

# Complement C5 Mediates Experimental Tubulointerstitial Fibrosis

Peter Boor,<sup>\*†</sup> Andrzej Konieczny,<sup>\*</sup> Luigi Villa,<sup>\*</sup> Anna-Lisa Schult,<sup>\*</sup> Eva Bücher,<sup>\*</sup> Song Rong,<sup>\*</sup> Uta Kunter,<sup>\*</sup> Claudia R.C. van Roeyen,<sup>\*</sup> Thomas Polakowski,<sup>‡</sup> Heiko Hawlisch,<sup>‡</sup> Sonja Hillebrandt,<sup>§</sup> Frank Lammert,<sup>§</sup> Frank Eitner,<sup>\*</sup> Jürgen Floege,<sup>\*</sup> and Tammo Ostendorf<sup>\*</sup>

<sup>\*</sup>Division of Nephrology, Rheinische-Westfälische Technische Hochschule, University of Aachen, Aachen, Germany;

<sup>†</sup>Department of Clinical and Experimental Pharmacotherapy, Research Base of Slovak Medical University, Bratislava,

Slovakia; <sup>‡</sup>Jerini AG, Berlin, Germany; and <sup>§</sup>Department of Internal Medicine I, University Hospital Bonn, Bonn, Germany

Renal fibrosis is the final common pathway of most progressive renal diseases. C5 was recently identified as a risk factor for liver fibrosis. This study investigated the role of C5 in the development of renal tubulointerstitial fibrosis by (1) induction of renal fibrosis in wild-type and C5<sup>-/-</sup> mice by unilateral ureteral ligation (UUO) and (2) investigation of the effects of a C5a receptor antagonist (C5aRA) in UUO. In C5<sup>-/-</sup> mice, when compared with wild-type controls, markers of renal fibrosis (Sirius Red, type I collagen, fibronectin,  $\alpha$ -smooth muscle actin, vimentin, and infiltrating macrophages) were significantly reduced on day 5 of UUO. On day 10, fibronectin mRNA and protein expression were still reduced in the C5<sup>-/-</sup> mice. Cortical mRNA of all PDGF isoforms and of TGF- $\beta_1$  (*i.e.*, central mediators of renal disease) were significantly reduced in C5<sup>-/-</sup> mice when compared with controls. Renal tubular cell expression of the C5aR was sparse in normal cortex but markedly upregulated after UUO. Treatment of wild-type UUO mice with C5aRA also led to a significant reduction of cortical Sirius Red staining, fibronectin protein expression, and PDGF-B mRNA expression on day 5. Neither genetic C5 deficiency nor C5aRA treatment caused any histologic changes in the nonobstructed kidneys. In cultured murine cortical tubular cells, C5a stimulated production of TGF- $\beta_1$ , and this was inhibited by C5aRA. Using a combined genetic and pharmacologic approach, C5, in particular C5a, is identified as a novel profibrotic factor in renal disease and as a potential new therapeutic target.

*J Am Soc Nephrol* 18: 1508–1515, 2007. doi: 10.1681/ASN.2006121343

Interstitial renal fibrosis is the common end point of progressive kidney diseases. The identification of mediators that are involved in this process could lead to novel therapeutic approaches.

It is well established that activation of downstream components of the complement cascade such as C5, C6, or the membrane attack complex C5b-9 is involved in many renal diseases (reviewed in reference [1]). For example, elevated urinary C5b-9 concentrations are found in proteinuric patients (2), urinary C5b-9 was shown to reflect disease activity in an experimental model of membranous glomerulonephritis better than proteinuria (3), C5b-9 depletion in experimental nephropathy reduces proteinuria (4), and C5b-9 is formed at sites of tubulointerstitial injury (5,6).

In proteinuric animal models such as mesangioproliferative glomerulonephritis, Adriamycin nephropathy, five-sixths nephrectomy, and puromycin aminonucleoside nephrosis, comple-

ment depletion or the lack of C6 ameliorated tubulointerstitial injury (7–11). In experimental immune-complex glomerulonephritis, lack of the C5a receptor (C5aR) reduced infiltrating interstitial cells and tubulointerstitial damage but had no influence on glomerular injury, thereby pointing to a role of the anaphylatoxin C5a, a small peptide that is released from C5, in tubulointerstitial injury (12). Conversely, inhibition of the complement regulatory protein Crry aggravated tubular damage (13). Thus, complement proteins, derived either from the glomerular filtrate or from local production (14), seem to become activated in the tubular lumen in proteinuric glomerular disease and contribute to progressive tubulointerstitial injury and loss of function (reviewed in Sheerin and Sacks [14]).

In three models of nonproteinuric primary or secondary tubulointerstitial injury, namely unilateral ureteral obstruction (UUO), cyclosporine A nephropathy, and streptozotocin-induced diabetic nephropathy, genetic C6 deficiency did not affect the severity of the diseases (15). It was therefore concluded that in contrast to proteinuric states, C5b-9 does not have a significant impact on the progression of tubulointerstitial damage in nonproteinuric chronic renal disease (15).

Recently, we identified the gene that encoded complement factor C5 as a quantitative trait gene that modifies the course of hepatic fibrosis (16). On the basis of these studies, we hypothesized that C5 may play a similar role, independent of proteinuria,

Received December 12, 2006. Accepted February 7, 2007.

Published online ahead of print. Publication date available at [www.jasn.org](http://www.jasn.org).

P.B. and A.K. contributed equally to this study.

**Address correspondence to:** Dr. Tammo Ostendorf, Division of Nephrology and Immunology, University Hospital Aachen, Pauwelsstrasse 30, D-52074 Aachen, Germany. Phone: +49-241-8089424; Fax: +49-241-8082446; E-mail: [tostendorf@ukaachen.de](mailto:tostendorf@ukaachen.de)

in the kidney. We therefore chose a nonproteinuric model of primary tubulointerstitial damage, namely UUO, to investigate the effects of genetic C5 deficiency or pharmacologic inhibition of the C5aR on the development of tubulointerstitial damage and fibrosis.

## Materials and Methods

### C5aR Antagonist

The C5aR antagonist (C5aRA) JPE-1375 was provided by Jerini AG (Berlin, Germany) and has been characterized previously (17). In brief, JPE-1375 is a hexameric linear peptidomimetic molecule (molecular weight 955) that inhibits C5a binding to the human C5aR with an  $IC_{50}$  of 111 nM (in transfected HEK-293 cells). Functional inhibition of C5aR signaling was shown *in vitro* for the C5a-induced glucosaminidase release from human polymorphonuclear cells ( $IC_{50}$  41 nM) and for the inhibition of chemotaxis of murine macrophage-like J774A.1 cells ( $IC_{50}$  42 nM). JPE-1375 abolished the C5a-induced effect in an *in vivo* model of an immune complex-mediated peritonitis, the reverse passive Arthus reaction (17), and in experimental kidney transplantation (unpublished data, F. Gueler, 2006). Potent inhibition of human C5aR and 17-fold higher activity on murine C5aR (17) makes JPE-1375 particularly suitable for testing in murine models.

### Cell Culture Experiments

Murine cortical tubular (MCT) cells were provided by F. Strutz (University of Göttingen, Göttingen, Germany). To address the expression of C5aR (CD88) *in vitro*, MCT cells were seeded on glass coverslips in six-well plates, grown to subconfluence, and subsequently serum-starved for 24 h. Then, the glass coverslips were removed, dried, fixed in 100% acetone for 10 min, dried again, and then stained as described in Renal Morphology and Immunohistochemistry. For examination of the effect of C5a on TGF- $\beta_1$  production, MCT cells were seeded into 25-mm<sup>2</sup> flasks, grown to subconfluence, and then serum-starved for 36 h. Cells were subsequently stimulated for 24 h with recombinant human C5a (rhC5a; 25 nM) or the combination of rhC5a (25 nM) and C5aRA (2.5  $\mu$ M). Specific binding and activity of rhC5a on murine C5aR was demonstrated previously (unpublished data, H.H., 2004) (18,19). Thereafter, cell culture supernatants were collected for TGF- $\beta_1$  measurements using a commercially available kit (R&D Systems, Wiesbaden-Nordenstadt, Germany). All cell culture experiments were performed in triplicate.

### Experimental Model and Experimental Design

All animal experiments were approved by the local review boards. The animals were held in rooms with constant temperature and humidity, 12-h/12-h daylight cycle, with *ad libitum* access to drinking water and food. All animals were obtained from The Jackson Laboratory (Bar Harbor, ME).

In the first approach, 7-wk-old male C5 deficient (C5<sup>-/-</sup>) mice (strain B10.D2-Hc<sup>0</sup>/oSnJ) and age-matched wild-type controls (C57BL/10SnJ) underwent UUO. Renal tissue (for histologic evaluation and RNA isolation) from both the obstructed and contralateral kidney was obtained when the mice were killed, on day 5 (C5<sup>-/-</sup>,  $n = 7$ ; wild-type,  $n = 8$ ) and day 10 (C5<sup>-/-</sup>,  $n = 5$ ; wild-type,  $n = 7$ ) after UUO.

In the second experiment, 7-wk-old male C57BL/10SnJ mice underwent UUO with a subsequent intraperitoneal implantation of an ALZET micro-osmotic pump (model 1002; pump rate 0.25  $\mu$ l/h; DURECT Corp., Cupertino, CA). The pumps were filled with either C5aRA ( $n = 8$ ) or vehicle (0.9% NaCl in 15% vol/vol ethanol;  $n = 8$ ). Daily C5aRA dosage was  $0.63 \pm 0.05$  mg/kg body wt. A similar dosage was previ-

ously demonstrated to be effective in mice (17). Blood and kidney tissue were obtained as described on day 5 after disease induction.

### Renal Morphology and Immunohistochemistry

For the evaluation of fibrosis, renal tissue was stained with Sirius Red in 4- $\mu$ m sections of kidney biopsies, fixed in methyl Carnoy's solution, and embedded in paraffin. For evaluation of Sirius Red and the various immunohistochemical stains listed in the next paragraph, computer-based morphometry (Soft Imaging System, Münster, Germany) was performed by an observer who was unaware of the origin of the slides. The percentage of positively stained area was calculated from 20 cortical fields (0.093 mm<sup>2</sup> each) per section, representing nearly the whole cortex.

Indirect immunoperoxidase procedure was performed as described previously (20). The following primary antibodies were used: Horseradish peroxidase-coupled murine mAb (clone 1A4) to human  $\alpha$ -smooth muscle actin ( $\alpha$ -SMA; DAKO A/S, Glostrup, Denmark), rabbit polyclonal antibody to rat fibronectin (Chemicon, Temecula, CA), goat polyclonal antibody to human collagen type I (Southern Biotechnology Associates, Birmingham, AL), rat mAb to murine macrophage antigen F4/80 (MCAP497, clone A3-1; Serotec Ltd., Oxford, UK), and chicken polyclonal antibody to mouse C5aR (BMA Biomedicals AG, Augst, Switzerland). All secondary antibodies used were biotinylated and affinity-purified (Vector, Burlingame, CA): Rabbit anti-goat IgG, goat anti-rabbit IgG, mouse-adsorbed rabbit anti-rat IgG, and donkey anti-chicken IgG. Negative controls were performed as described previously (21).

### RNA Extraction and Analyses

Total RNA was extracted from renal cortex using the RNeasy Mini Kit (Qiagen, Hilden, Germany). From total RNA, cDNA was synthesized and real-time quantitative reverse transcriptase-PCR was performed as described previously (22). Sequences of primers and probes used are listed in Table 1. Messenger RNA for each sample was normalized to the housekeeping gene for glyceraldehyde-3-phosphate-dehydrogenase. Results are given as relative mRNA expression normalized to the expression in contralateral healthy kidneys.

### Statistical Analyses

All values are expressed as means  $\pm$  SD. Groups were compared using unpaired *t* test or Mann-Whitney *U* test, where appropriate (both two-tailed). For *in vitro* experiments, ANOVA with least significant difference *post hoc* test was used. Statistical significance was defined as  $P < 0.05$ .

## Results

### C5 Deficiency Reduces Interstitial Fibrosis and Macrophage Influx after UUO

On day 5 after UUO, wild-type mice were characterized by widespread renal tubulointerstitial damage and fibrosis, as evidenced by increased Sirius Red-positive area and increases in type I collagen, fibronectin,  $\alpha$ -SMA, and macrophage staining (Figure 1). In comparison with wild-type mice, the C5<sup>-/-</sup> mice exhibited reduced renal cortical scarring as assessed by Sirius Red staining, collagen type I, and fibronectin immunostaining (-53, -52, and -42%, respectively; Figure 1). On day 10 after UUO, most of these differences persisted numerically but lost statistical significance as a result of high scatter, except for fibronectin expression, which remained significantly decreased (-43%) in the C5<sup>-/-</sup> mice (Figure 1). Consistent with the latter, renal cortical mRNA levels of fibronectin were significantly

Table 1. Sequences of primers and probes used for real-time reverse transcriptase-PCR<sup>a</sup>

Gene	Forward Primer (5' to 3')	Reverse Primer (5' to 3')	Taqman Probe (5' to 3')
mGAPDH	GGCAAATTCAACGGCACAGT	AGATGGTGATGGGCTTCCC	AAGGCCGAGAATGGGAAGCTTGTGCATC
mCol IV	GGCGGTACACAGTCAGACCAT	TGGTGTGCATCACGAAGGA	TCCGCAGTGCCTAACGGTTGGT
mFibr	GATGGAATCCGGGAGCTTTT	TGCAAGGCAACCACACTGAC	CCGGCCTGAGGCCCTGCAG
mPDGF-A	ACTTCCTGATCTGGCCCCC	TGAAGGCTGGCACTTGACG	CAGTGCAGCGCTTCACCTCCACA
mPDGF-B	CCATCCGCTCCTTTG	AAGTCCAGCTCAGCCCCAT	CGCCTGCTGCACAGAGACTCCGTA
mPDGF-C	TCGATGGCAGGTGGACTTG	ATACAGGAAAGCCTTGCCCAA	CAGCCTCTACAAGCCAACATGGCAGCT
mPDGF-D	TGTGAGGTGATTGCTCTCATCTC	TGTGAGGTGATTGCTCTCATCTC	TTGCGCAATGCCAACCTCAGGAG
mVim	GCACCCTGCAGTCATTGAGA	GCAAGGATTCACCTTTCGGTT	SYBR Green Kit
mTGF-β <sub>1</sub>	GGACTCTCCACCTGCAAGACC	GGATGGCTTCGATGCGC	SYBR Green Kit
mGAPDH	GGCAAATTCAACGGCACAGT	AGATGGTGATGGGCTTCCC	SYBR Green Kit

<sup>a</sup>mGAPDH, mouse glyceraldehyde-3-phosphate dehydrogenase; mCol IV, mouse type IV collagen; mFibr, mouse fibronectin; mVim, mouse vimentin; mTGF-β<sub>1</sub>, mouse TGF-β<sub>1</sub>; mPDGF-A, -B, -C, and -D, mouse PDGF-A, -B, -C, and -D.

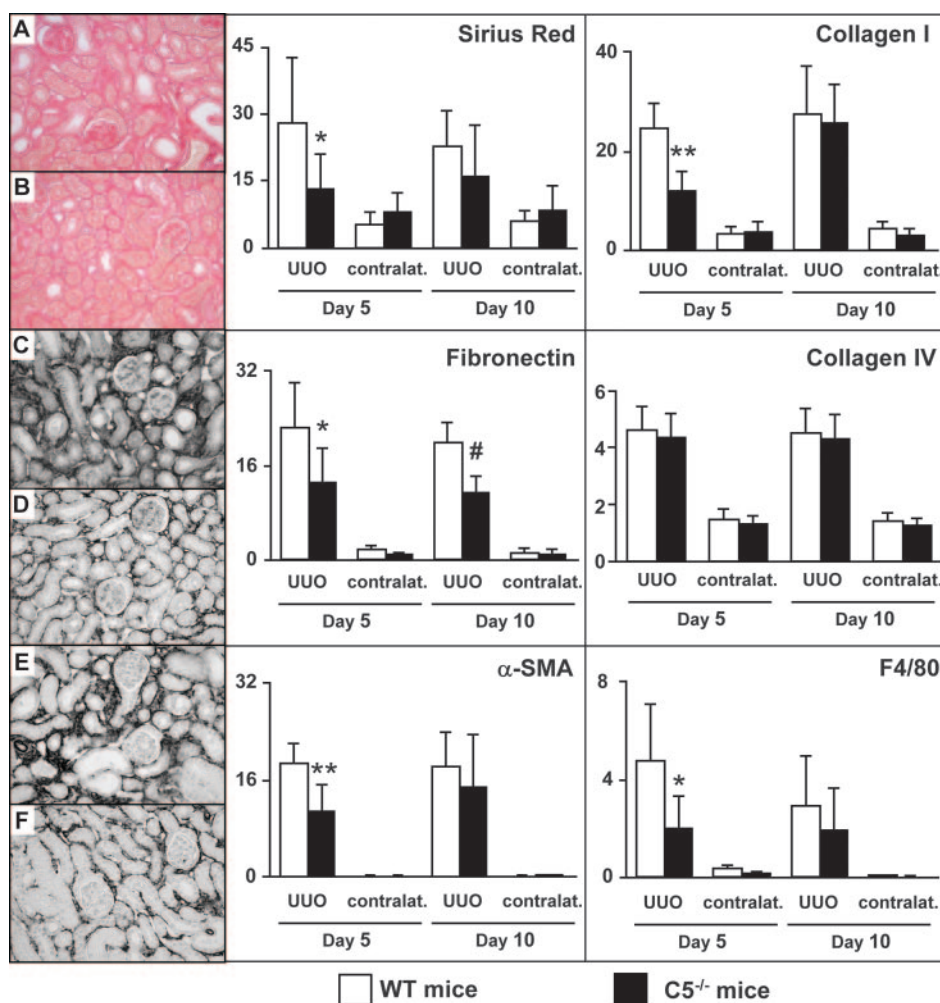


Figure 1. Representative images of obstructed kidneys at day 5 in wild-type (WT; A, C, and E) and C5 deficient (C5<sup>-/-</sup>; B, D, and F) mice: Staining for Sirius Red (A and B), fibronectin (C and D), and α-smooth muscle actin (α-SMA; E and F). Diagrams show data obtained by computer-based morphometric analysis on days 5 and 10 after the induction of unilateral ureteral obstruction (UUO) in obstructed and in healthy contralateral kidneys. Y axes give the relative area (%) of tissue that stained positively. Data are means ± SD. \*P < 0.05 versus WT day 5; \*\*P < 0.01 versus WT day 5; #P < 0.05 versus WT day 10. F4/80, staining for macrophages; contralat., contralateral healthy kidney. Magnification, ×100.



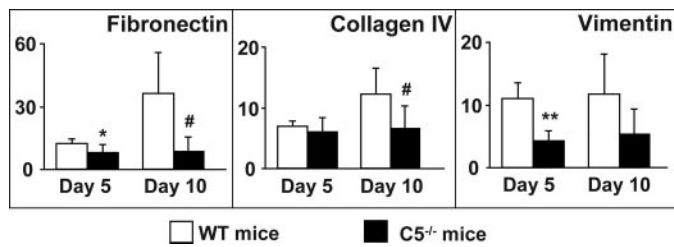


Figure 2. Expression of fibronectin, type IV collagen, and vimentin as assessed by real-time reverse transcriptase–PCR in WT and C5<sup>-/-</sup> mice on days 5 and 10 after UUO. Y axes depict the mRNA expression of the appropriate gene relative to non-obstructed kidneys, which were pooled and arbitrarily set as 1. Data are means ± SD. \*P < 0.05 versus WT day 5; \*\*P < 0.01 versus WT day 5; #P < 0.05 versus WT day 10.

lower in C5<sup>-/-</sup> mice compared with wild-type mice, both on day 5 (–33%) and 10 (–76%) after UUO (Figure 2). Expression of the type IV collagen mRNA was not affected by the lack of C5 after 5 d of UUO but was reduced by approximately 50% after 10 d (Figure 2); however, no differences were found on protein level (Figure 1).

Activation of renal interstitial fibroblasts and epithelial-to-mesenchymal transition (EMT) was assessed by the tubulointerstitial *de novo* expression of  $\alpha$ -SMA and vimentin. Compared with wild-type mice, C5<sup>-/-</sup> mice expressed significantly less renal cortical  $\alpha$ -SMA on day 5, whereas significance of the difference was lost on day 10 (Figure 1). Relative cortical mRNA expression of vimentin was also reduced in C5<sup>-/-</sup> mice on both days 5 (–62%) and 10 (–55%; Figure 2). Lack of C5 after UUO did not affect the tubular expression of E-cadherin, another EMT marker (data not shown).

On day 5 of UUO, macrophage influx was reduced by 57% in C5<sup>-/-</sup> mice. In the later phase of the disease, no difference between the animals persisted (Figure 1).

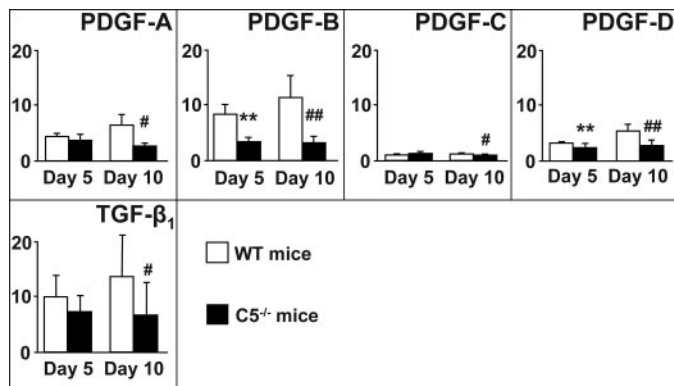


Figure 3. Expression of PDGF-A to -D and TGF- $\beta_1$  in WT and C5<sup>-/-</sup> mice on days 5 and 10 after UUO. Y axes depict the mRNA expression of the appropriate gene relative to non-obstructed kidneys, which were pooled and arbitrarily set as 1. Data are means ± SD. \*P < 0.05 versus WT day 5; \*\*P < 0.01 versus WT day 5; #P < 0.05 versus WT day 10.

### Renal Cortical Expression of all PDGF Isoforms and TGF- $\beta_1$ Are Reduced in C5<sup>-/-</sup> Mice after UUO

PDGF is an important mediator of renal fibrosis (22–26). In comparison with nonobstructed kidneys, the expression of PDGF-A, -B, and -D mRNA increased 4.5-, 8.3-, and 3.2-fold, respectively, in obstructed kidneys of wild-type mice on day 5 and 6.3-, 11.4-, and 5.5-fold, respectively, on day 10 (Figure 3). In contrast, PDGF-C mRNA exhibited very little change (Figure 3). C5 deficiency in the obstructed kidney led to reduced cortical mRNA expression of both PDGF receptor  $\beta$  agonists (PDGF-B and -D [–61 and –29%, respectively]) as compared with wild-type mice. On day 10, the relative expression of all PDGF isoform mRNA was reduced in C5<sup>-/-</sup> mice by –20% (PDGF-C) to –50% (PDGF-D; Figure 3). The expression of mRNA for TGF- $\beta_1$ , another key mediator of fibrosis, was also reduced in C5<sup>-/-</sup> mice when compared with wild-type mice on day 5 by 27% and on day 10 by 50% (Figure 3).

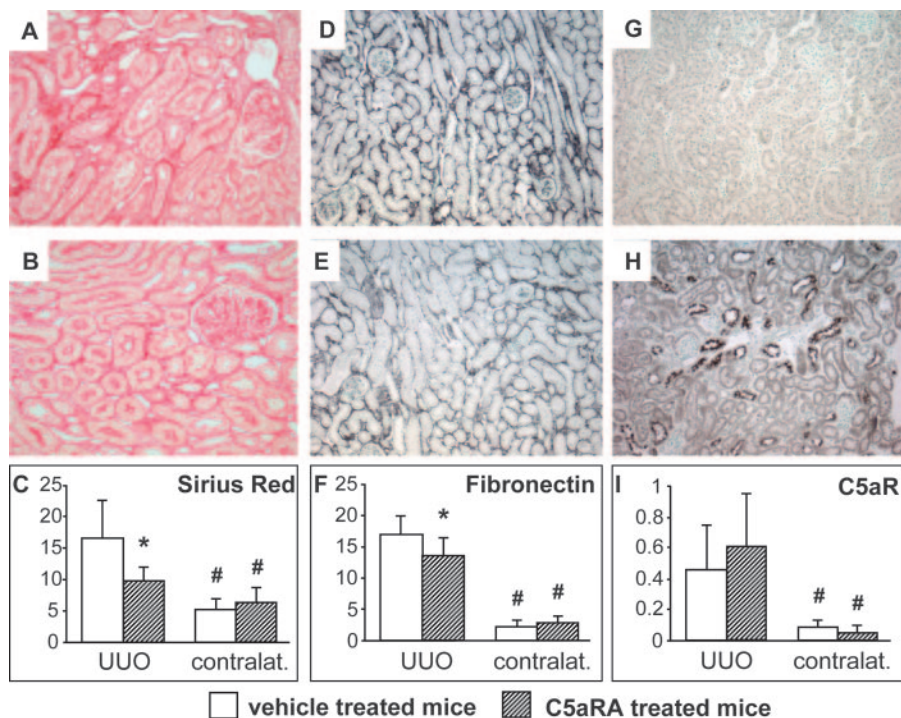
### Renal Expression of C5aR Is Upregulated after UUO

The expression of the C5aR in healthy mouse kidneys was sparse and localized to the apical side of tubular epithelial cells. Five days after UUO, a >16- and 22-fold increase in C5aR expression was detected in vehicle- and C5aRA-treated mice, respectively (Figure 4). Upregulated C5aR expression was mainly localized to the luminal side of tubular cells, but some tubular cells showed also lateral or diffuse positivity. Upregulated expression was particularly prominent in dilated tubules but occasionally also occurred in tubule segments that appeared normal by light microscopy (Figure 4, H and I). Treatment with C5aRA did not significantly affect the expression of the receptor in healthy or obstructed kidneys (Figure 4, G and I).

### Treatment with C5aRA Ameliorates Renal Scarring and PDGF-B Expression after UUO

Treatment with C5aRA after UUO reduced the Sirius Red-positive area and fibronectin expression by 41 and 21%, respectively (Figure 4, A through F). C5aRA-treated mice, when compared with vehicle-treated mice, also exhibited numerically lower renal cortical accumulation of type I collagen (6.9 ± 2.2 versus 9.1 ± 2.3% positively stained area, respectively; P = 0.06) and lower tubulointerstitial expression of  $\alpha$ -SMA (5.1 ± 3.1 versus 8.8 ± 6.0% positively stained area, respectively; P = 0.14). C5aRA treatment did not significantly influence renal cortical macrophage influx (2.4 ± 0.5 versus 3.0 ± 1.4% positively stained area in C5aRA- and vehicle-treated mice, respectively) or collagen type IV expression (3.5 ± 0.7 versus 4.1 ± 0.7% positively stained area in C5aRA- and vehicle-treated mice, respectively).

At the mRNA level, renal cortical expression of type IV collagen and fibronectin was not significantly influenced by C5aRA treatment (type IV collagen 6.7 ± 3.2 versus 8.1 ± 4.8 relative mRNA expression in C5aRA- and vehicle-treated mice, respectively; fibronectin 7.6 ± 3.1 versus 11.1 ± 4.6, respectively). PDGF-B mRNA was decreased in mice that were treated with C5aRA when compared with vehicle-treated mice (5.7 ± 2.4 versus 12.3 ± 7.0 relative mRNA expression; P < 0.05), whereas the other PDGF isoforms were not affected by the treatment. The expression of TGF- $\beta_1$  mRNA decreased mildly in mice that received C5aRA (3.7 ± 2.5 versus 4.5 ± 3.3 relative mRNA expression in C5aRA-treated versus vehicle-treated mice, respectively; NS).



**Figure 4.** Representative pictures of obstructed kidneys at day 5 in vehicle-treated (A, D, and G) and C5a receptor antagonist (C5aRA)-treated (B, E, and H) mice, stained with Sirius Red (A and B) and for fibronectin (D and E). C and F show data obtained by computer-based morphometric analysis of Sirius Red and fibronectin staining, respectively, on day 5 after the induction of UUO. (G) C5aR is expressed focally in some tubular cells of nonobstructed, contralateral kidney. (H) C5aR expression is markedly upregulated 5 d after UUO. (I) Computer-based morphometric analysis of C5aR expression in obstructed and nonobstructed kidneys in both vehicle- and C5aRA-treated groups. Y axes depict the relative area (%) of tissue that stained positively with Sirius Red and for fibronectin or C5aR. Data are means  $\pm$  SD. \* $P < 0.05$  versus vehicle; # $P \leq 0.01$  versus appropriate UUO kidney. Magnification,  $\times 100$ .

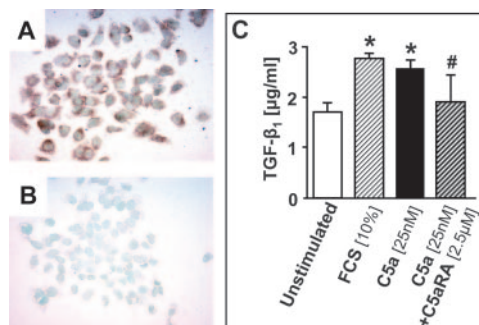
#### Effect of C5 Deficiency and C5aRA Treatment on Nonobstructed Kidneys and on Body Weight

In contralateral, nonobstructed kidneys, C5 deficiency did not affect most histologic parameters in comparison with non-manipulated wild-type mice (data not shown); however, on day 5, decreased macrophage counts and fibronectin immunoreactivity were noted in the nonobstructed kidneys of C5<sup>-/-</sup> mice ( $0.12 \pm 0.11$  versus  $0.33 \pm 0.18\%$  positively stained area for F4/80 in C5<sup>-/-</sup> and wild-type mice, respectively;  $0.89 \pm 0.35$  versus  $1.75 \pm 0.63\%$  positively stained area for fibronectin in C5<sup>-/-</sup> and wild-type mice, respectively; both  $P < 0.05$ ). These results probably represent a statistical chance observation. Treatment with C5aRA did not induce any detectable changes in nonobstructed kidneys (data not shown).

C5<sup>-/-</sup> mice exhibited between 13 and 19% lower body weight when compared with wild-type mice before UUO and on days 5 and 10 after UUO (data not shown;  $P < 0.01$  for all groups). The body weight of wild-type mice that were treated with vehicle or C5aRA was similar before disease induction ( $25 \pm 2$  versus  $25 \pm 2$  g, respectively) but significantly higher in the vehicle group after 5 d of UUO ( $24 \pm 1$  versus  $22 \pm 1$  g, in vehicle- and C5aRA-treated mice, respectively;  $P < 0.01$ ).

#### Effects of C5a on Murine Cortical Tubular Cells In Vitro

We first showed by immunocytochemistry that cultured MCT cells expressed C5aR (Figure 5, A and B). Addition of C5a



**Figure 5.** Murine cortical tubule (MCT) cells expressed C5aR *in vitro* (A). The appropriate negative control is shown in B. (C) Concentration of TGF-β<sub>1</sub> in cell culture supernatant of MCT cells that were serum-starved (unstimulated) or stimulated with 10% FCS, 25 nM C5a, or 25 nM C5a plus 2.5 M C5aRA for 24 h. \* $P < 0.01$  versus unstimulated cells; # $P < 0.05$  versus C5a-stimulated cells. Magnification,  $\times 200$ .

to the cell culture medium of serum-starved MCT cells stimulated their production of TGF-β<sub>1</sub> to a level comparable to cells that were grown in full medium, and this effect was inhibited by addition of C5aRA (Figure 5C). In contrast to TGF-β<sub>1</sub> production, mRNA levels of PDGF-B and -D in MCT cells were not affected by C5a (data not shown).

## Discussion

The major and novel finding of this study was that in the early phase of nonproteinuric renal damage, C5 deficiency led to a significant amelioration of major components of renal fibrosis: Fibroblast activation, extracellular matrix protein deposition, macrophage influx, and some markers of EMT. Our findings in mice that had UUO and were treated with a C5aRA additionally suggest that the anaphylatoxin C5a is the central mediator of this C5 effect. Therefore, the interaction of C5a with the C5aR (CD88) seems to be a key mediator of fibrosis in nonproteinuric conditions.

Our data are consistent with earlier findings showing that C6 deficiency does not affect the course of UUO (15) in contrast to proteinuric renal diseases, where C6 and thus C5b-9 contributed to tubulointerstitial damage (8–10). These latter findings, however, do not exclude an additional role of C5a in mediating renal interstitial fibrosis.

On day 10 after UUO, many of the C5-mediated effects lost statistical significance. This may relate to the progressive course of renal damage in the UUO model and to the redundancy of the various biologic systems involved in renal fibrosis (reviewed in Klahr and Morissey [27]). Indeed, many previous intervention studies in the UUO model have shown as well that the effect of the therapeutic intervention was ultimately lost as postrenal obstruction led to progressive destruction of the renal architecture (28–30).

Until now, there were no published data on the role of C5a in renal fibrosis. However, the first data emerged recently in liver and lung fibrosis models. We previously described that specific C5aR inhibition in a murine model of liver fibrosis significantly ameliorated the progress of fibrosis and reduced hepatic collagen content and expression as well as mRNA levels of tissue inhibitor of matrix metalloproteinases type 1 (16). Similar to our results, fibrosis was markedly attenuated in C5<sup>-/-</sup> mice with bleomycin-induced pulmonary fibrosis (31). In this study, pulmonary accumulation of collagen types I and III was significantly decreased in C5<sup>-/-</sup> mice, and the expression of TGF- $\beta_1$  and matrix metalloproteinase-3 were downregulated as well (31). These findings confirm and extend our hypothesis that C5a has a central role in the process of fibrosis in general (*i.e.*, independent of the tissue or organ) (16).

There are several possible mechanisms for how blockade of the C5a–C5aR interaction might ameliorate renal tubulointerstitial fibrosis. First, in mouse kidneys, tubular cells seem to be the only renal cell type that expresses C5aR, as shown in Figure 4 and previously described in BALB/c mice (32). This is similar to the C5aR expression pattern described in human kidney tissues (33,34). Our observation of a pronounced upregulation of tubular cell C5aR expression after UUO (Figure 4) provides a mechanistic basis *in situ* for exaggerated C5a signaling. Our data extend previous observation in an *in vitro* model of liver fibrogenesis using hepatic stellate cells, the principal cells of hepatic fibrosis, where C5aR was also greatly upregulated (approximately nine-fold) on day 5 of secondary culture of these cells, and the high C5aR levels were maintained thereafter (16).

A second profibrotic mechanism downstream of the C5aR may involve the production of mediators, such as TGF- $\beta$  and PDGF-B and -D, all of which have been shown to be crucially involved in renal scarring (22–26,35). *In vivo*, at least at the mRNA level, the considerable overexpression of both PDGF-B and -D and TGF- $\beta_1$  in ob-

structed kidneys was potently downregulated in C5<sup>-/-</sup> mice and in C5aRA-treated mice. Similar observations were made *in vitro*, where murine tubular cells that were stimulated with C5a produced significant amounts of TGF- $\beta_1$  protein. The amounts produced were in the same range as after stimulation with angiotensin II (36). We recently described that in mesangioproliferative glomerulonephritis, PDGF-B acts downstream of TGF- $\beta$  (37). Given the lack of a C5a effect on tubular cell PDGF-B and -D mRNA expression, our data suggest that C5a induces TGF- $\beta_1$  on injured tubular cells, which in turn mediates profibrotic effects directly or *via* the upregulation of PDGF-B and -D chains in neighboring cells such as fibroblasts. The expression of PDGF chains and receptors by interstitial fibroblasts in obstructed murine kidneys has been documented previously (38). The potent effects of C5a on renal fibronectin overproduction and accumulation after UUO (Figures 1 and 4) is in line with downstream effects of PDGF. In cultured hepatic stellate cells, activation of the C5aR induced fibronectin production, which was not mediated through TGF- $\beta_1$  (39).

A third mechanism that contributes to C5a-mediated tubulointerstitial renal fibrosis may be the inhibitory effect of C5a on the polarization of T-helper cells to T<sub>H1</sub> cells (40,41). The following shift to T<sub>H2</sub> cells, with their cytokine response pattern (*e.g.*, IL-13 and TGF- $\beta$  release), acts in a profibrotic manner (42).

Fourth, effects of C5a on kidney hemodynamics might also play a role. Intravenous infusion of human recombinant C5a in rats significantly decreased renal blood flow and GFR in healthy and obstructed kidneys without affecting BP or diuresis. These effects were mediated mainly *via* secretion and synthesis of leukotrienes by polymorphonuclear leukocytes (43). UUO is characterized by an early prominent and persistent vasoconstriction (27). Therefore, mice that lack C5 or receive C5aRA might have profited from normalized hemodynamics, in particular in early stages of obstruction.

A recently described second receptor for C5a, C5L2, has been shown to be expressed in human kidney (40). The exact renal C5L2-expressing cells, however, still have to be identified. C5L2 has been shown to be a C5a decoy receptor, thereby limiting the proinflammatory C5a response (44,45). It therefore seems unlikely that this receptor modulates any effects of the C5aRA in our model.

Monocyte/macrophage influx is a widely recognized contributory factor in the development of renal tubulointerstitial fibrosis (46), and chemotactic properties of C5a on leukocytes (including macrophages) are well described (46,47). Consistent with this, we observed fewer tubulointerstitial macrophages in the obstructed kidneys of C5<sup>-/-</sup> mice as compared with wild-type mice. However, treatment with C5aRA, although effective in reducing fibrosis, had only minor effects on the renal macrophage influx (Figure 4). The resultant uncertainty about the role of C5a-mediated leukocyte chemotaxis in renal fibrosis is reminiscent of data in a murine model of asthma induced by ovalbumin sensitization. In this model, inhibition of C5 and C5aR with mAb potently improved lower airway function; however, anti-C5aR treatment did not significantly influence the intrapulmonary inflammatory response, as assessed by white blood cell counts in the bronchoalveolar lavage (48). In UUO, other potent chemotactic factors, such as monocyte chemoattractant protein-1 and osteopontin, are overexpressed as well (27), and their action could have substituted for that of C5a. Another explanation that might contribute to a differential effect of C5 deficiency *versus* C5aRA is the



chemotactic activity of C5b-9, as described in lungs (49) as well as in kidney (9,10). Although the numbers of infiltrating macrophages did not differ, their activation state might have done so. C5a was shown to induce TNF- $\alpha$  production in inflammation-primed macrophages (50). In turn, macrophages that produce TNF- $\alpha$ , as well as TGF- $\beta$  and PDGF-B, promote fibrotic remodeling in UUO (35,51).

In addition to decreasing markers of renal fibrosis and affecting macrophage influx, the decreased cortical vimentin mRNA levels in obstructed kidneys of C5<sup>-/-</sup> mice supports the notion of decreased EMT. In contrast to vimentin, we observed no difference in E-cadherin expression between the groups. This is similar to our recent data with specific PDGF-D inhibition in established tubulointerstitial fibrosis (23) and consistent with other studies showing that development of EMT is not proceeding uniformly (52).

## Conclusion

This is the first study to identify C5, in particular the anaphylatoxin C5a, as a novel profibrotic factor in renal disease. Our study points to a new therapeutic target in the treatment of tubulointerstitial fibrosis, a common end-condition characteristic for all progressive renal diseases.

## Acknowledgments

This study was supported by a stipend from the German Academic Exchange Service to P.B., a stipend from the Italian Society of Nephrology to L.V., and grants of the German Research Foundation to T.O. and F.E. (OS 196/1-2) and F.L. (LA 997/4-1).

The technical help of Gabriele Dietzel, Andrea Cosler, and Lydia Zimmermanns is gratefully acknowledged.

## Disclosures

None.

## References

- Berger SP, Roos A, Daha MR: Complement and the kidney: What the nephrologist needs to know in 2006? *Nephrol Dial Transplant* 20: 2613–2619, 2005
- Morita Y, Ikeguchi H, Nakamura J, Hotta N, Yuzawa Y, Matsuo S: Complement activation products in the urine from proteinuric patients. *J Am Soc Nephrol* 11: 700–707, 2000
- Pruchno CJ, Burns MM, Schulze M, Johnson RJ, Baker PJ, Alpers CE, Couser WG: Urinary excretion of the C5b-9 membrane attack complex of complement is a marker of immune disease activity in autologous immune complex nephritis. *Am J Pathol* 138: 203–211, 1991
- Couser WG, Ochi RF, Baker PJ, Schulze M, Campbell C, Johnson RJ: C6 depletion reduces proteinuria in experimental nephropathy induced by a nonglomerular antigen. *J Am Soc Nephrol* 2: 894–901, 1991
- Mosolits S, Magyarlaki T, Nagy J: Membrane attack complex and membrane cofactor protein are related to tubulointerstitial inflammation in various human glomerulopathies. *Nephron* 75: 179–187, 1997
- Nath KA, Hostetter MK, Hostetter TH: Pathophysiology of chronic tubulo-interstitial disease in rats. Interactions of dietary acid load, ammonia, and complement component C3. *J Clin Invest* 76: 667–675, 1985
- Morita Y, Nomura A, Yuzawa Y, Nishikawa K, Hotta N, Shimizu F, Matsuo S: The role of complement in the pathogenesis of tubulointerstitial lesions in rat mesangial proliferative glomerulonephritis. *J Am Soc Nephrol* 8: 1363–1372, 1997
- Nangaku M, Pippin J, Couser WG: Complement membrane attack complex (C5b-9) mediates interstitial disease in experimental nephrotic syndrome. *J Am Soc Nephrol* 10: 2323–2331, 1999
- Nangaku M, Pippin J, Couser WG: C6 mediates chronic progression of tubulointerstitial damage in rats with remnant kidneys. *J Am Soc Nephrol* 13: 928–936, 2002
- Rangan GK, Pippin JW, Couser WG: C5b-9 regulates peritubular myofibroblast accumulation in experimental focal segmental glomerulosclerosis. *Kidney Int* 66: 1838–1848, 2004
- Nomura A, Morita Y, Maruyama S, Hotta N, Nadai M, Wang L, Hasegawa T, Matsuo S: Role of complement in acute tubulointerstitial injury of rats with aminonucleoside nephrosis. *Am J Pathol* 151: 539–547, 1997
- Welch TR, Frenzke M, Witte D, Davis AE: C5a is important in the tubulointerstitial component of experimental immune complex glomerulonephritis. *Clin Exp Immunol* 130: 43–48, 2002
- Hori Y, Yamada K, Hanafusa N, Okuda T, Okada N, Miyata T, Couser WG, Kurokawa K, Fujita T, Nangaku M: Crry, a complement regulatory protein, modulates renal interstitial disease induced by proteinuria. *Kidney Int* 56: 2096–2106, 1999
- Sheerin NS, Sacks SH: Leaked protein and interstitial damage in the kidney: Is complement the missing link? *Clin Exp Immunol* 130: 1–3, 2002
- Rangan GK, Pippin JW, Coombes JD, Couser WG: C5b-9 does not mediate chronic tubulointerstitial disease in the absence of proteinuria. *Kidney Int* 67: 492–503, 2005
- Hillebrandt S, Wasmuth HE, Weiskirchen R, Hellerbrand C, Keppeler H, Werth A, Schirin-Sokhan R, Wilkens G, Geier A, Lorenzen J, Kohl J, Gressner AM, Matern S, Lammer F: Complement factor 5 is a quantitative trait gene that modifies liver fibrogenesis in mice and humans. *Nat Genet* 37: 835–843, 2005
- Schnatbaum K, Locardi E, Scharn D, Richter U, Hawlisch H, Knolle J, Polakowski T: Peptidomimetic C5a receptor antagonists with hydrophobic substitutions at the C-terminus: Increased receptor specificity and in vivo activity. *Bioorg Med Chem Lett* 16: 5088–5092, 2006
- Heller T, Hennecke M, Baumann U, Gessner JE, zu Vilsendorf AM, Baensch M, Boulay F, Kola A, Klos A, Bautsch W, Kohl J: Selection of a C5a receptor antagonist from phage libraries attenuating the inflammatory response in immune complex disease and ischemia/reperfusion injury. *J Immunol* 163: 985–994, 1999
- Woodruff TM, Strachan AJ, Sanderson SD, Monk PN, Wong AK, Fairlie DP, Taylor SM: Species dependence for binding of small molecule agonist and antagonists to the C5a receptor on polymorphonuclear leukocytes. *Inflammation* 25: 171–177, 2001
- Floege J, Hackmann B, Kliem V, Kriz W, Alpers CE, Johnson RJ, Kuhn KW, Koch KM, Brunkhorst R: Age-related glomerulosclerosis and interstitial fibrosis in Milan normotensive rats: A podocyte disease. *Kidney Int* 51: 230–243, 1997
- Burg M, Ostendorf T, Mooney A, Koch KM, Floege J: Treatment of experimental mesangioproliferative glomerulonephritis with non-anticoagulant heparin: Therapeutic efficacy and safety. *Lab Invest* 76: 505–516, 1997

22. Ostendorf T, Rong S, Boor P, Wiedemann S, Kunter U, Haubold U, van Roeyen CR, Eitner F, Kawachi H, Starling G, Alvarez E, Smithson G, Floege J: Antagonism of PDGF-D by human antibody CR002 prevents renal scarring in experimental glomerulonephritis. *J Am Soc Nephrol* 17: 1054–1062, 2006
23. Boor P, Konieczny A, Villa L, Kunter U, van Roeyen CR, Larochelle WJ, Smithson G, Arrol S, Ostendorf T, Floege J: PDGF-D inhibition by CR002 ameliorates tubulointerstitial fibrosis following experimental glomerulonephritis. *Nephrol Dial Transplant* February 17, 2007 [epub ahead of print]
24. Eitner F, Floege J: Therapeutic targets for prevention and regression of progressive fibrosing renal diseases. *Curr Opin Investig Drugs* 6: 255–261, 2005
25. Ostendorf T, Kunter U, Grone HJ, Bahlmann F, Kawachi H, Shimizu F, Koch KM, Janjic N, Floege J: Specific antagonism of PDGF prevents renal scarring in experimental glomerulonephritis. *J Am Soc Nephrol* 12: 909–918, 2001
26. Tang WW, Ulich TR, Lacey DL, Hill DC, Qi M, Kaufman SA, Van GY, Tarpley JE, Yee JS: Platelet-derived growth factor-BB induces renal tubulointerstitial myofibroblast formation and tubulointerstitial fibrosis. *Am J Pathol* 148: 1169–1180, 1996
27. Klahr S, Morrissey J: Obstructive nephropathy and renal fibrosis. *Am J Physiol Renal Physiol* 283: F861–F875, 2002
28. Bascands JL, Schanstra JP: Obstructive nephropathy: Insights from genetically engineered animals. *Kidney Int* 68: 925–937, 2005
29. Oda T, Jung YO, Kim HS, Cai X, Lopez-Guisa JM, Ikeda Y, Eddy AA: PAI-1 deficiency attenuates the fibrogenic response to ureteral obstruction. *Kidney Int* 60: 587–596, 2001
30. Ophascharoensuk V, Fero ML, Hughes J, Roberts JM, Shankland SJ: The cyclin-dependent kinase inhibitor p27Kip1 safeguards against inflammatory injury. *Nat Med* 4: 575–580, 1998
31. Addis-Lieser E, Kohl J, Chiaramonte MG: Opposing regulatory roles of complement factor 5 in the development of bleomycin-induced pulmonary fibrosis. *J Immunol* 175: 1894–1902, 2005
32. Riedemann NC, Guo RF, Neff TA, Laudes IJ, Keller KA, Sarma VJ, Markiewski MM, Mastellos D, Strey CW, Pierson CL, Lambris JD, Zetoune FS, Ward PA: Increased C5a receptor expression in sepsis. *J Clin Invest* 110: 101–108, 2002
33. Fayyazi A, Scheel O, Werfel T, Schweyer S, Oppermann M, Gotze O, Radzun HJ, Zwirner J: The C5a receptor is expressed in normal renal proximal tubular but not in normal pulmonary or hepatic epithelial cells. *Immunology* 99: 38–45, 2000
34. Zahedi R, Braun M, Wetsel RA, Ault BH, Khan A, Welch TR, Frenzke M, Davis AE: The C5a receptor is expressed by human renal proximal tubular epithelial cells. *Clin Exp Immunol* 121: 226–233, 2000
35. Chevalier RL: Obstructive nephropathy: Towards biomarker discovery and gene therapy. *Nat Clin Pract Nephrol* 2: 157–168, 2006
36. Wolf G, Mueller E, Stahl RA, Ziyadeh FN: Angiotensin II-induced hypertrophy of cultured murine proximal tubular cells is mediated by endogenous transforming growth factor-beta. *J Clin Invest* 92: 1366–1372, 1993
37. Ostendorf T, Kunter U, van Roeyen C, Dooley S, Janjic N, Ruckman J, Eitner F, Floege J: The effects of platelet-derived growth factor antagonism in experimental glomerulonephritis are independent of the transforming growth factor-beta system. *J Am Soc Nephrol* 13: 658–667, 2002
38. Taneda S, Hudkins KL, Topouzis S, Gilbertson DG, Ophascharoensuk V, Truong L, Johnson RJ, Alpers CE: Obstructive uropathy in mice and humans: Potential role for PDGF-D in the progression of tubulointerstitial injury. *J Am Soc Nephrol* 14: 2544–2555, 2003
39. Schlaf G, Schmitz M, Heine I, Demberg T, Schieferdecker HL, Gotze O: Upregulation of fibronectin but not of entactin, collagen IV and smooth muscle actin by anaphylatoxin C5a in rat hepatic stellate cells. *Histol Histopathol* 19: 1165–1174, 2004
40. Wenderfer SE, Ke B, Hollmann TJ, Wetsel RA, Lan HY, Braun MC: C5a receptor deficiency attenuates T cell function and renal disease in MRLlpr mice. *J Am Soc Nephrol* 16: 3572–3582, 2005
41. Kohl J, Baelder R, Lewkowich IP, Pandey MK, Hawlisch H, Wang L, Best J, Herman NS, Sproles AA, Zwirner J, Whittsett JA, Gerard C, Sfyroera G, Lambris JD, Wills-Karp M: A regulatory role for the C5a anaphylatoxin in type 2 immunity in asthma. *J Clin Invest* 116: 783–796, 2006
42. Wynn TA: Fibrotic disease and the T(H)1/T(H)2 paradigm. *Nat Rev Immunol* 4: 583–594, 2004
43. Gulbins E, Schlottmann K, Rauterberg EW, Steinhausen M: Effects of rC5a on the circulation of normal and split hydronephrotic rat kidneys. *Am J Physiol* 265: F96–F103, 1993
44. Gerard NP, Lu B, Liu P, Craig S, Fujiwara Y, Okinaga S, Gerard C: An anti-inflammatory function for the complement anaphylatoxin C5a-binding protein, C5L2. *J Biol Chem* 280: 39677–39680, 2005
45. Johswich K, Martin M, Thalmann J, Rheinheimer C, Monk PN, Klos A: Ligand specificity of the anaphylatoxin C5L2 receptor and its regulation on myeloid and epithelial cell lines. *J Biol Chem* 281: 39088–39095, 2006
46. Sean Eardley K, Cockwell P: Macrophages and progressive tubulointerstitial disease. *Kidney Int* 68: 437–455, 2005
47. Fernandez HN, Henson PM, Otani A, Hugli TE: Chemotactic response to human C3a and C5a anaphylatoxins. I. Evaluation of C3a and C5a leukotaxis in vitro and under stimulated in vivo conditions. *J Immunol* 120: 109–115, 1978
48. Peng T, Hao L, Madri JA, Su X, Elias JA, Stahl GL, Squinto S, Wang Y: Role of C5 in the development of airway inflammation, airway hyperresponsiveness, and ongoing airway response. *J Clin Invest* 115: 1590–1600, 2005
49. Czermak BJ, Lentsch AB, Bless NM, Schmal H, Friedl HP, Ward PA: Synergistic enhancement of chemokine generation and lung injury by C5a or the membrane attack complex of complement. *Am J Pathol* 154: 1513–1524, 1999
50. Riedemann NC, Guo RF, Bernacki KD, Reuben JS, Laudes IJ, Neff TA, Gao H, Speyer C, Sarma VJ, Zetoune FS, Ward PA: Regulation by C5a of neutrophil activation during sepsis. *Immunity* 19: 193–202, 2003
51. Guo G, Morrissey J, McCracken R, Tolley T, Klahr S: Role of TNFR1 and TNFR2 receptors in tubulointerstitial fibrosis of obstructive nephropathy. *Am J Physiol* 277: F766–F772, 1999
52. Rastaldi MP, Ferrario F, Giardino L, Dell'Antonio G, Grillo C, Grillo P, Strutz F, Muller GA, Colasanti G, D'Amico G: Epithelial-mesenchymal transition of tubular epithelial cells in human renal biopsies. *Kidney Int* 62: 137–146, 2002