

C3a Mediates Epithelial-to-Mesenchymal Transition in Proteinuric Nephropathy

Ziyong Tang,^{*†} Bao Lu,[‡] Ellen Hatch,[§] Steven H. Sacks,^{*} and Neil S. Sheerin[§]

^{*}King's College London, Department of Nephrology and Transplantation, Guy's Hospital London, London, United Kingdom; [†]Department of Nephrology, Peking University Third Hospital, Beijing, China; [‡]Division of Pulmonary Medicine and Ina Sue Perlmutter Laboratory, Children's Hospital Boston, Harvard Medical School, Boston, Massachusetts; and [§]Institute of Cellular Medicine, Newcastle University, Newcastle upon Tyne, United Kingdom

ABSTRACT

Tubulointerstitial inflammation and progressive fibrosis are common pathways that lead to kidney failure in proteinuric nephropathies. Activation of the complement system has been implicated in the development of tubulointerstitial injury in clinical and animal studies, but the mechanism by which complement induces kidney injury is not fully understood. Here, we studied the effect of complement on the phenotype of tubular epithelial cells. Tubular epithelial cells exposed to serum proteins adopted phenotypic and functional characteristics of mesenchymal cells. Expression of E-cadherin protein decreased and expression of both α -smooth muscle actin protein and collagen I mRNA increased. Exposure of the cells to the complement anaphylotoxin C3a induced similar features. Treating with a C3a receptor (C3aR) antagonist prevented both C3a- and serum-induced epithelial-to-mesenchymal transition. In the adriamycin-induced proteinuria model, C3aR-deficient mice demonstrated less injury, preserved renal function, and improved survival compared with wild-type mice. Furthermore, the kidneys of C3aR-deficient mice had significantly less interstitial collagen I and α -smooth muscle actin. In summary, the complement anaphylotoxin C3a is an important mediator of glomerular and tubulointerstitial injury and can induce tubular epithelial-to-mesenchymal transition.

J Am Soc Nephrol 20: 593–603, 2009. doi: 10.1681/ASN.2008040434

In patients with glomerular disease, the presence of tubulointerstitial fibrosis^{1–3} on renal biopsy and heavy proteinuria⁴ both are strong predictors of a poor renal prognosis. With the exception of minimal-change nephrotic syndrome, in which the duration of proteinuria is usually short, there is evidence from experimental studies and clinical observation that proteinuria is responsible, at least in part, for injury to the tubulointerstitial compartment.^{5,6} Interventions that reduce the level of proteinuria, for example inhibition of the renin-angiotensin system, reduce the degree of tubulointerstitial disease⁷; however, it is not known how proteinuria damages the tubulointerstitial compartment. Tubular epithelial cells are exposed to filtered serum proteins. Absorption of excess protein may be toxic to epithelial cells, increase

cell metabolism, and worsen tubular ischemia. Alternatively, specific components of the glomerular filtrate, for example transferrin, lipid-conjugated albumin, and lipoproteins, may be toxic to the tubular epithelium.⁸ Proteins of the complement system are also filtered,^{9–11} and there is evidence that they contribute to renal tubulointerstitial injury.

Received April 29, 2008. Accepted November 3, 2008.

Published online ahead of print. Publication date available at www.jasn.org.

Correspondence: Dr. Neil Sheerin, School of Clinical Medical Sciences, Newcastle University, Framlington Place, Newcastle upon Tyne, NE2 4HH, UK. Phone: 0191-222-7146; Fax: 0191-222-0723; E-mail neil.sheerin@ncl.ac.uk

Copyright © 2009 by the American Society of Nephrology

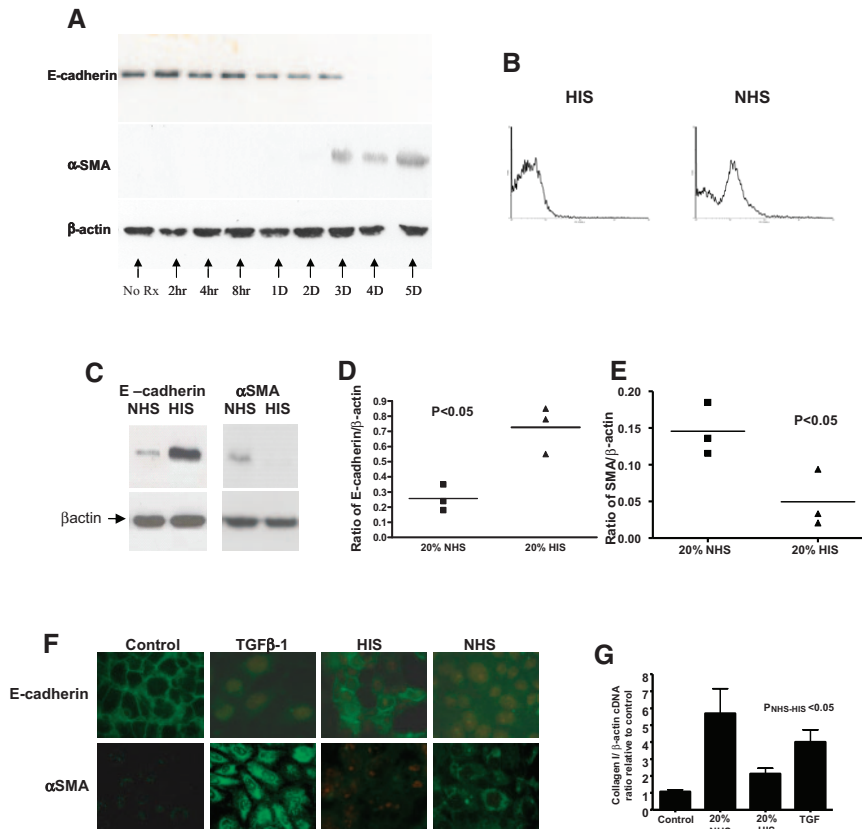


Figure 1. Induction of EMT in PTECs by serum proteins. (A) The potential of the human PTEC cell line HKC8 to undergo EMT was demonstrated by downregulation of E-cadherin and upregulation of α -SMA proteins by Western blotting in response to TGF- β 1. Control cells were grown in medium without TGF- β 1 (No Rx). Representative blot from two experiments. (B) FACS analysis detected C3d deposited on the surface of PTECs after exposure to NHS but not HIS. (C) Incubation with NHS (5 d) reduced E-cadherin expression and increased α -SMA expression by Western blot. (D and E) β -Actin was used to control for protein loading. The ratio of E-cadherin and α -SMA to β -actin was measured on triplicate cultures. (F) Loss of E-cadherin expression was seen by immunofluorescence after exposure of cells to both TGF- β 1 and NHS but not exposure to HIS. The reciprocal change in α -SMA expression was seen (representative of two experiments). (G) By real-time PCR (RT-PCR) analysis, incubation for 5 d with TGF- β or NHS increased the level of collagen I mRNA in PTECs ($n = 3$ per experiment \pm SEM). This did not occur with HIS.

The complement system is an important component of the innate immune system, comprising more than 30 soluble and membrane-bound proteins.^{12,13} It is activated through three distinct pathways: The classical, alternative, and mannose-binding lectin pathways. Once activated, complement has cytolytic effects through C5b-9, opsonises pathogens, and augments adaptive immune responses. Activation also generates the anaphylatoxins C3a and C5a, which have potent proinflammatory effects.

There is evidence that complement is activated in tubules during proteinuria¹⁴ as a result of intrinsic convertase activity in the apical membranes of the proximal tubules.¹⁵ There is a spatial association between complement deposition and tissue injury in the kidneys of patients with proteinuric nephropathies.¹⁶ In experimental proteinuria, genetic deficiency,^{17–19} or depletion or inhibition^{20,21} of complement reduces the level of tubulointerstitial injury and preserves renal function.²² These data support a role for both C5b-9¹⁹ and the anaphylatoxin C5a.²³

During the development of tubulointerstitial injury cells, myofibroblasts and matrix accumulate in the interstitium. Myofibroblasts have both smooth muscle cell- and fibroblast-like properties and express α -smooth muscle actin (α -SMA). Their presence within the renal tubulointerstitium is associated with a worse renal prognosis.^{24,25} Myofibroblasts may be derived from activation of resident renal fibroblasts, the bone marrow, or epithelial cells that change their phenotype and undergo epithelial-to-mesenchymal transition (EMT).^{26,27} There is increasing evidence that EMT is important in the development of interstitial fibrosis not only in the kidney^{27,28} but also in the liver²⁹ and lung.^{30,31} EMT can be induced in proximal tubular epithelial cells (PTECs) by growth factors, in particular TGF- β 1, whereas other proteins, for example bone morphogenic protein 7, prevent EMT.²⁶ The key events in EMT have been defined: Downregulation of epithelial proteins, particularly the junctional protein E-cadherin, increased cell motility, breakdown of the basement membrane, invasion of the interstitium, and increased matrix production.³²

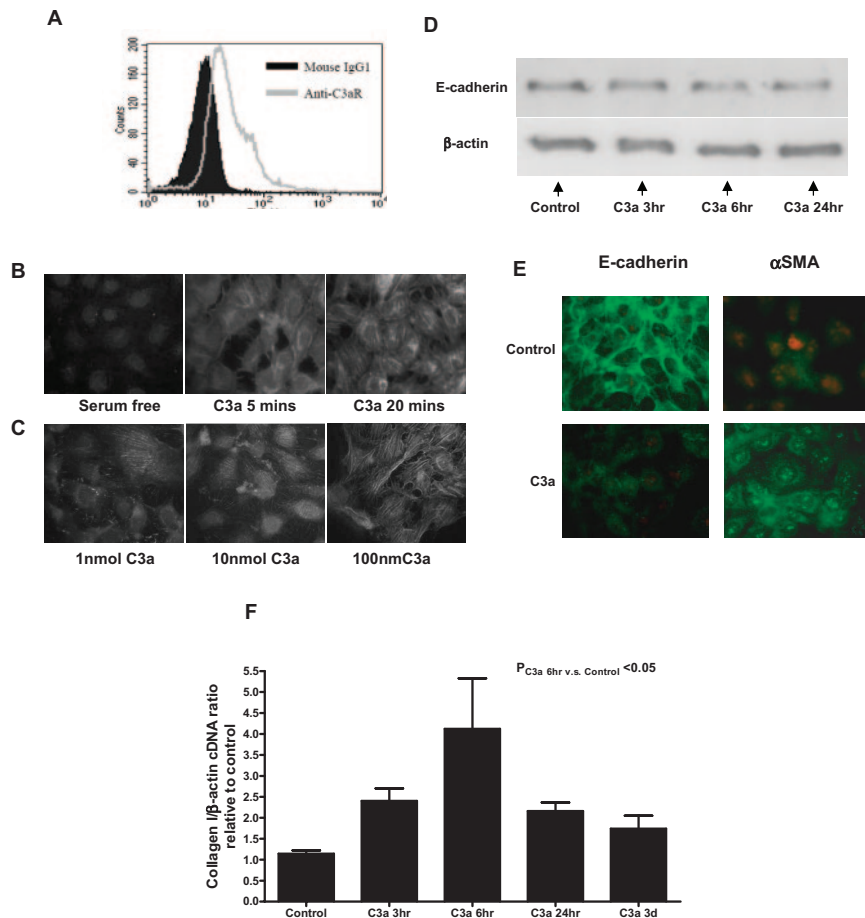


Figure 2. Induction of EMT in PTECs by C3a. (A) Expression of C3aR on PTECs by FACS using monoclonal mouse anti-human C3aR clone 8H1. (B and C) Incubation of PTECs with C3a induced a time-dependent (50 nmol/L; B) and concentration-dependent (C) actin reorganization with the development of stress fibers. Filamentous actin was detected by Texas Red phalloidin staining. (D) C3a also caused a reduction in E-cadherin expression by Western blot analysis (representative of two experiments). (E) This was also evident by immunocytochemistry with loss of the typical circumferential staining for E-cadherin after incubation of cells in 50 nmol/L C3a for 24 h. In addition, α -SMA expression was increased under these conditions (representative of two experiments). (F) RT-PCR analysis of collagen I mRNA levels in PTEC showed an increase after 6 h of incubation with 50 nmol/L C3a ($P < 0.05$), which declined after longer incubation ($n = 3$ per experiment \pm SEM). Data are representative of three experiments.

Although EMT can be induced by TGF- β 1 alone, it is likely that *in vivo* the induction of EMT is more complex. *In vitro* factors such as exposure to myeloma light chains³³ or hypoxia³⁴ can induce EMT, whereas immunosuppressive drugs³⁵ can prevent EMT. In studies of proteinuria in C3-deficient mice or C6-deficient rats, there was a reduction in the number of interstitial myofibroblasts, suggesting that complement activation may have a role in EMT.³⁶

In this study, we define the effect of complement activation on PTEC phenotype, in particular the role of the anaphylotoxin C3a, which can effectively induce EMT *in vitro*. This observation is extended to an animal model of proteinuria. Mice with a targeted deletion in the C3a Receptor (C3aR) show a marked reduction in the number of interstitial α -SMA-expressing myofibroblasts compared with wild-type mice and are protected from the development of tubulointerstitial injury.

RESULTS

Complement Activation Induces EMT

Consistent with existing literature, treatment of the human PTEC cell line HKC8³⁷ with TGF- β 1 induced EMT, with a change in cell shape to a spindle, fibroblast-like form, and actin stress fiber formation (data not shown). In addition, there was a time-dependent decrease in E-cadherin expression and increase in α -SMA expression (Figure 1A). When exposed to serum proteins (20% normal human serum [NHS]), PTECs activated complement as demonstrated by the detection of C3d on the cell surface by FACS staining (Figure 1B). This treatment was not toxic to cells (data not shown). PTECs incubated in 20% NHS did not show morphologic changes; however, suppression of E-cadherin and increase in α -SMA expression occurred (Figure 1C). This was not due to growth factors, because heat-inactivated serum (HIS), which lacks complement activity but would contain heat-stable

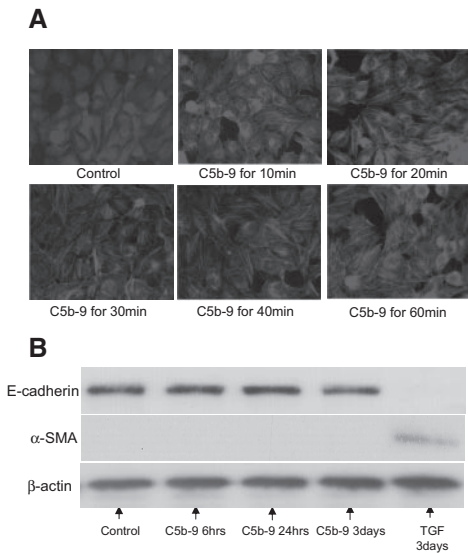


Figure 3. C5b-9 induces actin reorganization but not EMT. (A) Treatment of PTECs with sublytic concentrations of C5b-9 induces the formation of actin stress fibers in a time-dependent manner. (B) No reduction in E-cadherin or increase in α -SMA expression was seen with C5b-9 treatment. Representative data from two experiments.

growth factors,³⁸ did not have this effect. Densitometric analysis of the Western blots demonstrated that the effect of NHS was reproducible and significant (Figure 1, D and E).

Immunocytochemical staining of NHS-treated PTECs confirmed a reduction in E-cadherin expression and increase in α -SMA expression. This occurred with TGF- β 1 treatment but not with HIS treatment (Figure 1F). Collagen I gene expression, assessed by real-time PCR, was increased four-fold in PTECs after treatment with TGF- β 1 (Figure 1G). An equivalent significant increase was seen after incubation of PTECs in NHS but not HIS.

Anaphylotoxin C3a but not C5b-9 Induces EMT

We next considered which effector limb of complement caused EMT. It has been reported that PTECs express the anaphylotoxin receptor C3aR. This was confirmed on HKC8 cells by FACS (Figure 2A). PTECs treated with C3a showed a time- and dosage-dependent formation of actin stress fibers (Figure 2B). This change was evident after 5 min and maximal after 20 min. This occurred at C3a concentrations >10 nM (Figure 2C). Treatment of PTECs with C3a reduced E-cadherin expression, evident by Western blotting (Figure 2D) and immunocytochemistry (Figure 2E). In addition, C3a induced expression of α -SMA (Figure 2E) and a time-dependent increase in collagen I mRNA (Figure 2F). The changes induced by serum could therefore be replicated by C3a alone. In contrast, C5b-9 induced only actin reorganization but did not induce other features of EMT (Figure 3).

Inhibition of C3a Signaling Blocks Serum-Induced EMT

The effect of C3a on PTEC phenotype could be prevented by the C3aR antagonist SB290157. Treatment of PTECs with

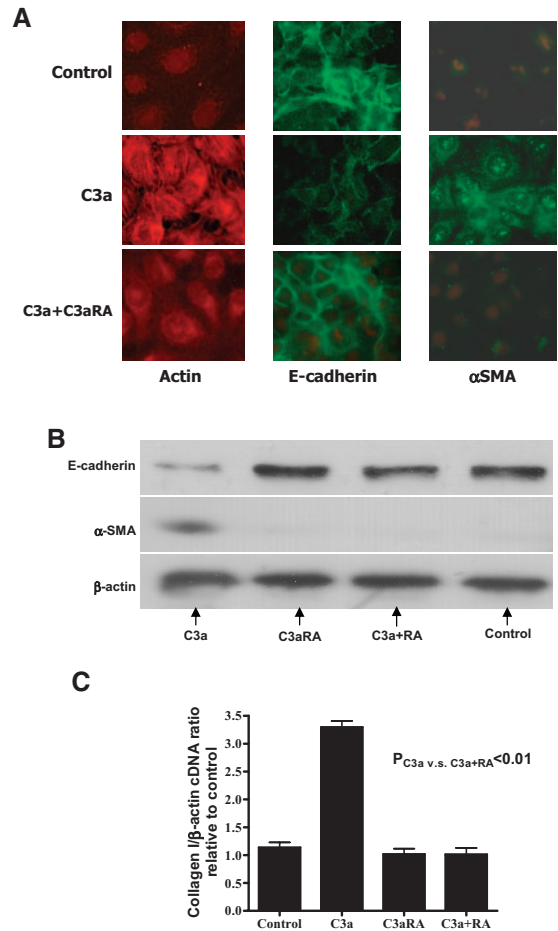


Figure 4. The C3aR antagonist SB 290157 blocked the effect of C3a on PTEC phenotype. (A) In control conditions, no actin stress fibers were evident; strong E-cadherin staining but absent α -SMA staining was evident. On exposure to 50 nmol/L C3a, stress fibers formed, E-cadherin expression was reduced, and α -SMA expression occurred. These changes were prevented by preincubation with 1 μ M C3aR antagonist SB290157. (B) The same changes were evident on Western blotting of whole-cell lysates from C3a-treated cells (50 nmol/L, 24 h). C3aR antagonist treatment alone did not alter expression of either E-cadherin or α -SMA. Representative blot from two experiments. (C) Collagen I mRNA levels were increased by C3a (50 nmol/L for 6 h). This was prevented by pretreatment with antagonist ($n = 3$ per condition \pm SEM).

C3a induced actin reorganization, loss of E-cadherin, and an increase in α -SMA expression, all of which were prevented by pretreatment with SB290157 (Figure 4, A and B). This inhibitor also blocked the increase in collagen I mRNA (Figure 4C).

To confirm the importance of C3a in serum-mediated EMT, we pretreated cells with SB290157 before exposure to NHS. The changes induced by serum, reduced E-cadherin, increased α -SMA (Figure 5A), and increased collagen I mRNA (Figure 5B) were prevented by SB290157, confirming the importance of the C3a–C3aR interaction.

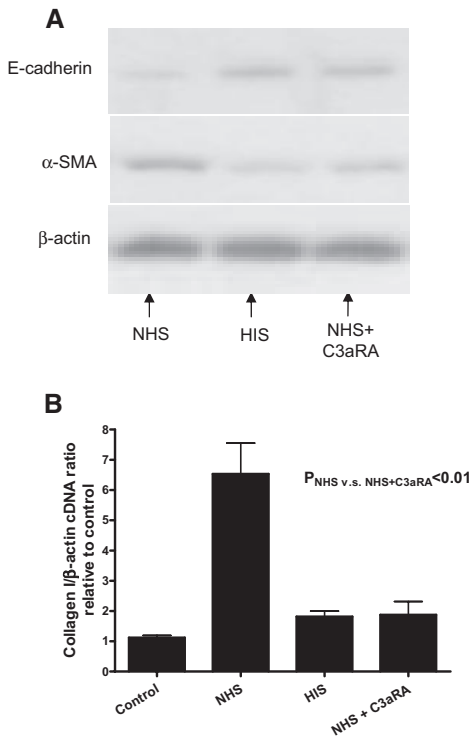


Figure 5. C3aR antagonist prevents serum protein-induced EMT. (A) Incubation of PTECs in 20% NHS for 5 d as a source of complement proteins induced changes typical of EMT (loss of E-cadherin and increased α -SMA). These changes were prevented by the presence of a C3aR antagonist (C3aRA; SB290157). Representative blot from two experiments. (B) Incubation in 20% NHS for 5 d also increased collagen I mRNA levels, demonstrated by RT-PCR. This increase was prevented by the presence of C3aRA ($n = 3$ per group \pm SEM).

Effect of C3a on the Development of Renal Injury In Vivo

We next explored whether C3a affected the development of proteinuria-mediated renal injury. Proteinuria was induced with adriamycin in wild-type (wild-type ADR; $n = 14$) and C3aR $^{-/-}$ mice (C3aR $^{-/-}$ ADR; $n = 8$). Albuminuria was evident in both groups of mice 1 wk after disease induction and was maximal after 2 wk (saline-injected control mice 0.013 ± 0.009 mg/24 h; wild-type ADR mice 57.200 ± 11.500 mg/24 h; C3aR $^{-/-}$ ADR mice 27.900 ± 6.900 mg/24 h). The level of albuminuria was significantly greater in the wild-type mice (Figure 6A). There was also greater mortality in the wild-type mice with 50% dying during the 6-wk protocol (Figure 6B). Mice died at 2 to 3 wk, when albuminuria was greatest. Blood urea nitrogen (BUN) concentration was high in mice immediately before death, confirming that death was due to renal failure. BUN concentration in the surviving mice was greater in the wild-type mice, consistent with more severe renal disease in that group (Figure 6C).

Periodic acid-Schiff (PAS) staining was performed to assess histologic renal injury (Figure 7, A–C). In the kidneys from wild-type ADR mice, the glomeruli were abnormal, with evi-

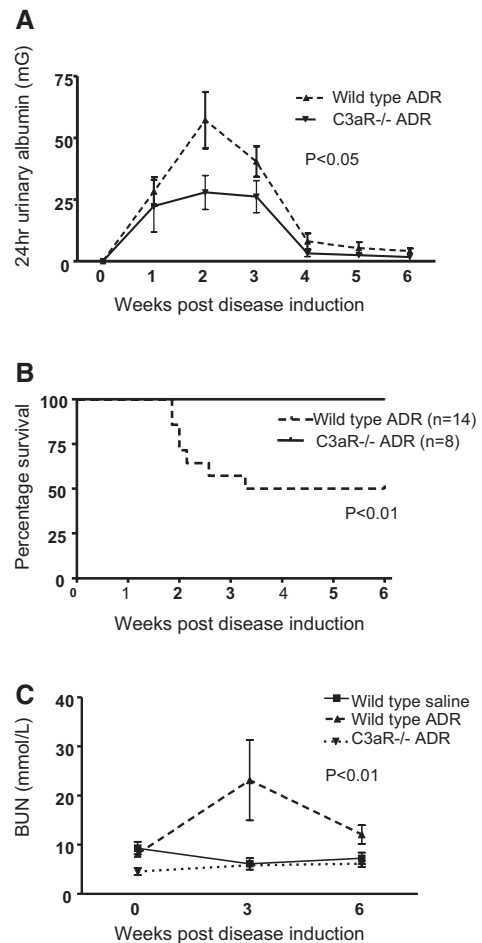


Figure 6. Functional analysis of adriamycin nephropathy in C3aR $^{-/-}$ mice. (A) Twenty-four-hour urinary albumin excretion was measured immediately before and at weekly intervals after the induction of adriamycin nephropathy in wild-type (dashed line) and C3aR $^{-/-}$ (solid line) mice. Points represent mean \pm SEM. (B) Kaplan Meyer survival analysis of wild-type and C3aR $^{-/-}$ mice after the induction of adriamycin nephropathy. (C) BUN concentration (mmol/L) was measured in serum samples taken immediately before and 3 and 6 wk after the induction of adriamycin nephropathy in wild-type and C3aR $^{-/-}$ mice. BUN concentration in saline-injected control mice (dotted line) is shown for comparison. P values shown in figure are for comparison of wild-type with C3aR $^{-/-}$ mice.

dence of glomerular sclerosis. The ratio of PAS-positive area to total glomerular tuft area was used as a measure of glomerular injury (Figure 7D). There was a significant increase in this ratio in wild-type ADR mice compared with saline-injected controls ($P < 0.01$). This change was significantly less in the C3aR $^{-/-}$ ADR mice compared with wild-type ADR mice ($P < 0.01$) and was not greater than saline-injected control mice. The greater histologic injury to the glomerulus of wild-type ADR mice compared with C3aR $^{-/-}$ mice is consistent with the higher level of albuminuria.

Wild-type ADR mice had more severe tubulointerstitial disease. The ratio of tubular diameter to tubular epithelial cell height

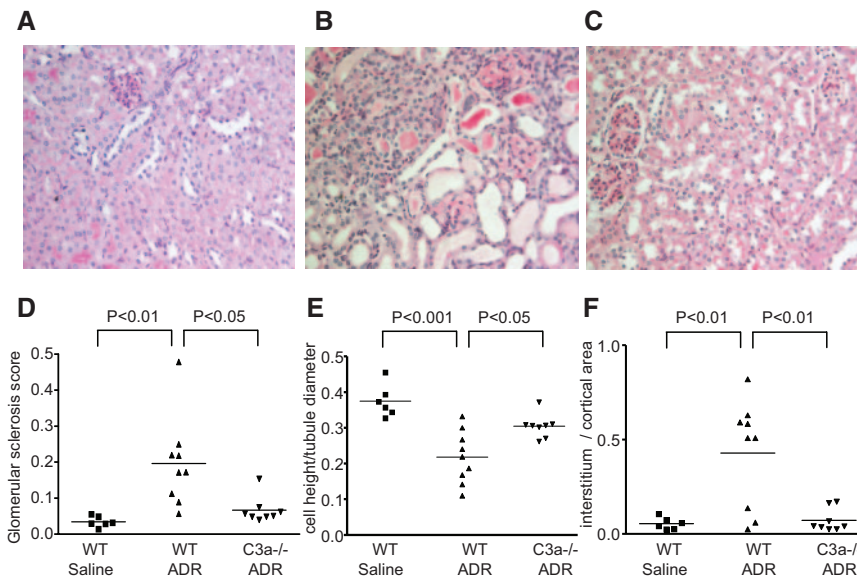


Figure 7. Histologic analysis of injury in wild-type and C3aR^{-/-} mice. (A through C) Representative histologic sections from saline-injected control mice (A), wild-type ADR mice (B), and C3aR^{-/-} ADR mice (C) 6 wk after injection. (D) The ratio of sclerotic, PAS-positive area to total glomerular tuft area was calculated in 20 equatorial glomeruli per mouse, and the mean is shown. This was used as a measure of glomerular injury. (E) The ratio of tubular cell height to tubular diameter was calculated for 50 tubules in 10 nonoverlapping cortical fields, and the mean is shown. The ratio was used as a measure of tubular epithelial cell injury. (F) The ratio of interstitial area to cortical area was calculated for five nonoverlapping fields for each mouse, and the mean is presented. Significant differences are shown. Magnification, $\times 400$.

was used as a measure of tubular injury (Figure 7E). This ratio was significantly reduced in both wild-type ADR and C3aR^{-/-} mice, consistent with proteinuria-induced tubular epithelial cell injury ($P < 0.01$, for both comparisons by *t* test); however, the degree of reduction was significantly greater in the wild-type ADR mice than in the C3aR^{-/-} ADR mice ($P < 0.01$), suggesting that the C3aR was involved in the tubular injury that occurred. The degree of interstitial expansion was also greater in the wild-type ADR mice than both saline-injected controls and C3aR^{-/-} mice (Figure 7F). The interstitial volume in the C3aR^{-/-} ADR mice was not significantly different from that of saline control mice.

C3aR-Dependent Accumulation of Interstitial Collagen I, Myofibroblasts, and Macrophages

In the kidneys of saline-injected control mice, there was little collagen I staining (Figure 8A); however, in the wild-type ADR mice, this was significantly increased (Figure 8, B and D) with staining around damaged tubules. Although the collagen I staining was increased in C3aR^{-/-} ADR mice (Figure 8C) compared with the saline-injected controls ($P < 0.05$), this was significantly less than in the wild-type ADR mice ($P < 0.005$).

In the kidney of saline-injected control mice, α -SMA staining was evident only in blood vessel walls (Figure 8E). In the wild-type ADR mice, there was staining for α -SMA around damaged tubules, consistent with accumulation of myofibroblasts (Figure 8F). More α -SMA staining was present in the C3aR^{-/-} mice than in saline-injected control mice ($P < 0.05$), but this was significantly less than in wild-type ADR mice ($P < 0.001$); therefore, myofibroblast accumulation in the interstitium of proteinuric

mice is dependent at least in part on C3aR activation (Figure 8, G and H).

In the kidneys of saline-injected mice, few macrophages were seen (Figure 8I). In the wild-type ADR mice, a significant infiltration was seen, in keeping with previous reports in this model (Figure 8J). The number of macrophages was significantly less in the C3aR^{-/-} ADR mice (Figure 8, K and L), suggesting that C3a is involved in macrophage accumulation either directly, through macrophage C3aR, or indirectly, by stimulating chemokine production.

C3aR Staining and C3a Generation in Proteinuria

Immunohistochemical staining for C3aR in saline-injected control mice demonstrated presence of receptor on interstitial cells and weak staining on the apical borders of tubules (Figure 9A); however, in the ADR group, strong staining was evident both in the glomerular mesangium and in damaged tubules (Figure 9B). No staining was seen in C3aR^{-/-} mice. C3a was detected in the urine by ELISA and increased in parallel with the level of albuminuria (Figure 9C). The urinary C3a concentration was similar in the wild-type ADR mice and the C3aR^{-/-} ADR mice; therefore, in the proteinuric wild-type mice, both receptor and ligand are present.

DISCUSSION

It is established that complement is activated in the kidney and present in the urine of patients with proteinuria.^{10,14,39} In many

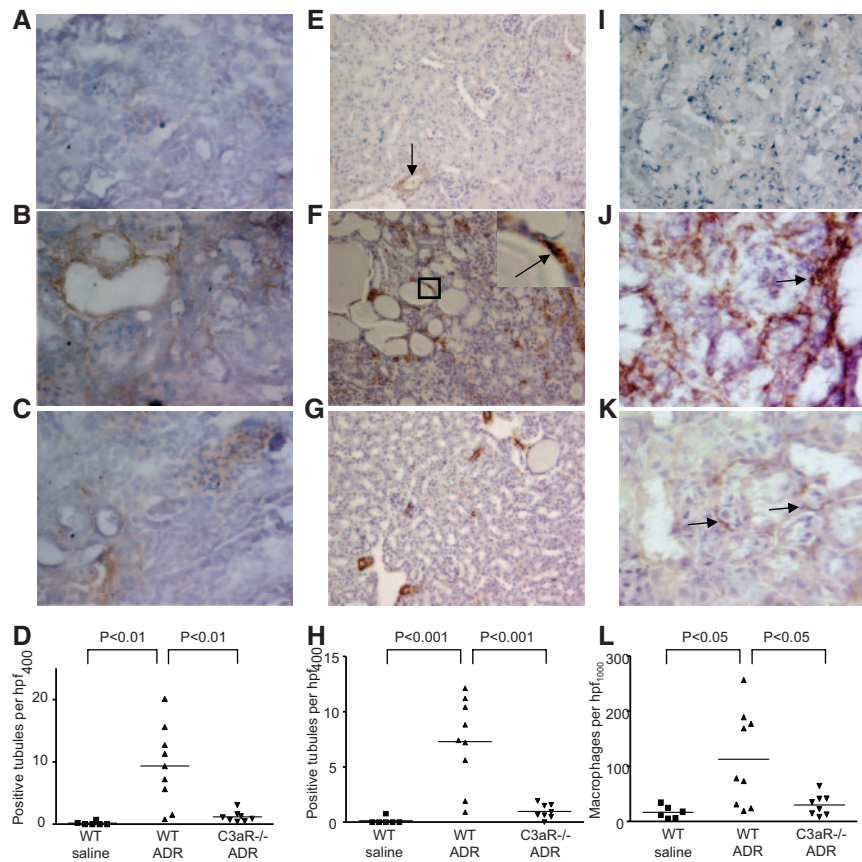


Figure 8. Collagen I and α -SMA staining. (A) Collagen I staining in the kidney of saline-injected control mice. No staining is seen. (B) After the induction of adriamycin nephropathy in wild-type mice, extensive collagen staining is seen around damaged tubules. (C) Less collagen I staining is present in the C3aR^{-/-} mice with adriamycin nephropathy. (D) The mean number of tubules surrounded by collagen I staining per $\times 400$ field was calculated on 10 nonoverlapping cortical fields for each mouse. (E) α -SMA staining is limited to blood vessel walls in saline-injected control sections (arrow). (F) Increased staining for α -SMA is seen in wild-type ADR mice, particularly around damaged tubules. α -SMA-positive tubular cells can be seen (inset, arrow). (G) Less staining is present in C3aR^{-/-} ADR mice. (H) The mean number of tubules surrounded by α -SMA staining per $\times 400$ field was calculated on 10 nonoverlapping cortical fields for each mouse. Significance values are shown in D and H. (I) Very few macrophages are seen in saline-injected control sections. (J) Infiltration of macrophages into the interstitium was seen in wild-type ADR mice. (K) Fewer macrophages are seen in the kidney of C3aR^{-/-} mice. (L) The number of macrophages per high power ($\times 1000$) field was counted in 20 nonoverlapping cortical fields. The mean is shown in all graphs. Magnifications: $\times 400$ in A through C; $\times 250$ in E through G; $\times 1000$ in I through K.

animal models, complement deficiency,^{17–19,23,36} depletion,²¹ or inhibition^{21,22} reduces the level of renal injury. This is the first report that the anaphylotoxin C3a is an important mediator of proteinuria-associated renal injury.

Expansion of the interstitium, with collagen deposition and cellular infiltration, is the hallmark of many progressive renal diseases. Myofibroblasts are found in the expanded interstitium and seem to be important in the loss of renal function, their presence predicting a poor renal prognosis.^{24,25} The origin of myofibroblasts is still controversial, but there is good evidence that some are derived from the tubular epithelium through EMT.^{27,40}

In proteinuria, tubular epithelial cells activate complement through the alternative pathway generating anaphylatoxins and C5b-9. We used NHS to model this *in vitro*. This may not

accurately reflect protein concentration or the duration of exposure that would occur *in vivo*. Nevertheless, PTECs activated complement, and this induced EMT. The failure of HIS to change PTEC phenotype is evidence that EMT was mediated by complement. This was confirmed by demonstrating that the same changes in PTEC phenotype occurred on exposure to purified C3a. In addition, the effects of serum could be inhibited by blocking the C3aR, providing direct evidence that complement C3a is important in this process.

It was shown previously by immunochemistry, *in situ* hybridization, and functional studies that C3aR is present on PTECs. Although some recent studies questioned the specificity of the antibodies used for immunolocalization,⁴¹ it is clear that PTECs do have the capacity to respond to C3a. Previous studies showed that C3a can induce TGF- β 1,⁴² collagen I,⁴³

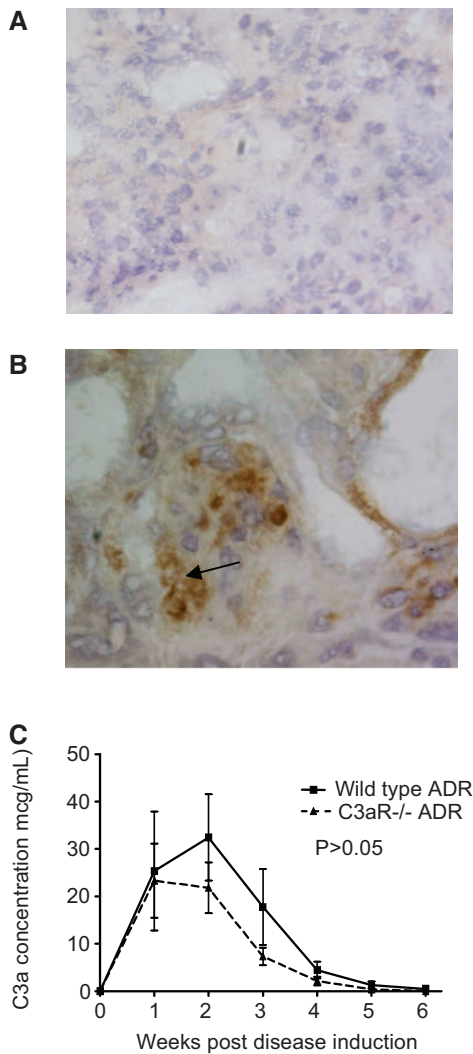


Figure 9. Localization of C3aR and C3a generation in adriamycin nephropathy. (A) No staining for C3aR was seen in C3aR^{-/-} ADR mice, confirming the specificity of the antibody used. (B) In wild-type ADR mice, both glomerular, predominantly mesangial (arrow), and tubular epithelial cell staining for C3aR was seen. (C) Urinary C3a concentration was measured by ELISA. A significant increase in urinary C3a concentration was seen in both wild-type ADR and C3aR^{-/-} ADR mice. Magnification, $\times 1000$.

and chemokine synthesis⁴⁴ in PTECs, and we have now shown that C3a can also induce EMT. The capacity of C3a to induce a change in the phenotype of other renal cells has also been reported. Wan *et al.*⁴⁵ demonstrated that C3a can change the phenotype of mesangial cells, which may have implications for understanding the effect of C3a in this mouse model of proteinuria.

We then investigated, using the adriamycin model of proteinuric nephropathy, whether these effects of C3a on PTEC phenotype had relevance *in vivo*. There are two distinct components to renal injury in adriamycin nephropathy. First is the glomerular injury that is thought to be due to toxic effects of adriamycin on the glomerular epithelial cell; however, it is

clear from this and other studies that immune factors, including complement activation¹⁷ and T lymphocytes,⁴⁶ are important in the development of glomerular injury. In this study, both the level of albuminuria, an indicator of disruption of the glomerular filtration barrier, and the degree of glomerular sclerosis were reduced by the absence of C3aR. Complement is activated in the glomerulus in adriamycin nephropathy by the alternative pathway.⁴⁷ C3aR expression in the glomerulus has been described in several studies^{43,48} and is clearly present in the glomeruli of mice with adriamycin nephropathy, primarily in a mesangial distribution. It is likely that an interaction between glomerular C3aR and locally generated C3a is responsible for the greater glomerular damage observed.

Similar to previous studies of adriamycin nephropathy C3a-deficient mice,^{17,49} the degree of tubulointerstitial injury was less in the C3aR-deficient mice. In addition, the deposition of interstitial collagen I and accumulation of myofibroblasts were significantly reduced in the absence of C3aR. This is consistent with the *in vitro* finding that C3a can induce EMT; however, C3a has many other possible actions, illustrated by the attenuated macrophage infiltrate in the absence of C3aR. C3aR deficiency therefore may influence myofibroblast accumulation by other mechanisms, for example infiltration of bone marrow-derived fibrocytes. A previous study of proteinuric C6-deficient rats demonstrated a reduction in interstitial myofibroblasts, implicating C5b-9 in this process. In our *in vitro* system, C5b-9 could induce some phenotypic changes in cells (actin reorganization) but did not downregulate E-cadherin or upregulate α -SMA, in contrast to C3a, which could induce all of these changes.

There is clearly a role for C3a in the development of renal tubulointerstitial injury, either directly or indirectly. It is possible that the reduced tubulointerstitial injury seen in the C3aR-deficient mice is a consequence of the less severe glomerular injury and reduced proteinuria; however, significant proteinuria still develops in the absence of C3aR, but the changes in the tubulointerstitial compartment are dramatically reduced. In addition, the reduction in tubulointerstitial injury seen in this study in the absence of C3aR is similar to that seen in studies in which complement is inhibited or depleted and in which the level of proteinuria is not affected.^{18–22} Overall, this suggests that C3a is contributing directly to tubulointerstitial injury. A role for C3a in renal injury is supported by the presence of C3a in the urine of proteinuric mice, the concentration increasing in parallel with albuminuria. In addition, C3aR expression increases on the tubular epithelium during disease development. This is consistent with localization of C3aR message by *in situ* hybridization during the development of murine lupus nephritis.⁵⁰ The factors regulating C3aR expression are being defined and are critical to understanding function.⁵¹

The result of the interaction between C3a and its receptor in this model of proteinuria is greater injury, reduced renal function, and consequently a higher mortality. This study provides important mechanistic information, enhancing our under-

standing of how complement causes injury. This may allow targeting of therapy at this effector limb of complement, which may prove both safer and more achievable than complete complement inhibition. In addition, it may provide insight into the mechanism of progressive, fibrotic diseases in other organs in which EMT has been implicated.

CONCISE METHODS

Reagents

The following antibodies were used in this study: Mouse anti-human E-cadherin (BD Biosciences, Oxford, UK), mouse anti- α -SMA (Sigma-Aldrich, Dorset, UK), mouse anti-human C3aR (BD Biosciences), monoclonal rat anti-mouse C3a (two clones; BD Biosciences), rat anti-mouse F4/80 (AbD Serotec, Oxfordshire, UK), rabbit anti-C3d (Dako UK Ltd., Cambridgeshire, UK), mouse anti-human β -actin (Sigma Aldrich), chicken anti-mouse C3aR (BMA Biomedicals, Augst, Switzerland), rabbit anti-mouse collagen I (Chemicon, Temecula, CA), AlexaFluor 488-conjugated goat anti-mouse IgG (Invitrogen, Paisley, UK), biotinylated goat anti-chicken Ig (Vector Laboratories, Burlingame, CA), and horseradish peroxidase (HRP)-conjugated rabbit anti-mouse IgG (Dako). Isotype control antibodies were purchased from Sigma. Human C3a was purchased from Cortex Biochem (Concord, Massachusetts) and C5b6, C7, C8, and C9 from Merck (Nottingham, UK). TGF- β 1 was from R&D Systems (Abingdon, UK). The C3aR-deficient mice were generated by homologous recombination and were provided by Dr. Bao Lu (Children's Hospital, Harvard Medical School, Boston, MA).⁵² Mice were backcrossed for 10 generations onto a Balb/c background. Age- and gender-matched control Balb/c mice were purchased from Harlan (Bicester, UK). Pooled normal human serum was collected from four volunteers and stored at -80°C until use. Serum was heat inactivated at 56°C for 30 min.

In Vitro Study of EMT

The renal proximal tubular cell line HKC8³⁷ was grown to subconfluence and treated with serum, TGF- β 1, or isolated complement proteins as described for each experiment. For Western blotting, cells were grown in six-well plates and lysed with 300 μl of lysis buffer with 1% protease inhibitor mix (both from Perbio, Northumberland, UK). Protein concentration was measured (BCA kit; Perbio), and 10 μg of cellular protein was separated on 10% polyacrylamide gels under reducing conditions. Proteins were transferred to a nitrocellulose membrane and blotted with anti-E-cadherin and anti- α -SMA. Membranes were stripped and reprobed with anti- β -actin. Cells were grown on glass coverslips, fixed in acetone at -20°C for 5 min, and then stained with either E-cadherin or α -SMA followed by a fluorochrome-conjugated secondary antibody.

Induction and Analysis of Adriamycin Nephropathy

All animal procedures were performed in accordance with United Kingdom Home Office regulations. Six- to 8-wk-old female C3aR $-/-$ or wild-type Balb/c mice were administered an injection of 10 mg/kg body wt Adriamycin (or equivalent volume of normal sal-

ine) *via* the tail vein.^{39,49} Mice were housed in metabolic cages for 24 h before disease induction and at weekly intervals thereafter to collect urine. Blood samples were taken before and at 3 and 6 wk after disease induction. Six weeks after disease induction, mice were killed and the kidneys were harvested for analysis. Urine albumin concentration was measured by radial immunodiffusion as described previously.⁵³ BUN was measured using a commercial kit (Sigma Aldrich) according to the manufacturer's instructions. Urinary C3a concentration was measured by ELISA using paired antibodies and purified mouse C3a (all from BD Biosciences).

Histologic Analysis

Histologic injury was assessed on PAS-stained sections using Lucia software (Jencons-PLS, East Sussex, UK). The glomerular capillary tuft area and the area occupied by PAS-positive sclerotic segments were calculated. The ratio of sclerotic area to tuft area was used as an index of glomerular sclerosis.^{46,54} The ratio of tubular epithelial cell height to tubule diameter was used as the index of tubular atrophy and dilation. Line morphometric measurements were used to measure the cross-sectional diameter and the tubular cell height of an individual cortical tubule. The cortical interstitial volume was defined as the peritubular space including peritubular capillaries. This area was quantified in five nonoverlapping, random cortical fields on each section. The ratio of interstitial volume to the total area was used as an index for interstitial expansion.⁵⁴ Histologic analysis was performed on $\times 400$ magnification images.

Immunohistochemistry

Collagen I staining was performed on frozen sections with 1:200 rabbit anti-mouse collagen I followed by in 1:200 HRP-conjugated goat anti-rabbit IgG. α -SMA was performed on formalin-fixed sections with α -SMA antibody (1:50,000 in PBS for 60 min) then in goat anti-mouse polymer conjugated with HRP (Envision; Dako). Antibody binding was visualized with DAB. C3aR was detected on frozen sections using chicken anti-mouse C3aR, biotinylated goat anti-chicken Ig, and streptavidin-HRP. After immunochemical staining for α -SMA and collagen I, the degree of staining was assessed by counting the number of tubules with surrounding collagen I or α -SMA staining per $\times 400$ field. Macrophages were detected using rat anti-mouse F4/80 and an HRP-conjugated secondary antibody.

PCR Analysis

Total RNA was extracted from cortex and reverse-transcribed. Semi-quantitative real-time PCR was used to quantify collagen I mRNA and β -actin mRNA using Taqman gene expression assays (Applied Biosystems, Warrington, UK). The ratio of collagen I to β -actin was calculated for each sample and normalized to a saline-injected control.

Statistical Analysis

Results are expressed as arithmetic means \pm SEM. Statistical analysis was performed using the statistical analysis option in the Prism 4 software. *T* test, one-way ANOVA (with Bonferroni post test for multiple comparisons) or two-way ANOVA was used. Log rank test was

used to compare survival. $P < 0.05$ was regarded as significantly different.

ACKNOWLEDGMENTS

Z.T. was supported by a traveling fellowship from the KC Wong Foundation.

DISCLOSURES

None.

REFERENCES

- Wehrmann M, Bohle A, Bogenschutz O, Eissele R, Freislederer A, Ohlschlegel C, Schumm G, Batz C, Gartner HV: Long-term prognosis of chronic idiopathic membranous glomerulonephritis: An analysis of 334 cases with particular regard to tubulo-interstitial changes. *Clin Nephrol* 31: 67–76, 1989
- Bohle A, Mackensen-Haen S, von Gise H: Significance of tubulointerstitial changes in the renal cortex for the excretory function and concentration ability of the kidney: A morphometric contribution. *Am J Nephrol* 7: 421–433, 1987
- Mackensen-Haen S, Bohle A, Christensen J, Wehrmann M, Kendziorra H, Kokot F: The consequences for renal function of widening of the interstitium and changes in the tubular epithelium of the renal cortex and outer medulla in various renal diseases. *Clin Nephrol* 37: 70–77, 1992
- Peterson JC, Adler S, Burkart JM, Greene T, Hebert LA, Hunsicker LG, King AJ, Klahr S, Massry SG, Seifter JL: Blood pressure control, proteinuria, and the progression of renal disease. The Modification of Diet in Renal Disease Study. *Ann Intern Med* 123: 754–762, 1995
- Eddy AA: Interstitial nephritis induced by protein-overload proteinuria. *Am J Pathol* 135: 719–733, 1989
- Remuzzi G, Benigni A, Remuzzi A: Mechanisms of progression and regression of renal lesions of chronic nephropathies and diabetes. *J Clin Invest* 116: 288–296, 2006
- Eddy AA, McCulloch L, Liu E, Adams J: A relationship between proteinuria and acute tubulointerstitial disease in rats with experimental nephrotic syndrome. *Am J Pathol* 138: 1111–1123, 1991
- Zandi-Nejad K, Eddy AA, Glassock RJ, Brenner BM: Why is proteinuria an ominous biomarker of progressive kidney disease? *Kidney Int Suppl* S76–S89, 2004
- Schulze M, Donadio JV Jr, Pruchno CJ, Baker PJ, Johnson RJ, Stahl RA, Watkins S, Martin DC, Wurzner R, Gotze O, Couser WG: Elevated urinary excretion of the C5b-9 complex in membranous nephropathy. *Kidney Int* 40: 533–538, 1991
- 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
- Abbate M, Zoja C, Corna D, Rottoli D, Zanchi C, Azzollini N, Tomasoni S, Berlinger S, Noris M, Morigi M, Remuzzi G: Complement-mediated dysfunction of glomerular filtration barrier accelerates progressive renal injury. *J Am Soc Nephrol* 19: 1158–1167, 2008
- Walport MJ: Complement. Second of two parts. *N Engl J Med* 344: 1140–1144, 2001
- Walport MJ: Complement. First of two parts. *N Engl J Med* 344: 1058–1066, 2001
- Ogrodowski JL, Hebert LA, Sedmak D, Cosio FG, Tamerius J, Kolb W: Measurement of SC5b-9 in urine in patients with the nephrotic syndrome. *Kidney Int* 40: 1141–1147, 1991
- Camussi G, Tetta C, Mazzucco G, Vercellone A: The brush border of proximal tubules of normal human kidney activates the alternative pathway of the complement system in vitro. *Ann NY Acad Sci* 420: 321–324, 1983
- 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
- Turnberg D, Lewis M, Moss J, Xu Y, Botto M, Cook HT: Complement activation contributes to both glomerular and tubulointerstitial damage in adriamycin nephropathy in mice. *J Immunol* 177: 4094–4102, 2006
- 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
- 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
- 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
- He C, Imai M, Song H, Quigg RJ, Tomlinson S: Complement inhibitors targeted to the proximal tubule prevent injury in experimental nephrotic syndrome and demonstrate a key role for C5b-9. *J Immunol* 174: 5750–5757, 2005
- Welch TR, Frenze M, Witte D, Davis AE: C5a is important in the tubulointerstitial component of experimental immune complex glomerulonephritis. *Clin Exp Immunol* 130: 43–48, 2002
- Goumenos DS, Brown CB, Shortland J, el Nahas AM: Myofibroblasts, predictors of progression of mesangial IgA nephropathy? *Nephrol Dial Transplant* 9: 1418–1425, 1994
- Roberts IS, Burrows C, Shanks JH, Venning M, McWilliam LJ: Interstitial myofibroblasts: Predictors of progression in membranous nephropathy. *J Clin Pathol* 50: 123–127, 1997
- Kalluri R, Neilson EG: Epithelial-mesenchymal transition and its implications for fibrosis. *J Clin Invest* 112: 1776–1784, 2003
- Iwano M, Plieth D, Danoff TM, Xue C, Okada H, Neilson EG: Evidence that fibroblasts derive from epithelium during tissue fibrosis. *J Clin Invest* 110: 341–350, 2002
- Zeisberg M, Hanai J, Sugimoto H, Mammoto T, Charytan D, Strutz F, Kalluri R: BMP-7 counteracts TGF-beta1-induced epithelial-to-mesenchymal transition and reverses chronic renal injury. *Nat Med* 9: 964–968, 2003
- Robertson H, Kirby JA, Yip WW, Jones DE, Burt AD: Biliary epithelial-mesenchymal transition in posttransplantation recurrence of primary biliary cirrhosis. *Hepatology* 45: 977–981, 2007
- Willis BC, Borok Z: TGF-beta-induced EMT: Mechanisms and implications for fibrotic lung disease. *Am J Physiol Lung Cell Mol Physiol* 293: L525–L534, 2007
- Willis BC, Liebler JM, Luby-Phelps K, Nicholson AG, Crandall ED, du Bois RM, Borok Z: Induction of epithelial-mesenchymal transition in alveolar epithelial cells by transforming growth factor-beta1: Potential role in idiopathic pulmonary fibrosis. *Am J Pathol* 166: 1321–1332, 2005
- Yang J, Liu Y: Dissection of key events in tubular epithelial to myofibroblast transition and its implications in renal interstitial fibrosis. *Am J Pathol* 159: 1465–1475, 2001
- Li M, Hering-Smith KS, Simon EE, Batuman V: Myeloma light chains induce epithelial-mesenchymal transition in human renal proximal tubule epithelial cells. *Nephrol Dial Transplant* 23: 860–870, 2008
- Manotham K, Tanaka T, Matsumoto M, Ohse T, Inagi R, Miyata T,

- Kurokawa K, Fujita T, Ingelfinger JR, Nangaku M: Transdifferentiation of cultured tubular cells induced by hypoxia. *Kidney Int* 65: 871–880, 2004
35. Copeland JW, Beaumont BW, Merriees MJ, Pilmore HL: Epithelial-to-mesenchymal transition of human proximal tubular epithelial cells: Effects of rapamycin, mycophenolate, cyclosporin, azathioprine, and methylprednisolone. *Transplantation* 83: 809–814, 2007
 36. Rangan GK, Pippin JW, Couser WG: C5b-9 regulates peritubular myofibroblast accumulation in experimental focal segmental glomerulosclerosis. *Kidney Int* 66: 1838–1848, 2004
 37. Racusen LC, Monteil C, Sgrignoli A, Lucskay M, Marouillat S, Rhim JG, Morin JP: Cell lines with extended *in vitro* growth potential from human renal proximal tubule: Characterization, response to inducers, and comparison with established cell lines. *J Lab Clin Med* 129: 318–329, 1997
 38. Moses HL, Branum EL, Proper JA, Robinson RA: Transforming growth factor production by chemically transformed cells. *Cancer Res* 41: 2842–2848, 1981
 39. Abe K, Li K, Sacks SH, Sheerin NS: The membrane attack complex, C5b-9, up regulates collagen gene expression in renal tubular epithelial cells. *Clin Exp Immunol* 136: 60–66, 2004
 40. Ng YY, Huang TP, Yang WC, Chen ZP, Yang AH, Mu W, Nikolic-Paterson DJ, Atkins RC, Lan HY: Tubular epithelial-myofibroblast transdifferentiation in progressive tubulointerstitial fibrosis in 5/6 nephrectomized rats. *Kidney Int* 54: 864–876, 1998
 41. Kiafard Z, Tschernig T, Schweyer S, Bley A, Neumann D, Zwirner J: Use of monoclonal antibodies to assess expression of anaphylatoxin receptors in tubular epithelial cells of human, murine and rat kidneys. *Immunobiology* 212: 129–139, 2007
 42. Peake PW, O'Grady S, Pussell BA, Charlesworth JA: C3a is made by proximal tubular HK-2 cells and activates them via the C3a receptor. *Kidney Int* 56: 1729–1736, 1999
 43. Braun MC, Reins RY, Li TB, Hollmann TJ, Dutta R, Rick WA, Teng BB, Ke B: Renal expression of the C3a receptor and functional responses of primary human proximal tubular epithelial cells. *J Immunol* 173: 4190–4196, 2004
 44. Thurman JM, Lenderink AM, Royer PA, Coleman KE, Zhou J, Lambris JD, Nemenoff RA, Quigg RJ, Holers VM: C3a is required for the production of CXC chemokines by tubular epithelial cells after renal ischemia/reperfusion. *J Immunol* 178: 1819–1828, 2007
 45. Wan JX, Fukuda N, Endo M, Tahira Y, Yao EH, Matsuda H, Ueno T, Matsumoto K: Complement 3 is involved in changing the phenotype of human glomerular mesangial cells. *J Cell Physiol* 213: 495–501, 2007
 46. Wang Y, Wang YP, Tay YC, Harris DC: Role of CD8(+) cells in the progression of murine adriamycin nephropathy. *Kidney Int* 59: 941–949, 2001
 47. Lenderink AM, Liegel K, Ljubanovic D, Coleman KE, Gilkeson GS, Holers VM, Thurman JM: The alternative pathway of complement is activated in the glomeruli and tubulointerstitium of mice with adriamycin nephropathy. *Am J Physiol Renal Physiol* 293: F555–F564, 2007
 48. Mizuno M, Blanchin S, Gasque P, Nishikawa K, Matsuo S: High levels of complement C3a receptor in the glomeruli in lupus nephritis. *Am J Kidney Dis* 49: 598–606, 2007
 49. Sheerin NS, Riskey P, Abe K, Tang Z, Wong W, Lin T, Sacks SH: Synthesis of complement protein C3 in the kidney is an important mediator of local tissue injury. *FASEB J* 22: 1065–1072, 2008
 50. Bao L, Osawe I, Haas M, Quigg RJ: Signaling through up-regulated C3a receptor is key to the development of experimental lupus nephritis. *J Immunol* 175: 1947–1955, 2005
 51. Schaefer M, Konrad S, Thalmann J, Rheinheimer C, Johswich K, Sohns B, Klos A: The transcription factors AP-1 and Ets are regulators of C3a receptor expression. *J Biol Chem* 280: 42113–42123, 2005
 52. Humbles AA, Lu B, Nilsson CA, Lilly C, Israel E, Fujiwara Y, Gerard NP, Gerard C: A role for the C3a anaphylatoxin receptor in the effector phase of asthma. *Nature* 406: 998–1001, 2000
 53. Robson MG, Cook HT, Botto M, Taylor PR, Busso N, Salvi R, Pusey CD, Walport MJ, Davies KA: Accelerated nephrotoxic nephritis is exacerbated in C1q-deficient mice. *J Immunol* 166: 6820–6828, 2001
 54. Kairaitis L, Wang Y, Zheng L, Tay YC, Wang Y, Harris DC: Blockade of CD40-CD40 ligand protects against renal injury in chronic proteinuric renal disease. *Kidney Int* 64: 1265–1272, 2003

See related editorial, "Proteinuria and Epithelia-to-Mesenchymal Transition: The Role of Complement," on pages 459–460.