM) for 12–36 h and supernatant samples were taken. Human interferon γ (IFN γ) (R&D Systems, Wiesbaden, Germany) was used with final concentrations from 0.1 to 100 ng/ml. Brefeldin A (BFA) (Sigma), diluted in ethanol, was used at 5 ng/ml and applied for 0–8 h.

Primary rat podocytes were cultivated from rats on embryonic day 18. Kidneys were removed and washed twice with Hank's Balanced Salt Solution (HBSS) (Gibco). Papain replaced HBSS

for enzymatic tissue digestion. After 30 min of shaking at 37°C, digestion was stopped by three RPMI washes. Kidneys were homogenized and passed through a 40 µm cell strainer. Cells were seeded at 20,000 cells/ml in RPMI 1640 medium, containing 10% FBS, penicillin-streptomycin 100 U/ml, sodium pyruvate and HEPES (all Gibco) at 37°C. Differentiation occurred after 2 weeks.

Quantitative PCR (qPCR) and Conventional PCR

RNA was then isolated using an innuPrep RNA Mini Kit (Analytik Jena, Biometra, Jena, Germany). RNA concentration was measured with a NanoDrop ND-1000 spectrophotometer (Thermo Scientific) (47). Two micrograms of RNA was converted to cDNA using a cDNA synthesis kit (Life Technologies, Carlsbad, CA, USA). qPCR was performed using SYBR green (Sigma), according to manufacturer's instructions on the StepOnePlus real-time PCR system (Applied Biosystems, Life Technologies) (95°C 20 s, 60°C 20 s, 72°C 20 s). Sequences were obtained from the Harvard Primer bank (<http://pga.mgh.harvard.edu/primerbank/>) and purchased from Life Technologies: for human CFH forward: CCTGATCGCAAGAAAGACCAG and reverse: ACTGAACGGAATTAGGTC CAAAT and for C3 forward: CCAAGAGCTCAAGGTGAGGG and reverse: GGAACGGACAACGAGGACTT. The primer sequences for rat CFH forward TTAGGCTGGCAGTTGGATCT, CFH reverse TCCACCCATCTGCATCACAT C3 forward TCA GGGTCCCAGCTACTAGT, and C3 reverse AGTCTCTTCACT CTCCAGCC. Relative expression was determined as compared to 18S controls.

In conventional (reverse transcriptase) PCR the following primers from Life Technologies were used for C3: GCT GCTCCTGCTACTAACCCA (forward), AAAGGCAGTT CCCTCCACTTT (reverse), and for CFH: ACATTACTTC ATTCCCGTTGTC (forward), ATACTCCAGTTTCCCATC CCAA (reverse). PCR was accomplished using dNTPs (Life Technologies), Dream Taq Polymerase-set (Fermentas, Thermo Scientific), and Biometra Thermocycler (Labpreco, Horsham, PA, USA) (94°C 2 min; 94°C 1 min, 60°C 1 min, 72°C 1 min x 34; 72°C 2 min). PCR-product was loaded onto a 1.5% agarose gel with a 100 bp DNA Ladder (Biomol, Hamburg, Germany). Additional primer sequences are shown in **Supplementary Table 1**.

Immunofluorescence

Cells were grown on coverslips and fixed in 4% paraformaldehyde (PFA) (Sigma), as previously described (48). Cells were blocked with 3% bovine serum albumin, and permeabilized with 0.05% Triton-X 100 (both Sigma), when needed. Images were taken using the Leica confocal imaging spectrophotometer system (TCS-SP2) or Leica TCS SP5 (Leica, Wetzlar, Germany). Imaging was done with HCX PL FLUOTAR L 40x 0.6 objective lens with an optical zoom of 40, or with 63x HCX PL APO Lbd. Bl. Oil. Microscopes were used at the Wolfson Imaging Facility, University of Bristol or at the University Microscope Imaging Facility (UMIF), University Medical Center Hamburg-Eppendorf.

Western Blotting

Lysates were made with RIPA lysis buffer (Sigma), gel electrophoresed (4–12% Gels, Invitrogen, Carlsbad, CA, USA). Samples were boiled with 4×NuPAGE LDS Sample Buffer (Invitrogen) and 0.1 M dithiothreitol and blotted onto PVDF (Merck Millipore) membranes as previously described (47, 48). Membranes were blocked with Odyssey® Blocking Buffer (Licor), 60 min at room temperature prior to overnight primary antibody incubation at 4°C. Seebblue protein standard (Invitrogen) was used as marker. Blots were washed and incubated with appropriate secondary antibody before being imaged using an Odyssey® Clx Blot Scanner for IRDye-antibodies. Densitometry was performed using Quantity One software (v4.6.5, BioRad Laboratories, Hercules, CA, USA).

Cofactor Assay

Cofactor activity of secreted CFH was tested as previously described (49, 50). In short, together with recombinant C3b (final concentration 6 µg/ml) and recombinant CFI (final concentration 40 µg/ml) podocyte supernatant (48 h in SFM) was incubated at 37°C. Samples were taken from the reaction after 0, 5, and 20 min. The addition of reduced SDS sample buffer (Invitrogen) and boiling the samples for 10 min stopped the reaction. The samples were then separated by SDS page, as described for Western blotting. C3 split products were detected with anti C3 antibody. Recombinant CFH (final concentration 33 µg/ml) was used in the assay for positive control.

Complement Challenge Assays

Human podocytes were washed in PBS prior to treatment with 30% rabbit serum in gelatin veronal buffer (GVB) (Sigma) to set up antigen antibody complexes on the cell surface. Complement was then activated by adding 20% CFH-deficient human serum (Complement Technology, Tyler, TX, USA) in GVB for 30 min (51). Complement activation was then detected by Western blot against C5b-9.

Rat podocytes were serum starved for 24 h and incubated with anti-Fx1A-IgG (0.5 mg/ml), produced as previously described (52). Briefly, sheep were immunized with isolated brush border proteins, and serum was taken. IgG was isolated with Nab™ Spin Kit for antibody purification (Thermo Scientific). After incubation for 20 min at room temperature, 2.5% NHS, or 2.5% C3-depleted human serum (Calbiochem, Merck Millipore), was added for 40 min at 37°C. Cells were harvested for Western blotting.

C3 Convertase Assay

The C3 convertase (MicroVue, Quidel, San Diego, CA, USA) assay was used following the manufacturer's instructions. C3a was measured at 450 nm using ultra-microplate reader EL808 (BioTek) and KC Junior software (v.1.41.6). A ratio of sample and untreated control was calculated. Supernatants were taken from plates with defined cell number and measured within 2 h.

Factor H Re-synthesis Assay

Human podocytes were grown for immunofluorescence and treated with trypsin (Gibco) 10 μ g/ml for 15 min at 37°C (53). Cells were then incubated with SFM for 24 h.

Fluorescence *in situ* Hybridization

Kidneys from wildtype untreated C57BL/6 mice were dissected. Kidneys were fixed in RNase free 4% PFA overnight at 4°C and cryopreserved in 30% sucrose, cryo-sectioned (14 μ m) and stored frozen at -20°C until use. The Affymetrix Quantigene View RNA (Affymetrix, Santa Clara, CA, USA) *in situ* hybridization system was used as per manufacturer's instructions. Sections were thawed and dried at 60°C prior to Protease Q (20 min, 40°C) treatment. Probes for CFH (accession No: NM_009888, Catalog No VB1-16095) and C3 (accession No: NM_009778, Catalog No VB1-13781), purchased from Affymetrix, were applied at 40°C for 4 h. A no probe control was run alongside each experiment. The probe was labeled using fast red dye (Affymetrix). After washing, slides were blocked in DAKO blocking reagent (Dako, Hamburg, Germany). Rabbit anti-laminin antibody was diluted in antibody diluting reagent (Dako) and incubated overnight at 4°C. Secondary antibody (1:200) diluted in antibody diluting reagent, was added after washing, and incubated for 3 h. DAPI nuclear counter stain was applied prior to mounting using Fluoromount and then imaged using Leica SP5.

Statistical Analysis

Statistical analyses and graphs were carried out using PRISM (Version 5, GraphPad Software). Results were considered significant when $p < 0.05$. Images were analyzed with ImageJ.

RESULTS

Human Podocytes Express and Secrete Complement Factors C3 and CFH *in vitro*

To evaluate the potential of podocytes to build complement components we analyzed conditionally immortalized human podocytes. Conventional reverse transcriptase PCR identified mRNA for the activating key component C3 (PCR product 783 base-pairs [bp]) and also for CFH (320 bp) (**Figure 1A**). RNA was also identified for the early activating complement proteins C1q, C1r, C1s, C2, C4, C5; the alternative pathway activators factor B, factor D, properdin and the regulators CD55, CD59, and CD46 (MCP) (**Supplementary Figure 1**). As CFH is the most important soluble inhibitor of the alternative pathway, and C3 is a key component in early complement activation, our subsequent work focused specifically on these two proteins. After 24 h of incubation in SFM, C3 and CFH were detected with Western blots of the whole cell lysates and in the cell culture-supernatant. Podocytes showed the expression of two products of the C3 and the CFH-gene, which probably corresponds to the α -chain of C3 and factor H-like protein (FHL) (**Figure 1B**). Complement proteins for C2, C5, and the regulatory components CFH, CD46, CD55, and CD59 were also detected (data not shown). The expression of C3 and CFH could also be determined in immunofluorescence (**Figures 1C–E**). Both proteins were detected on the surface of non-permeabilized podocytes.

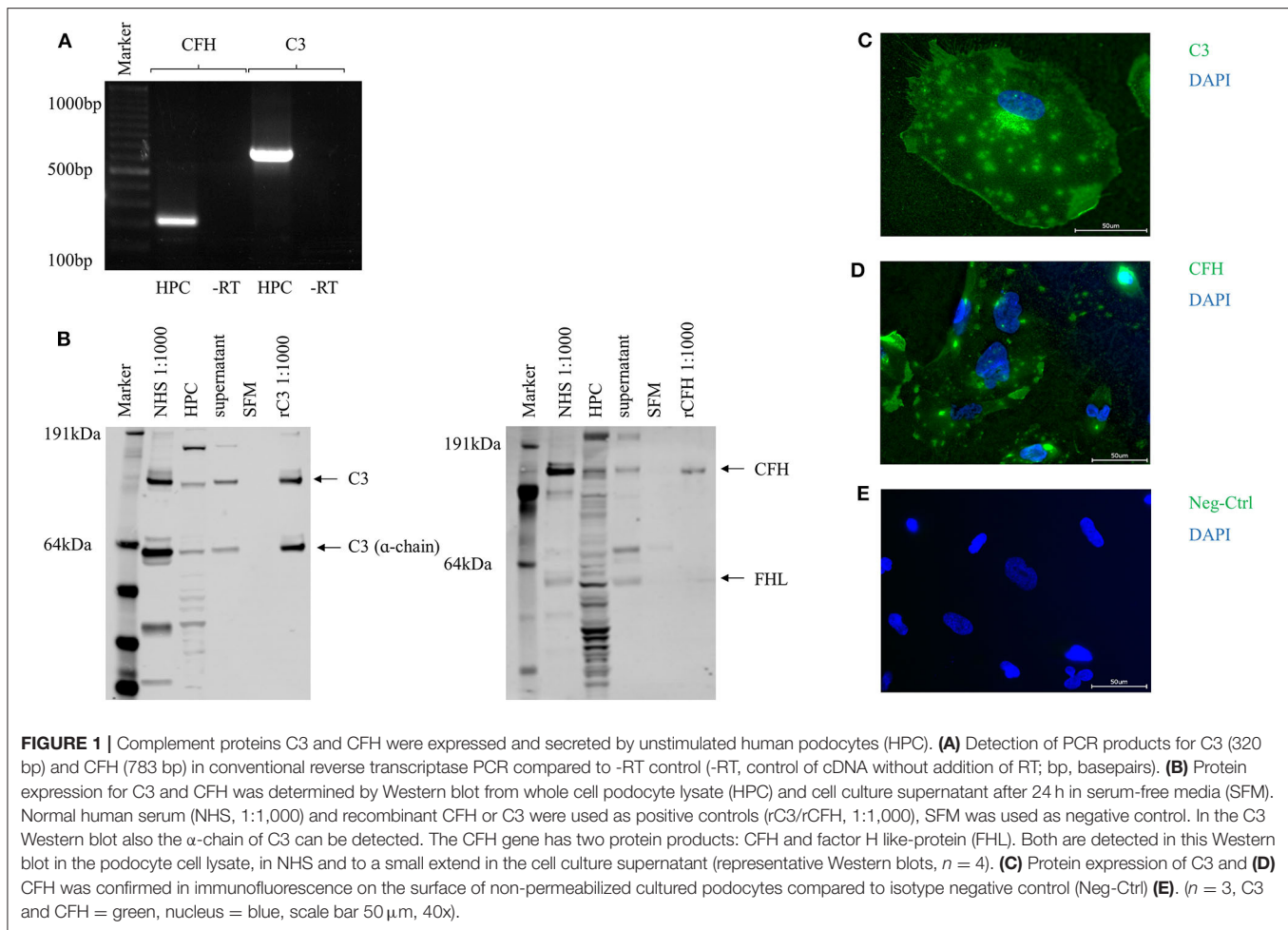
Production and Secretion of Podocyte Complement Components Is an Active Process

Secreted CFH circulates throughout the body and can bind to most cells by binding to the cellular glycocalyx. This regulates uncontrolled complement activation directly on the cell surface. CFH glycocalyx binding sites can be degraded temporarily by treatment with low dose trypsin (54). To see whether the podocytes are capable of replacing removed CFH from the surface we treated differentiated podocytes with low dose trypsin to remove surface-bound CFH. Cells were then allowed to recover in SFM. CFH was detected again on the surface of the podocytes 24 h later, showing that these cells can produce and replace CFH (**Figure 2A**).

IFN γ has previously been shown to increase cellular synthesis of complement proteins in a variety of cell lines (55–57). Therefore, human podocytes were treated with IFN γ at different time points and concentrations. Stimulation with IFN γ significantly enhanced human podocyte mRNA expression of C3 and CFH (**Figure 2B**). It also increased the expression of both proteins in whole cell lysates (data not shown). Furthermore, secreted C3 and CFH were increased after stimulation with IFN γ in a time- and dose-dependent manner (**Figures 2C–E**). This suggests that podocytes are capable of producing complement proteins as part of a pro-inflammatory response.

Expression of Complement Factor C3 and CFH Varies in Cultured Human Podocytes and Glomerular Endothelial Cells

Podocytes are always affected in proteinuric glomerulopathies. Nevertheless, within the glomerulus there are other cell types, which can contribute to the local complement production. Glomerular endothelial cells have direct contact with serum-based complement activation and complement products, and we have previously shown that podocyte-derived VEGF regulated expression of protective complement regulators on glomerular endothelial cells (51). To determine the cell specific production of complement proteins, we compared the expression of CFH and C3 in conditionally immortalized human glomerular endothelial cells (CiGenC) (45) and podocytes (44). Cultivated podocytes produced significantly more C3 mRNA, but less CFH mRNA compared to endothelial cells (**Figures 3A,B**). At the protein level for C3 and CFH, there was a slight, but non-significant, higher expression of C3 in podocytes (**Figures 3C,D**). The expression of CFH was significantly lower in podocytes according to protein quantification (**Figures 3C,E**). Complement activation may happen on any glomerular cell. Hence, the secretion of produced complement products is important. From the results in mRNA production, a comparison of secretion of complement proteins C3 and CFH showed a significant higher secretion of C3 (**Figures 3F,G**) and a lower secretion of CFH (**Figures 3F,H**) in podocytes compared to endothelial cells. Therefore, we could show that complement production and secretion profiles may differ in different intraglomerular cell types. As podocytes are the main target in PAN and in



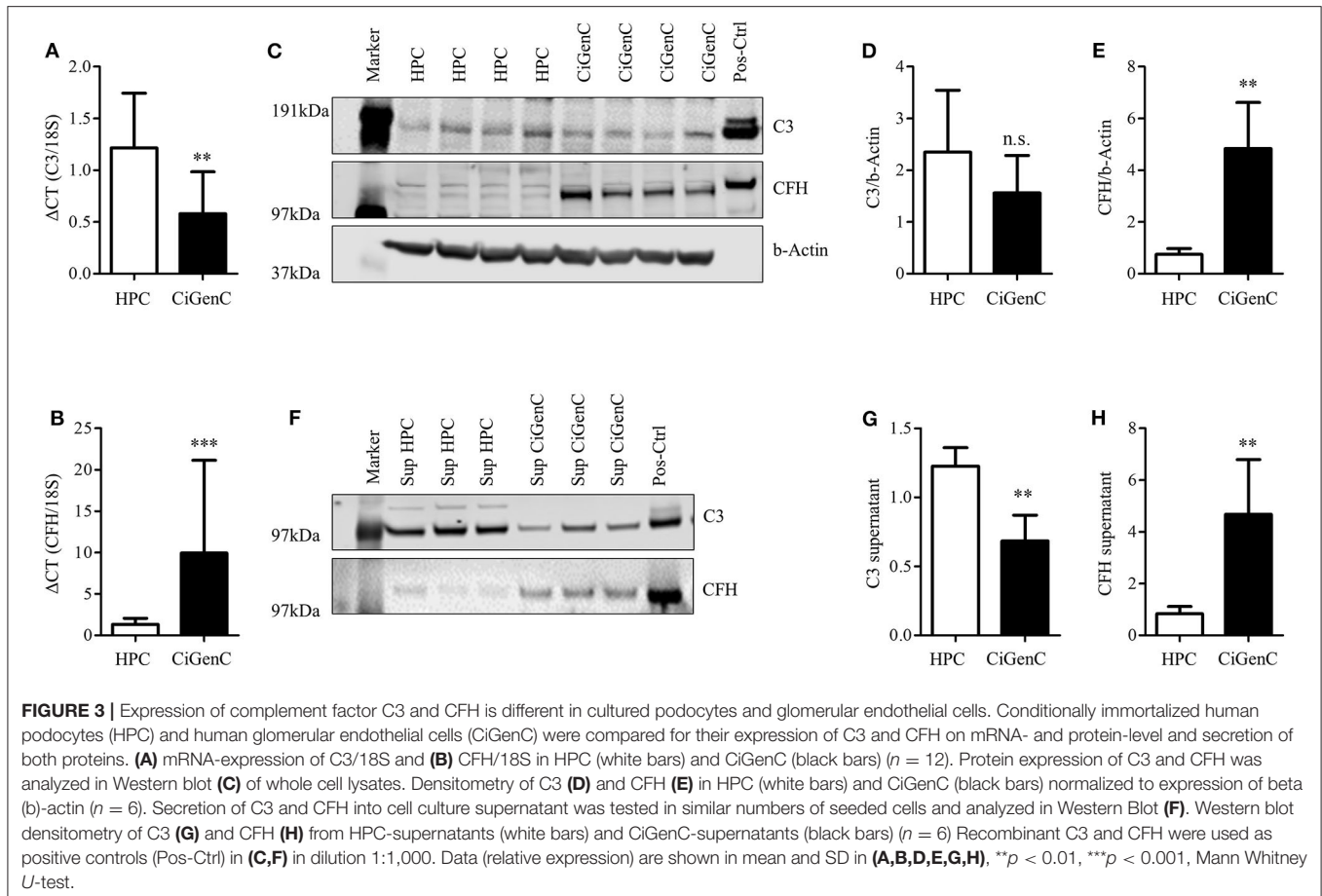
most proteinuric glomerulopathies, we further analyzed the complement expression in podocytes.

Podocyte-Derived CFH Is Efficient in Complement Control

One of the major functions of CFH is its cofactor activity in assisting CFI. With the help of CFH, CFI is able to split and inactivate C3b. The generation of C3b is a key central step in the activation of the complement system. It is required to build the C5 convertase. Therefore, the inactivation of C3b is a principal step in complement system regulation. The functional activity of secreted CFH was tested in a cofactor assay. Podocyte supernatant, which contains secreted CFH, was incubated with recombinant CFI and C3b. Together with CFI the supernatant was able to split C3b. This was evidenced by the reduction of the C3b α -chain and the detection of the cleavage products of α' 68- and α' 43-kD in Western blot (**Figure 4A**). This indicates that podocyte-derived CFH is sufficient to act as a cofactor for CFI cleaving C3b. CD46 as a membrane bound factor also is known to split C3b together with CFI. There is an ongoing discussion about shed CD46, which might in our case also act as a cofactor for CFI and might contribute to the splitting of C3b. We could never

detect any CD46 in the cell culture supernatant (**Figure 4B**), even though there was a positive signal in the cell lysate. There was also no detectable induction of CD46 within the cell or in the supernatant after stimulation with IFN γ (different to CFH). CFH seems to be crucial for the cofactor activity in the supernatant. Nevertheless, the contribution of other complement regulators cannot be excluded in this setting.

Finally, complement activation on normal human podocytes and podocytes isolated from a patient with diagnosed aHUS was compared. aHUS is a rare disorder of complement regulation, which results in kidney impairment, thrombocytopenia and anemia. A mutation in regulatory complement genes is found in many patients with this disease. We used podocytes from a patient with a known Arg1182Ser (G3546T) CFH mutation. This mutation prevents CFH from binding to the cell surface and partially to C3b (46). This is why cells from this patient cannot regulate complement activation on the surface. In a complement challenge assay, there was significantly more C5b-9 on the surface of cells containing the CFH mutation, compared to not diseased podocytes. This suggests that the secreted podocyte CFH contributed to complement regulation in the normal podocyte cell line but the mutated CFH



C3. C3a formation occurs through activation and cleavage of C3, which is catalyzed by C3 convertase. This indicates that, under specific circumstances, podocytes are capable of producing C3 splitting enzymes, e.g., a C3 convertase.

C3 and CFH Are Processed in Golgi Apparatus

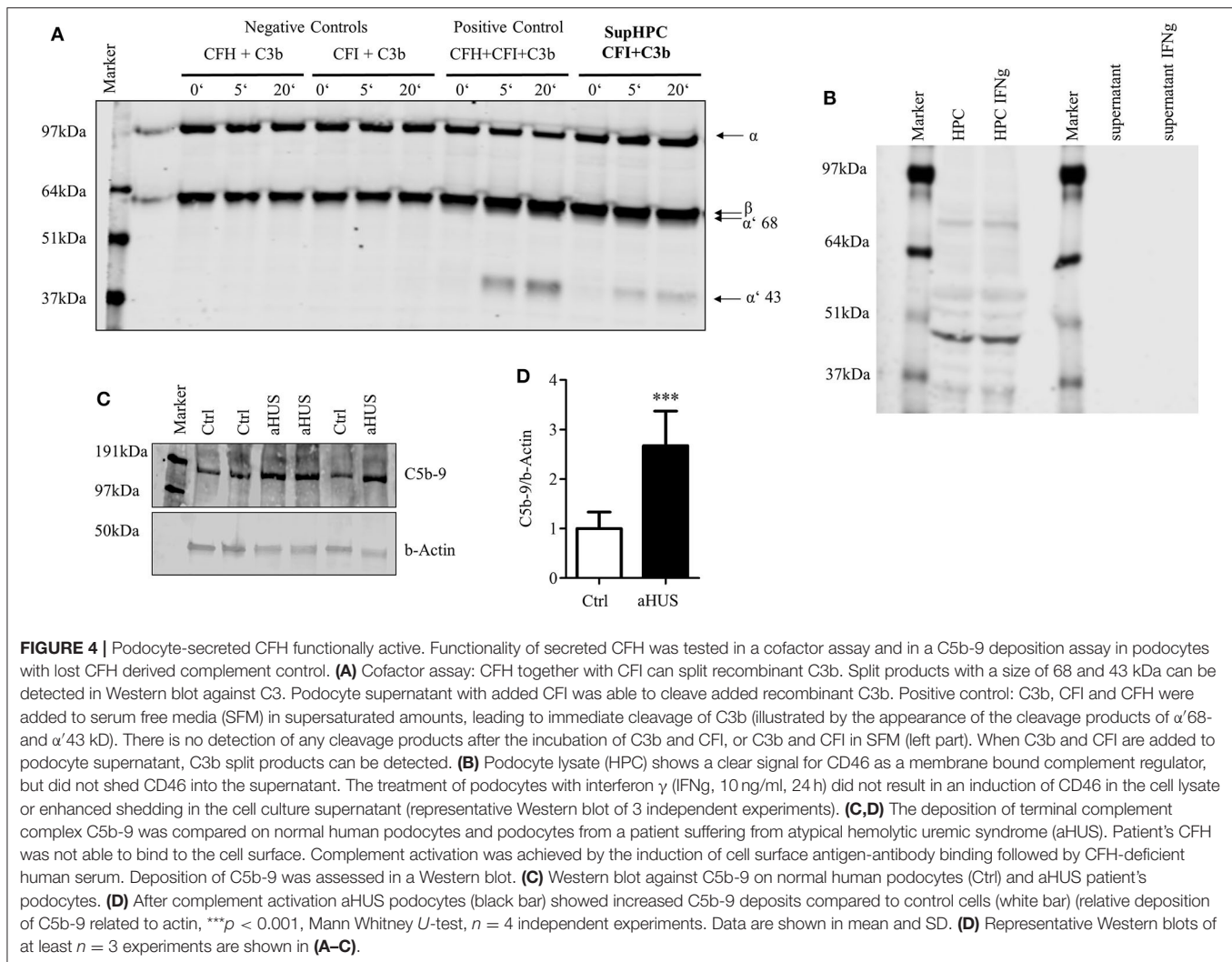
To confirm that the cultured podocyte can actively secrete complement components, we investigated the intracellular localization of C3 and CFH. Podocytes were co-stained for C3, CFH, and the Golgi apparatus marker giantin. CFH and C3 co-localized with giantin (**Figure 6A**, detailed split images can be seen in **Supplementary Figure 3**). Negative controls showed no staining (data not shown). Brefeldin A (BFA), a general inhibitor of exocytosis, leads to a retrograde transport of proteins and a disruption of the Golgi apparatus. Incubation with BFA reduced the secretion of C3 in podocytes (**Figures 6B,C**). The influence of BFA on CFH secretion could not be measured, because CFH was barely detectable in the supernatant before 12 h of incubation, and treatment with BFA for longer than 8 h resulted in apoptosis of these cells. Treatment with BFA for 4 h led to collapse of the Golgi stacks and C3 and CFH moved from the perinucleus to a more diffuse cytoplasmic distribution (**Figure 6D**). These results

indicate that complement proteins are processed in the Golgi apparatus in podocytes.

To determine the specific transportation pathways, we analyzed the co-localization of complement C3 and CFH with microtubules. Microtubules play an important role in intracellular transportation pathways and can be visualized by the staining of tubulin. We found a clear co-localization of C3 and CFH with tubulin. C3 and CFH were partly found in small round structures, which might be transportation vesicles (**Figure 6E**). These results indicate that podocyte-produced complement proteins are processed in the Golgi apparatus, packed in secretion vesicles and moved to the surface along the microtubules.

Glomerular C3 and CFH Is Upregulated in PAN

To evaluate if glomerular damage also results in the regulation of complement genes, PAN was induced in male Sprague-Dawley rats. PAN is a well-characterized and established model to mimic proteinuric diseases. Puromycin induces injury of the podocyte actin cytoskeleton (60) leading to sustained foot process effacement, podocyte loss, glomerular sclerosis, and proteinuria. As we were interested in the balance between C3, the central activating component, and CFH, the most important inhibitor of the alternative pathway, we investigated glomerular damage and



mRNA expression of these two complement proteins in isolated glomeruli. After 14 days, PAN rats developed severe proteinuria (data not shown). Staining with Periodic Schiff Acid showed massive sclerosis and glomerular damage (**Figure 7A**). In these studies, we confirmed the results of Li et al. (35), concerning the glomerular upregulation of C3 mRNA in PAN (**Figure 7B**). In addition, we detected an increased induction of CFH in comparison to H₂O-treated controls (**Figure 7C**). As podocytes are the main target in PAN, we were extremely interested in the ability of podocytes to produce these critical opposing players in the complement system.

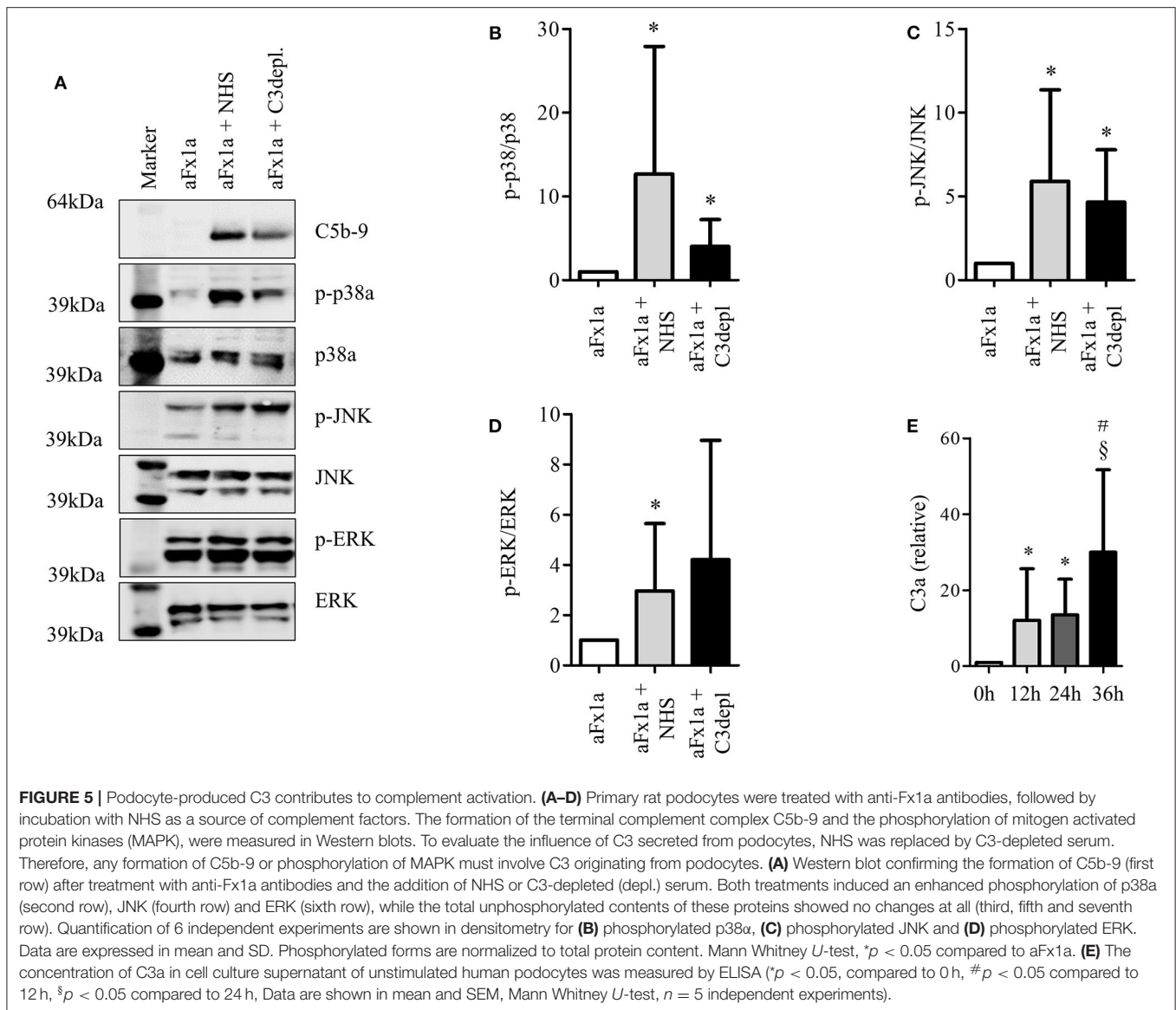
Podocytes Expressed CFH and C3 *in vivo*

Unfortunately, we were not able to show any positive staining for C3 or CFH protein in podocytes in murine or human tissue (data not shown). Therefore, to prove the origin of CFH and C3 production in glomeruli *in vivo*, fluorescence *in situ* hybridization (FISH) studies were performed in glomeruli of healthy C57Bl/6 mice. C3- and CFH-mRNA was identified in glomeruli of healthy C57Bl/6 mice (**Figures 7D,E**). Negative

control showed no staining of the mRNA signal (**Figure 7F**). The mRNA of C3 and CFH partially co-localized with podocyte nuclei. These findings are clear evidence that local glomerular complement production occurs within the glomeruli and in podocytes. Furthermore, there was C3 and CFH in tubular cells, indicating that podocytes are not the only source in the kidney.

DISCUSSION

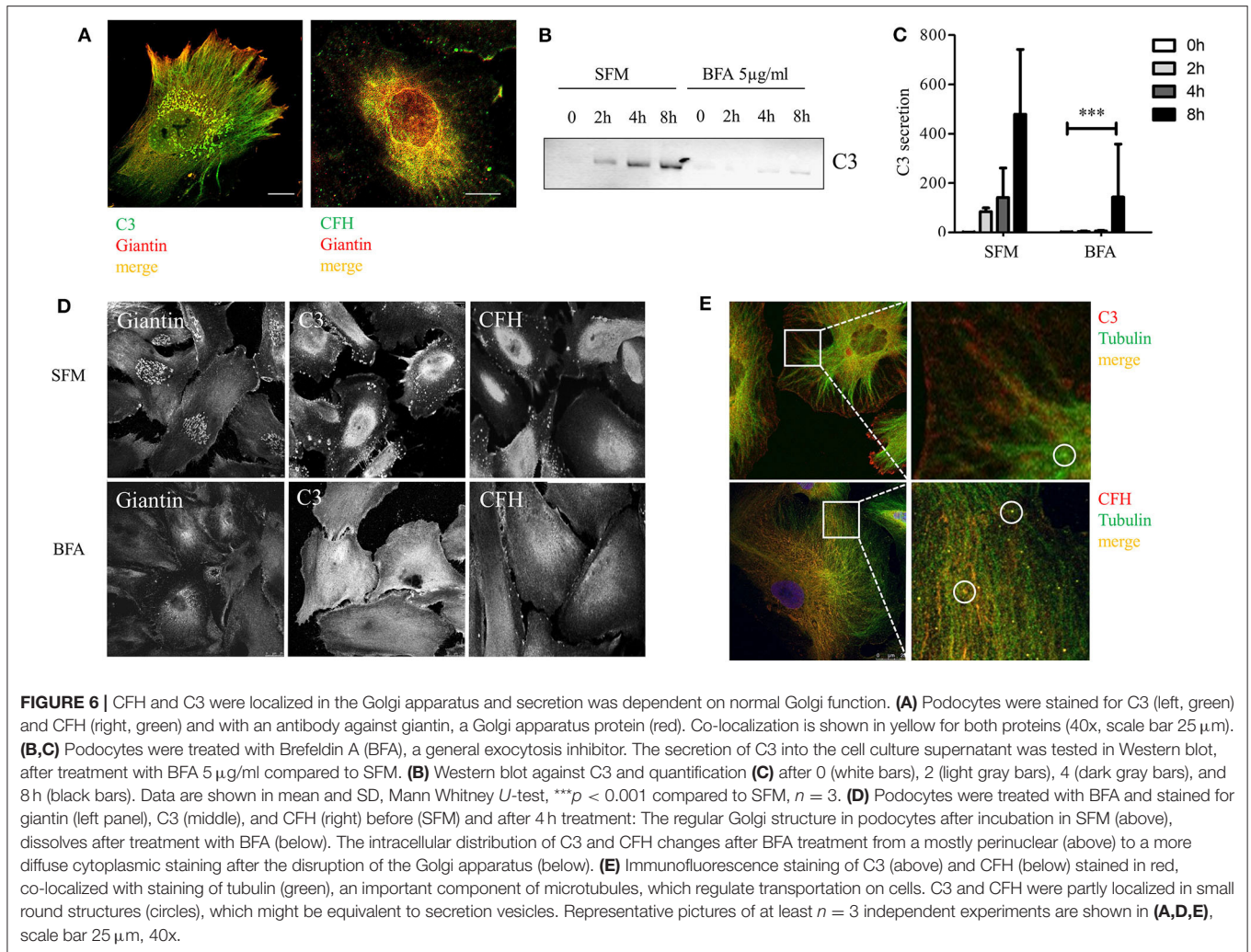
Podocytes are complex differentiated cells that are essential for the functionality of the glomerular filtration barrier and renal function. Damaged podocytes have very limited potential for recovery and regeneration (61). Therefore, the integrity of the podocyte and the resistance against damage due to local complement activation or against invading pathogens is important. The complement system is a crucial part of the innate immune system. It helps with the elimination of invading microorganisms and the removal of apoptotic cells. Nevertheless, it has also been shown to contribute to a growing list of inflammatory conditions (62). The kidney is particularly



sensitive to complement-mediated damage. In several systemic complement disorders, for example in diseases like aHUS or systemic lupus erythematosus (SLE), the kidney is a particular target. The reason for the kidney's unique susceptibility to complement-induced damage is not yet fully elucidated, but it could be influenced by its structural organization and functional duties (63). Renal perfusion contributes to the constant contact of the glomerulus with several circulating toxins or immune cells.

With the podocyte at the center of all forms of proteinuria, these cells were analyzed to determine their potential to produce complement proteins. Our studies showed that podocytes not only express and store complement proteins, they can also secrete functionally active C3 and CFH, which is enhanced by IFN γ , an immune-stimulating protein. Therefore, it is probable that podocytes influence local complement activation in situations

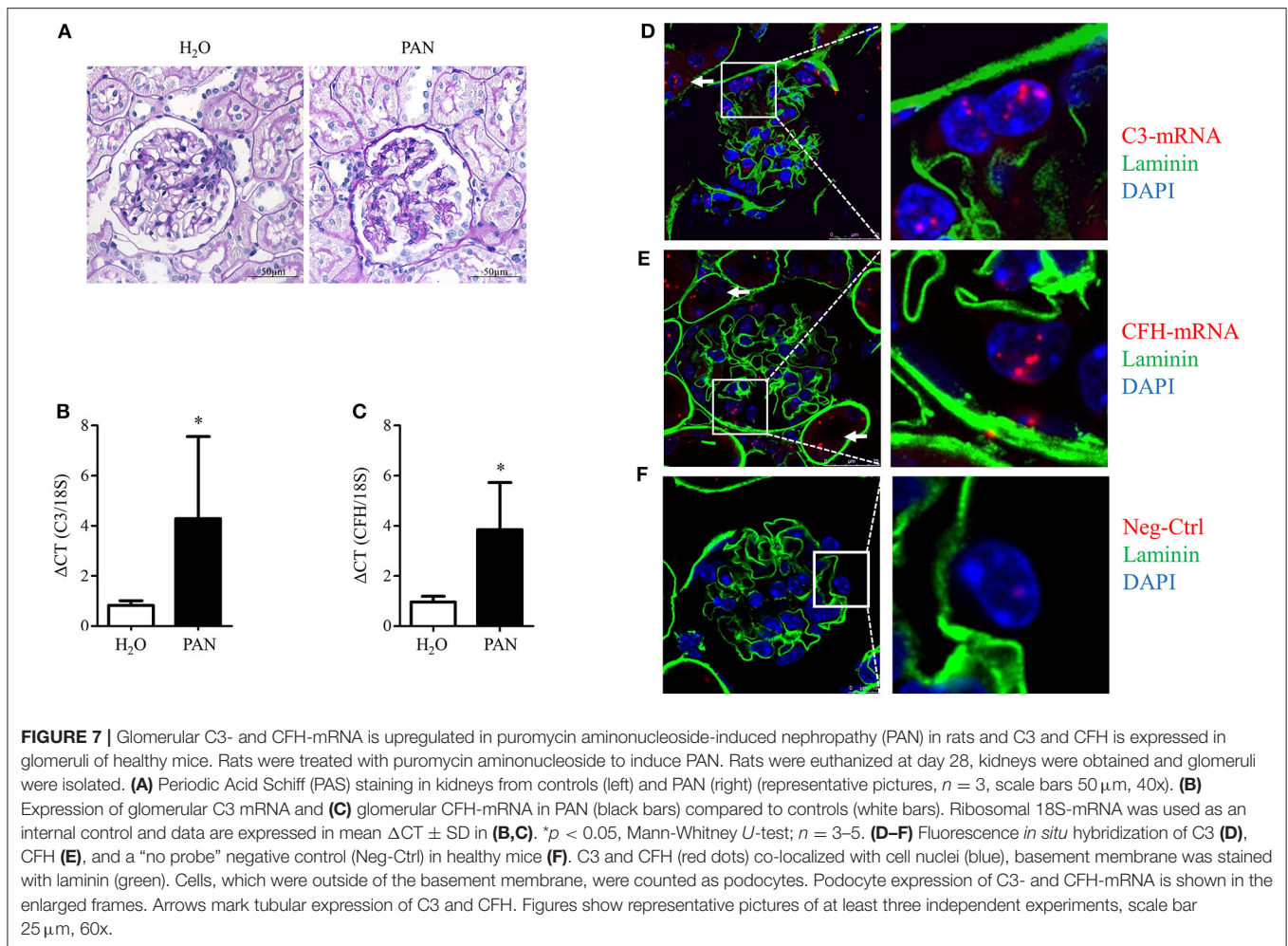
of immune-related challenge, for example in infection and inflammation. The need for resistance of kidney cells against invading microorganisms might be a good explanation for kidney cells' potential to express complement activating components. The kidney seems to have an activated complement profile under unstimulated conditions. These complement proteins might serve to remove immune complexes and provide protection against microorganisms of the urinary tract (64). Podocytes, sitting on the outside of the glomerular filtration barrier, are especially prone to contact with invasive pathogenic substances. Therefore, the expression of C3, as the key player in complement activation and particularly the secretion of C3a as a potent anaphylatoxin, could be crucially useful. On the other hand, the podocyte-produced C3 could also contribute to activation of the system due to a systemic complement disorder or secondary complement activation in inflammatory circumstances. This



might explain why podocytes are damaged in complement mediated diseases like C3 glomerulonephritis or aHUS.

It has been described previously how several complement components were locally produced by parenchymal cells of the glomeruli and in tubules. Li et al. (35) have already shown a glomerular C3 upregulation in an experimental proteinuric model. We confirmed their findings that podocytes are one source of C3 mRNA using FISH and indirectly via glomerular upregulation in PAN treated rats. We also showed that podocytes in culture are able to actively secrete C3, which contributed to external experimental complement activation. The detection of C3a indicated that podocyte-secreted C3 is functionally active and can induce complement activation. This might occur during local inflammatory conditions. With the generation of C3 and C3a, podocytes could activate complement upstream of C5b-9. Additionally, the secreted complement proteins could be washed into the urinary space. Several complement proteins have already been detected in urine (14, 65–72). The occurring protein leak in situations of proteinuria was previously thought to be responsible for this detection. Others have suspected production of C3 by tubular cells (73). Our work also detection. Others have

suspected production of C3 by tubular cells (73). Our work As has already been shown, kidney cells, and podocytes in particular, are affected directly by complement activation (74, 75). Therefore, defending mechanisms are essential to prevent permanent damage. Podocytes, like several other cells, produce membrane-bound regulators to protect themselves from complement attack (76). Membrane-bound regulators, such as CD46, CD55, and CD59, protect the respective regulator-expressing cell from the assembling of complement activation products on the cell surface. Additionally, there are circulating complement regulators like CFH, which predominantly regulate complement activation in the alternative pathway. CFH molecules are mainly produced in the liver. However, we also detected the expression of CFH *in vitro* and *in vivo* in podocytes. The podocyte-derived CFH acted as a cofactor for CFI to cleave C3b in a cofactor assay, showing that it was functionally active. A very important other mechanism of CFH in complement control is the decay of the convertase, which was not tested in detail in this study. Nevertheless, in a setting with functionally defective CFH, podocytes were not able to prevent the adhesion of C5b-9 during the complement attack.



It could be argued that aHUS is exclusively an endothelial cell disorder, but proteinuria can be a presenting clinical feature of this disease, confirming that podocytes are affected early in the disease (77). The detection of impaired podocyte CFH in one aHUS patient (78) supports the importance of podocyte-derived CFH in protecting the glomerulum from complement mediated injury. Further, other researchers have recently suggested that podocyte dysfunction can occur in acquired and genetic forms of complement-mediated aHUS, suggesting that podocytes do indeed play a role in this disease (79). Loss of local complement regulation may be important for those patients who require a second hit before manifestation of the disease. The local production of CFH also seems to be essential in SLE, where it is responsible for processing the immune complexes. The deficiency of CFH results in an accelerated development of lupus nephritis in an animal model for SLE (80). Furthermore, an important role of podocyte derived CFH development of lupus nephritis in an animal model for SLE (80). Furthermore, another indication that the podocyte is a local source for CFH is the detection of CFH in urine from healthy individuals (81). CFH, a 150 kDa serum protein, would not normally undergo

ultrafiltration. In this context, CFH may be produced on demand locally and play a regulatory role in urine even under normal circumstances. There is evidence for the local importance of CFH in other diseases as well. In membranous nephropathy, the ratio of urinary C5b-9 to CFH was associated with active glomerular disease highlighting the protective role of this protein in disease (81). Elevated urinary CFH levels have also been detected in other glomerulopathies (70, 82). In our opinion podocytes, in addition to other glomerular cells, are one source of CFH. Another source of this urinary CFH could be the tubular cells, which showed a positive CFH and C3 mRNA signal in FISH. Nevertheless, in glomerular diseases tubular cells are usually damaged later, while proteinuria indicates that glomerular cells are affected early on. This local glomerular interplay could be important concerning CFH production, since it has a complement regulatory role on basement membranes that do not express other complement regulators (83).

Endothelial cells in culture can produce and secrete complement factors such as C3 and CFH and can contribute to local glomerular complement activation. However, there was a variation in the level of expression in both factors between

podocytes and glomerular endothelial cells, suggesting distinct levels of regulation within different sections of the glomerulus. Even though this different expression level was described only *in vitro*, it indicates that glomerular cells might have different individual tasks. We could hypothesize that they have to work together to organize appropriate local complement activation. These glomerular cell types have to ensure that there is a distinct balance between activation and regulation of the cascade to prevent uncontrolled kidney damage. Our previous work has shown that there is a synergistic role between podocytes and endothelial cells in glomerular complement regulation (51).

The different expression levels of complement factors lead to another additional consideration: it has already been shown that there is an important crosstalk between podocytes and endothelial cells, which occurs particularly with regards to VEGF (4). On one hand, the production of different factors in different cells could contribute to complement activation on different levels. On the other hand, the secretion of complement factors might have additional novel roles for other surrounding cells. There is a growing body of evidence that C3 and C3a have roles extending beyond those of complement system activation. Rather than being just a pro-inflammatory effector system, complement is emerging as a central player in cell differentiation, proliferation, repair and induction in T-cells (84). The complement system is not only thought to repair and induction in T-cells (84). The complement system is not only thought intracellular complement—the so called *complosome*—seems to have an impact on normal cell physiology (85). Nevertheless, the meaning of local on normal cell physiology (85). Nevertheless, the meaning of local endothelial cells has not yet been uncovered. Even though, the podocyte might not be the only or main source of kidney derived complement proteins, it is not known, whether even low amounts of locally produced activating or regulating factors might have a relatively higher impact on local inflammation. The hypothesis, why we were not able to show any positive protein signal for C3 or CFH, might be due to the low expression of both proteins in podocytes compared to other cell-types or the concentration measured systemically in the blood. Another explanation might be that both proteins are not stored in huge amounts within the cells and might be secreted rapidly to regulate the local complement activation.

However, one of the most important conclusions resulting from our findings is that the podocyte seems to have more tasks than just mechanistic stabilization of the glomerular filter. There is a growing body of evidence that podocytes may act as immune modulating cells (38). Even though they are affected in this disease themselves, podocytes seem to play an important role in the regulation of complement factors that may prevent nephritis in SLE; they also influence innate and adaptive immunity. It has been shown that isolated glomeruli express TLRs, for example TLR4 (40), which is associated with the development of lupus nephritis. The activation of TLR4 (e.g., with lipopolysaccharide) induces the expression of various cytokines (e.g., CCL2, CCL7, CXCL1, CXCL5, CCL3, CCL5, and more) (37, 38). Therefore, the podocyte seems to be able to create an inflammatory milieu in certain circumstances.

Additionally, podocytes express the receptor for advanced glycation end products (RAGE), which is important in several immune reactions and which is upregulated in ADR-induced nephropathy (86). They are also capable of expressing the NLRP3 inflammasome, which is activated in the development of proteinuria in lupus nephritis (87).

With our findings we demonstrated that podocytes have a role beyond a purely stabilization function in the glomerulus. The podocyte might act as an immune modulating, and especially local complement modulating, cell. In states of proteinuria, primary or secondary complement activation can occur within the glomeruli and podocytes seem to be able to contribute to this complement activation by production of activating or inhibitory complement components. Even though there is increasing knowledge about the involvement of complement in kidney diseases, the new role of the podocyte as the local source of complement activators and regulators requires additional study in all causes of glomerular complement activation. Furthermore, our results underline a possible role of the podocyte as an immune modulating cell.

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author.

ETHICS STATEMENT

The animal study was reviewed and approved by Behörde für Gesundheit und Verbraucherschutz, Fachbereich Veterinärwesen.

AUTHOR CONTRIBUTIONS

AM, LK, CL, JO, and MS contributed conception and design of the study. AM, LK, JA, and HH performed the experiments. RH, GW, CM-S, RC, and LF designed parts of the experiments and wrote sections of the manuscript. All authors contributed to the manuscript revision, creation of the final manuscript, read, and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fimmu.2020.01833/full#supplementary-material>

Supplementary Figure 1 | Unstimulated human podocytes (HPC) express mRNA for various complement factors in conventional PCR (-RT, control of cDNA without addition of RT; bp, base pairs).

Supplementary Figure 2 | Schematic presentation of the assumed underlying mechanisms in the complement challenge assay. **(A)** The binding of anti-podocyte antibodies leads to the activation of the complement cascade via the classical pathway (after the addition of normal human serum as a complete source of complement factors). The complement activation and building of C5b-9 induces sub-lytic damage in this *in vitro* model, which can be shown with the detection of phosphorylation of mitogen activated protein kinases (MAPK). **(B)** The use of C3-depleted serum did not prevent the phosphorylation of MAPK, which proves that podocytes derived C3 replaces external C3.

Supplementary Figure 3 | Detailed split images: Podocytes were stained for C3 and CFH (green) and with an antibody against giantin, a Golgi apparatus protein (red). Co-localization is shown in yellow for both proteins (40x, scale bar 25 μ m).

Supplementary Table 1 | Primer sequences conventional PCR, primers were obtained from Sigma Aldrich.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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