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Complement Inhibitors Targeted to the Proximal Tubule Prevent Injury in Experimental Nephrotic Syndrome and Demonstrate a Key Role for C5b-9

Chun He,* Masaki Imai,* Hongbin Song,* Richard J. Quigg, † and Stephen Tomlinson2*

In glomerular diseases of diverse etiologies, dysfunction of the glomerular barrier to protein passage results in proteinuria, and proteinuria is considered an independent risk factor that plays a direct role in inflammation, interstitial fibrosis, and renal failure. The mechanism by which proteinuria leads to nephrotoxic injury is unclear, but a role for complement in mediating interstitial damage appears likely. We describe a strategy for Ag-specific targeting of complement inhibitors using a single chain Ab fragment and show that complement inhibitors targeted to the tubular epithelium protect against tubulointerstitial injury and renal dysfunction in a rat model of puromycin-induced nephrosis. The targeting of systemically administered complement inhibitors markedly enhanced their efficacy and obviated the need to systemically inhibit complement, thus reducing the risk of compromising host defense and immune homeostasis. Targeted inhibition of complement activation by Crry, and of membrane attack complex formation by CD59 was equally therapeutic, demonstrating that the MAC plays a key role in proteinuria-induced tubulointerstitial injury. CD59 activity was dependent on its being targeted to the site of complement activation, and this is the first report of specific inhibition of the MAC in vivo after systemic administration of inhibitor. The data establish the MAC is a valid target for pharmaceutical intervention in proteinuric disorders and provide an approach to investigate the role of the MAC in complement-dependent disease under clinically relevant conditions. The Journal of Immunology, 2005, 174: 5750–5757.

Most patients with glomerular proteinuria develop tubulointerstitial injury, and there is a well-established relationship between proteinuria, tubulointerstitial injury, and poor prognosis in kidney disease (1). Complement is implicated in the pathogenesis of proteinuria-associated tubulointerstitial injury (2, 3). During glomerular proteinuria, complement proteins are likely to be present in the glomerular filtrate, and proximal tubular cells from both rat and human kidneys activate complement (4, 5). There is also evidence that tubular cells are an important local source of complement, and proteinuric filtrate may contain nephrotoxic factors that activate tubular cells and up-regulate expression of complement proteins (2). Complement activation results in the formation of the C5 convertase, a central enzymatic complex that cleaves C3, leading to the generation of C3 opsonins and C3a. C5 convertase is also involved in the formation of the C5 convertase, an enzymatic complex that cleaves C5 to generate C5a and ultimately the membrane attack complex (MAC) or C5b-9. C3 opsonins, the proinflammatory C3a and C5a peptides, and the cytolitic and proinflammatory MAC are variously implicated in pathogenic mechanisms when complement is inappropriately activated.

Under normal circumstances, host cells are effectively protected from complement attack by membrane complement regulators that function by inhibiting either the C3 convertase or MAC formation. Human C3 inhibitors are CR1, decay-accelerating factor, and membrane cofactor protein. Rodents possess an additional C3 inhibitor termed Crry, a structural and functional analog of human CR1 (6). MAC formation is controlled by CD59 in both humans and rodents. Because complement inhibitors display species selective activity, the use of Crry and rodent CD59 in rodent models is appropriate.

Various types of complement inhibitory proteins, including several that are based on soluble forms of membrane complement inhibitors, are currently under investigation for therapy of inflammatory and ischemic disease and disease states associated with bioincompatibility (reviewed in Refs. 7–9). The best therapeutically characterized inhibitors of complement are soluble CR1 (sCR1) and an anti-C5 mAb that inhibits the generation of C5a and the MAC. Almost all previous therapeutic studies (in animal models and in the clinic) have been performed with systemic complement inhibitors, even though it is recognized that systemic suppression of the complement system may compromise host defense and immune homeostasis (7, 10, 11). Systemic inhibition at the C3 step is particularly undesirable due to important physiological functions of C3 and C5 activation products. Under conditions in which C3 and C5 activation products are also involved in disease pathogenesis, we hypothesize that appropriate targeting of a complement inhibitor that functions early in the pathway (such as Crry) will minimize systemic inhibition while maintaining an effective local concentration. In contrast, if the MAC plays a critical role in pathogenesis, complement inhibition late in the pathway (by CD59) will be advantageous because the generation of C3 and C5 activation products will not be altered. However, soluble CD59 is
not an effective inhibitor, and specific inhibition of the MAC after systemic administration has not been previously accomplished in vivo. Here, we explore an approach of targeting complement inhibitors to investigate therapeutic potential and pathogenic mechanisms in a well-characterized rat model of proteinuria-induced tubulointerstitial injury.

Materials and Methods

Animals, cells, and reagents

Normal rat serum was obtained from Cocalico Biologicals, and C6-deficient rat serum was a gift from Dr. W. M. Baldwin (Johns Hopkins University School of Medicine, Baltimore, MD). Mononuclear mouse anti-rat Crry, 512 (12), and mononuclear mouse anti-rat CD59, 6D1 (13), are described. Antiserum against rat C9 that recognizes MAC in rat tissue was a gift from Dr. Paul Morgan (University of Wales, Cardiff, U.K.). Anti-rat C3 serum was from MP Biologicals. Rabbit antiserum to rat Crry, human CD59 (cross-reactive with rat CD59), and K9/9 single-chain Fv (scFv) were prepared by Cocalico Biologicals. All secondary Abs were purchased from Sigma-Aldrich. Female Sprague-Dawley rats weighing 45–50 g were purchased from Harlan and were housed with free access to food and water.

Experimental protocols were conducted according to current guidelines presented in the National Institute of Health Guide for the care and use of laboratory animals. The rat proximal tubule epithelial cell (PTEC) line was obtained from American Type Culture Collection and cultured in DMEM with 10% FCS.

Recombinant DNA techniques

For the cloning of Ab variable region cDNA, total RNA was extracted from the hybridoma cell line expressing K9/9 mAb (14) using a RNA Extract Kit (Qiagen). Variable region cDNAs (V\textsubscript{L} and V\textsubscript{H} chains) were amplified by RT-PCR. The 5\textsuperscript{-} and 3\textsuperscript{-}primers used for V\textsubscript{L} chain were ATGAAGTGCGAGTGTGCTGC and ACTGGATGGTGGGAA GATGG. The 5\textsuperscript{-} and 3\textsuperscript{-}primers used for V\textsubscript{H} chain were ATGAAATGTCGCCGTAGGCCTGGTGGCTG and ACTGGATGGTGGGAA GATGG. PCR fragments were subcloned into TA cloning vector (Invitrogen) and VL and VH chains joined by a (G\textsubscript{S}8)\textsubscript{S} linker. For construction of targeted complement inhibitors, the K99 targeting scFv was linked to cDNA encoding an extracellular domain of rat Crry (five N-terminal short consensus complement inhibitors, the K9/9 targeting scFv was linked to cDNA encoding Crry). PCR fragments were subcloned into TA cloning vector (Invitrogen) and VL chain was verified. Antiserum against rat C9 that recognizes MAC in rat tissue was a gift from Dr. W. M. Baldwin (Johns Hopkins University School of Medicine, Baltimore, MD). Monoclonal mouse anti-rat C57BL/6 was a gift from Dr. Paul Morgan (University of Wales, Cardiff, U.K.). Anti-rat C9 that recognizes MAC in rat tissue was a gift from Dr. W. M. Baldwin (Johns Hopkins University School of Medicine, Baltimore, MD). Monoclonal mouse anti-rat C9 was a gift from Dr. W. M. Baldwin (Johns Hopkins University School of Medicine, Baltimore, MD). Monoclonal mouse anti-rat C9 was a gift from Dr. W. M. Baldwin (Johns Hopkins University School of Medicine, Baltimore, MD).

Expression and purification of recombinant proteins

For the cloning of Ab variable region cDNA, total RNA was extracted from the hybridoma cell line expressing K9/9 mAb (14) using a RNA Extract Kit (Qiagen). Variable region cDNAs (V\textsubscript{L} and V\textsubscript{H} chains) were amplified by RT-PCR. The 5\textsuperscript{-} and 3\textsuperscript{-}primers used for V\textsubscript{L} chain were ATGAAGTGCGAGTGTGCTGC and ACTGGATGGTGGGAA GATGG. The 5\textsuperscript{-} and 3\textsuperscript{-}primers used for V\textsubscript{H} chain were ATGAAATGTCGCCGTAGGCCTGGTGGCTG and ACTGGATGGTGGGAA GATGG. PCR fragments were subcloned into TA cloning vector (Invitrogen) and VL and VH chains joined by a (G\textsubscript{S}8)\textsubscript{S} linker. For construction of targeted complement inhibitors, the K99 targeting scFv was linked to cDNA encoding an extracellular domain of rat Crry (five N-terminal short consensus complement inhibitors, the K9/9 targeting scFv was linked to cDNA encoding Crry). PCR fragments were subcloned into TA cloning vector (Invitrogen) and VL chain was verified.

In vitro analysis of binding

Rat PTECs were detached with 0.05 M EDTA in PBS, washed twice, and resuspended to 1 × 10\textsuperscript{6} ml in DMEM. Cells were then sensitized to complement by incubation (4°C for 30 min) in 10% rabbit antiserum raised against rat 13762 mammary adenocarcinoma cells (16). After sensitization, an equal volume of rat serum diluted in DMEM was added, and cell viability was determined after 45 min at 37°C using the CytoTox 96 Non-Radioactive Cytotoxicity Assay Kit (Promega). Cell lysis assays were typically performed in 96-well ELISA plates in a final volume of 100 μl. To determine the effect of complement inhibitors on complement-mediated lysis, the recombinant proteins in PBS were added after Ab sensitization, and incubations were continued for a further 30 min at 4°C before addition of rat serum. In some experiments, cells were washed twice with DMEM before addition of rat serum. Percentage of inhibition of lysis was calculated as (A – B)/A, where A = percentage of cell lysis in 10% normal rat serum and B = percentage of cell lysis in 10% normal rat serum with recombinant protein. The effect of the complement-inhibitory proteins on C3 deposition was determined by flow cytometry. For flow cytometry, the procedure described above for cell lysis determinations was followed except that 10% C6-deficient rat serum was used in place of normal rat serum. After incubation in serum, the cells were washed, and C3 deposition was detected by means of anti-rat C3 Ab-FTTC by standard procedures (17).

In vivo blood clearance and biodistribution of radiolabeled recombinant proteins

For determination of rate of clearance of the circulation, iodinated recombinant proteins (1 × 10\textsuperscript{7} cpm) were injected into the tail vein; blood was collected at 30 min, 1 h, 2 h, 4 h, and 8 h after injection; and radioactivity in blood was counted. Counting half-life (t\textsubscript{1/2}) was calculated by standard methods (18). Biodistribution studies were performed using standard procedures for determining tissue distribution of injected radiolabeled proteins (18, 19). Briefly, 1 × 10\textsuperscript{7} cpm of 125I-labeled soluble Crry (sCrry; 1.2 × 10\textsuperscript{7} cpm/μg), targeted Crry (tCrry) (1.3 × 10\textsuperscript{7} cpm/μg), targeted CD59 (tCD59; 1.2 × 10\textsuperscript{7} cpm/μg), and targeting vehicle only (t-vehicle) (10.1 × 10\textsuperscript{7} cpm/μg) were injected into the tail vein of 40- to 50-g female rats. After 48 h, a blood sample was taken, and major organs were removed, shredded, washed in PBS containing 10 mM EDTA, weighed, and counted. Targeting specificity was determined as percentage of cpm per gram of tissue divided by total injected cpm per gram of body weight. Percentage = A/B × 100, where A = tissue cpm per gram of tissue weight and B = total cpm per gram of body weight. All animal studies were approved by the Institutional Animal Care and Use Committee institutional review board at Medical University of South Carolina.

Serum complement-inhibitory activity

Ab-sensitized sheep erythrocytes (1 × 10\textsuperscript{7}; Advanced Research Technologies) were incubated in serum dilutions in a final volume of 300 μl at 37°C for 1 h. Gelatin-veronal buffer-EDTA (Sigma-Aldrich) was then added, and hemolysis was determined by measuring OD\textsubscript{414} of supernatants (20). Total complement hemolytic activity (CH50) was defined as the reciprocal of the dilution of serum that lysed 50% of the erythrocytes; this measurement was used to compare serum complement activity. After i.p. injection of recombinant complement inhibitors (40 mg/kg), serum was collected at different times and percentage of complement inhibitory activity in sera was defined as the difference between CH50 of normal serum and CH50 of sample divided by CH50 of normal serum.

Rat model of puromycin aminonucleoside-induced nephrosis and experimental protocol

Proteinuria-induced tubulointerstitial injury was induced in female Sprague-Dawley rats (Harlan) weighing 45–50 g by single tail vein injection of 150 mg/kg puromycin aminonucleoside (PAN). Proteinuria develops by day 4. In a dose-response pilot study, 20 rats were divided into 10 groups of 2 animals. Nine groups received PAN, and a control group received saline. On days 4, 6, 8, and 10, control rats received an i.p. injection of PBS, and each group of PAN-treated rats received i.p. injections of varying doses (5–40 mg/kg) of sCrry or tCrry. After the final injection, rats were placed in metabolic cages, and urine was collected for 24 h. On day 11, blood was collected, rats were sacrificed, and kidneys were removed. In a larger therapeutic study, 24 rats were divided into 6 groups of 4 animals. Five groups received PAN, and a control group received saline. Control rats received i.p. injections of PBS, and PAN-treated rats received i.p. injections of varying doses (5–40 mg/kg) of sCrry or tCrry. After the final injection, rats were placed in metabolic cages, and urine was collected for 24 h. On day 11, blood was collected, rats were sacrificed, and kidneys were removed. In a larger therapeutic study, 24 rats were divided into 6 groups of 4 animals. Five groups received PAN, and a control group received saline. Control rats received i.p. injections of PBS, and PAN-treated rats received i.p. injections of PBS, sCrry, tCrry, tCD59, or targeting vehicle only (Table I) at 40 mg/kg using the same protocol described above for pilot study.

Renal function and histology

Urinary protein was measured using a bicinchoninic acid protein assay kit (Pierce). Creatinine clearance, a measure of renal function, was calculated after the measurement of rat serum and urine creatinine with a creatinine regent kit (Roche Pharmaceuticals) according to manufacturer’s instructions. For histological assessment of tubulointerstitial injury, paraffin-embedded kidney sections were stained with periodic acid-Schiff reagent, and tubulointerstitial injury was assessed in a blinded manner. Tubulointerstitial injury is defined as tubular dilatation, tubular atrophy, tubular cast formation, sloughing of tubular epithelial cells, or thickening of the tubular epithelium.
basement membrane and was scored on a scale of 0–4, as follows: 0, no injury; 1, <25% of the tubulointerstitium injured; 2: 25–50% of the tubulointerstitium injured; 3, 51–75% of the tubulointerstitium injured; 4: >75% of the tubulointerstitium injured.

**Immunofluorescence microscopy**

To investigate C3 and MAC deposition, frozen kidney sections were stained with anti-rat C3 or anti-rat C9 antisera together with appropriate FITC-labeled secondary Abs as described (21). The binding of targeted complement inhibitors in the kidney was similarly analyzed using rabbit antisera raised against K9/9 scFv. Digital images were acquired and optimized with Adobe Photoshop using identical settings. Neutrophil infiltration was also assessed by immunofluorescence microscopy using polymorphonuclear leukocyte-specific mouse anti-rat granulocyte mAb (BD Biosciences) and by measuring myeloperoxidase activity in kidney tissue samples as described (22).

**Statistics**

Data are presented as mean ± SD. Data were analyzed by global ANOVA followed by the post hoc test (Scheffe’s procedure). For dose-response experiments, group comparisons were done with the Kruskal-Wallis test.

**Results**

**Recombinant complement inhibitors**

The targeted complement-inhibitory proteins prepared were rat Crry and CD59 linked to a scFv-targeting moiety derived from K9/9 mAb that binds in vivo to an unidentified Ag on rat glomerular and proximal tubular epithelial cells (14). In addition to targeted Crry and CD59 (tCrry, tCD59), soluble untargeted forms of Crry and CD59 (sCrry, sCD59) were prepared, along with a K9/9 scFv which served as a t-vehicle. All purified proteins migrated as a single band of expected molecular mass after SDS-PAGE (data not shown).

**In vitro characterization of recombinant complement inhibitors**

The targeting and complement inhibitory function of the recombinant proteins in vitro was analyzed using rat kidney PTEC as target cells. To assess the cell surface binding of the recombinant proteins, the binding of radiolabeled proteins to PTEC was measured. Iodinated tCrry, tCD59, and t-vehicle all bound PTEC, and scFv specificity was demonstrated by the ability of unlabeled scFv to inhibit binding of labeled proteins (Fig. 1). To determine functional activity, PTEC were sensitized to complement with a polyclonal Ab and the effect of the recombinant proteins on rat complement-mediated cell lysis (Fig. 2, A and B) and on C3 deposition (Fig. 2C) was measured. tCrry, tCD59, and sCrry were equally effective at inhibiting rat complement-mediated lysis of PTEC. Untargeted sCD59 was a poor inhibitor of complement, and this result is consistent with previous data on sCD59 activity (21, 23). If complement inhibitors were removed after incubation with PTEC, but before the addition of rat serum, only tCrry and tCD59 were protective, demonstrating that the complement inhibitors were functional when membrane bound (data not shown). tCrry also inhibited complement activation and C3 deposition on PTEC, and as expected tCD59 had no effect on C3 deposition (Fig. 2C). The scFv targeting vehicle alone had no complement-inhibitory activity. Because sCD59 did not effectively inhibit complement in vitro, further in vivo characterization of sCD59 was not pursued.

**Biodistribution and pharmacokinetics of recombinant complement inhibitors**

To determine whether tCrry and tCD59 target to the rat kidney, we performed a biodistribution study using iodinated proteins. Forty-eight hours after tail vein injection of 8–10 μg radiolabeled tCrry, tCD59, or t-vehicle (at similar specific activities; see Materials and Methods), rats were killed, and the biodistribution of radiolabeled proteins was determined in the absence and presence of 100-fold excess of unlabeled protein (mean ± SD; n = 3).

**Table I. Treatment groups and resulting proteinuria**

<table>
<thead>
<tr>
<th>Group</th>
<th>PAN (mg/kg)</th>
<th>Therapy</th>
<th>Urinary Protein (mg/24 h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>150</td>
<td>PBS</td>
<td>134 ± 16.4</td>
</tr>
<tr>
<td>II</td>
<td>150</td>
<td>t-Vehicle</td>
<td>150 ± 15.8</td>
</tr>
<tr>
<td>III</td>
<td>150</td>
<td>sCrry</td>
<td>131 ± 7.5</td>
</tr>
<tr>
<td>IV</td>
<td>150</td>
<td>tCrry</td>
<td>142 ± 18.0</td>
</tr>
<tr>
<td>V</td>
<td>150</td>
<td>tCD59</td>
<td>148 ± 24.0</td>
</tr>
<tr>
<td>VI</td>
<td>Saline</td>
<td>PBS</td>
<td>0.56 ± 0.02</td>
</tr>
</tbody>
</table>

*Urinary protein was determined during a 24-h period after therapy and just before sacrifice. There was no significant difference between PAN-treated groups.
Methods), we measured the percentage of administered protein bound in the kidney and other selected organs. A significantly higher proportion of radioactivity was localized to the kidney compared with the other organs that were examined (Fig. 3). The radiolabeled proteins targeted the kidney in both healthy and PAN-treated proteinuric rats (described below) with somewhat higher levels of binding in proteinuric rats, perhaps reflecting increased access to targeting Ag on tubular cells (K9/9 mAb was shown previously to bind glomerular and tubular Ags in vivo; Ref. 14). Binding of sCrry in the kidney was not detected. Although a high concentration of targeted complement inhibitors remained in the kidneys at 48 h relative to other organs, the circulatory half-life of the proteins was short; the $t_{1/2}$ in healthy rats for tCrry, tCD59, sCrry, and t-vehicle was 38, 33, 34, and 18 min, respectively (Fig. 4). The $t_{1/2}$ in proteinuric rats was not significantly different.

Characterization of complement inhibitors in a rat model of tubulointerstitial injury

We next investigated the recombinant complement inhibitors therapeutically in a well-characterized rat model of nonimmunologic proteinuria-associated tubulointerstitial injury. Our protocol consisted of injecting rats with PAN to induce proteinuria on day 0, followed by i.p. injection of either PBS, complement inhibitor or t-vehicle on days 4, 6, 8, and 10. Urine was collected during a 24-h period after the final injection; on day 11, the rats were sacrificed, blood was collected, and the kidneys removed for analysis. Using this protocol, we initially performed a dose-response pilot study using tCrry and sCrry (Fig. 5). tCrry, but not sCrry, was therapeutically effective at the higher doses tested, and both creatinine clearance and tissue injury score were statistically different ($p < 0.05$ comparing treated and control groups). Based on the pilot data, a larger therapeutic study ($n = 4$) was undertaken using all of the recombinant proteins at a dose of 40 mg/kg (Table I). As expected, PAN induced heavy proteinuria, and in agreement with published data (24), proteinuria appeared before the first dose of...
complement inhibitor (day 4), indicating that the model is clinically relevant. Also, to address the most clinically relevant situation, we chose to administer individual inhibitors rather than the two combined at a time when proteinuria was evident. There was no significant difference in urinary protein levels between the treatment groups after the final therapeutic injection (Table I), and as expected PAN treatment significantly impaired renal function as measured by creatinine clearance. In proteinuric rats, relative creatinine clearance was 40% of normal, and clearance was not significantly improved with sCry treatment (Fig. 6). In contrast, creatinine clearance in proteinuric rats receiving either tCry or tCD59 was significantly improved and was 89 and 84% of normal, respectively (p < 0.01). Both tCry and tCD59 individually provided a level of protection that was not statistically different from injury seen in the control group (rats not receiving puromycin). Targeting vehicle alone had no effect on creatinine clearance. Histological examination of kidney sections prepared from rats treated with PAN and receiving no therapy revealed dilation of tubular lumina and tubular and epithelial cell degeneration as assessed by loss of brush border (Figs. 7 and 8). Minimal improvement was seen with sCry therapy. However, tubular dilation and degeneration was significantly and similarly suppressed in proteinuric rats receiving either tCry or tCD59 (Figs. 7 and 8). We did not measure creatinine clearances or perform survival renal biopsies to assess tubular injury scores before administration of targeted inhibitors, but we would expect these measurements to be relatively normal at this time even though proteinuria was present. Our data show that we prevented the observed pathophysiological effects seen in control treated proteinuric animals.

To investigate the functional consequence of the complement inhibitors in vivo, we examined C3 and MAC deposition on proximal tubules from rats in the different treatment groups. Proximal tubules of proteinuric rats that received no therapy revealed deposition of tubular lumina and tubular and epithelial cell degeneration as assessed by loss of brush border (Figs. 7 and 8). Minimal improvement was seen with sCry therapy. However, tubular dilation and degeneration was significantly and similarly suppressed in proteinuric rats receiving either tCry or tCD59 (Figs. 7 and 8). We did not measure creatinine clearances or perform survival renal biopsies to assess tubular injury scores before administration of targeted inhibitors, but we would expect these measurements to be relatively normal at this time even though proteinuria was present. Our data show that we prevented the observed pathophysiological effects seen in control treated proteinuric animals.

FIGURE 7. Histological assessment of tubulointerstitial injury. Periodic acid-Schiff reagent staining of kidney sections from different treatment groups. Representative images shown.

FIGURE 8. Semiquantitative analysis of tubulointerstitial injury as assessed by tubular dilation and degeneration. Injury was defined using a scale of 0–4 as defined in Materials and Methods.
difference in serum complement activity between healthy control mice and proteinuric mice treated with either PBS or the different inhibitors. Thus, neither ongoing disease nor inhibitor treatment affected serum complement levels (determined by hemolytic assay; data not shown).

**Discussion**

The therapeutic potential of complement inhibition for the treatment of inflammatory and ischemic disease and disease states associated with bioincompatibility is now well established. The vast majority of previous therapeutic studies (in animal models and in the clinic) have been performed with systemic complement inhibitors, even though there are concerns regarding bioavailability and the fact that systemic inhibition of complement may compromise host defense and immune homeostasis. Systemic inhibition at the C3 step is particularly undesirable due to important physiological functions of C3 and C5 activation products. The potential advantages of targeted complement inhibition have been discussed in the literature during the past few years, and in vitro studies have demonstrated the feasibility of a targeted approach to complement inhibition. More recently, specific in vivo targeting of a complement inhibitor (human decay-accelerating factor) to sites of complement deposition and to erythrocytes have been demonstrated in mice using a soluble CR2 (21) and an Ab fragment (25), respectively, but functional characterization in a model of disease has not been reported. Here, we show that appropriate targeting of a systemically administered complement inhibitor to a site of complement-dependent tissue injury both significantly enhances efficacy compared with an untargeted counterpart and obviates the need to systemically inhibit complement.

tCry and tCD59 individually provided similar levels of protection that was not statistically different from the control group. These data, coupled with a growing literature on the role of alternative pathway-activated C5b-9-mediated tubular injury in proteinuric conditions, makes it likely that the effect we observed was due to Cry preventing alternative pathway C3 activation and CD59 preventing C5b-9 generation, with either being effective and equivalent. Thus, in proteinuric tubulointerstitial injury, the effects of C5b-9 predominate, rather than the proinflammatory C3a, C3b, and C5a proteins, which likely reflects the inaccessibility of inflammatory cells bearing receptors for the latter, whereas C5b-9 can act directly on any cell.

For CD59 to function effectively it must be bound at the site of MAC formation. This functional constraint on CD59 has been shown in vitro (21, 23) and more recently in vivo by the demonstration that a membrane-inserting form of CD59 when injected intraarticularly ameliorated disease in a model of rheumatoid arthritis (26). We show here for the first time specific inhibition of the MAC in vivo after the systemic administration of an inhibitor.
and demonstrate that the MAC plays a key role in proteinuria-induced tubulointerstitial injury.

Specific targeting of systemically administered CD59 will provide the means to investigate the pathologic role of the MAC in various complement-dependent diseases under clinically relevant conditions. In this context, complement-deficient animals have provided for a much better understanding of complement-associated disease mechanisms, but the data can be at odds with results obtained from studies in which complement has been temporarily inhibited. Complement deficiency may influence development of other immune functions, with the possible modulation of compensatory pathways. Also, there is likely to be some remaining functionality after the administration of a complement inhibitor, and this may have a bearing on therapeutic outcome, because complement can have both protective and injurious effects during disease. Because most patients will have normal or only partially depleted complement levels, the availability of complement inhibitors that can be targeted to a site of disease and that can inhibit complement at different points in the pathway provides a clinically relevant means to study the role of complement in experimental disease.

To reiterate, for CD59-based inhibition, targeting is a requirement for effective function. Our demonstration of specific MAC inhibition in vivo after the systemic administration of inhibitor has important implications for proteinuric disorders and also other disease conditions in which the MAC has a major pathogenic role; a CD59-based inhibitor will not interfere with the generation of the complement activation products that are important in normal physiological processes (7, 10, 11). For complement-dependent disease processes involving early complement activation fragments, the targeting of C3 (and potentially C5) inhibitors offers advantages of improved efficacy and safety due to low levels of systemic inhibition. Indeed, a short circulatory half-life of a targeted complement inhibitor may be advantageous, and effective therapy was achieved in our model with very low levels of systemic complement inhibition. The rapid decline in serum complement inhibitory levels after i.p. injection indicate that the targeted inhibitors were effective due to their presence on the tubular surface for an extended period. Of note, however, are the immunofluorescence studies that show binding of the targeted constructs to tubules. Analysis was performed on tissue sections prepared from kidneys removed 7 days after onset of proteinuria and complement activation, and the intracellular staining pattern is indicative of active endocytosis.

The C3 inhibitor sCR1 has shown therapeutic benefit in a variety of animal models of disease, and therapeutically effective doses maintain systemic inhibition for \(>24\) h (27). A sialyl Lewis\(^x\) (sLe\(^x\)) glycosylated form of sCR1 has been shown to bind selectins in vitro, albeit with low affinity, and to localize to the vasculature in some models of inflammatory disease (28–32). However, sLe\(^x\) sCR1 and sCR1 have similar pharmacokinetics and serum inhibitory activity (33), and therapeutically effective doses maintain systemic inhibition. The therapeutic benefit of sLe\(^x\) sCR1 compared with sCR1 was seen in some models of inflammatory disease, and the available data indicate that an important benefit of sLe\(^x\) sCR1 is derived from its ability to act as both selectin antagonist and complement inhibitor (28, 33, 34).

Relevant to the work described here are variants of the PAN nephrosis model. Animals given either repeated s.c. doses or a single high dose of PAN directly into the jugular vein develop glomerulosclerosis as a late finding (35, 36). Given the benefit of blocking angiotensin II generation in the remnant kidney model, which is also characterized by chronic progressive renal insufficiency and glomerulosclerosis (37), angiotensin-converting enzyme inhibitors have been used in the PAN nephrosis model. A high dose of enalapril can reduce proteinuria in the early phase of disease, while at the same time lowering systemic blood pressure (38). With continued use, there is an improvement in the ultimate development of late phase proteinuria, glomerulosclerosis, and tubulointerstitial pathology, which is likely due to hemodynamic effects (reducing proteinuria), as well as prevention of direct angiotensin II actions (38–40). Based upon our data and the results of past studies, a viable explanation for the effectiveness of angiotensin-converting enzyme blockade is that by leading to reduced proteinuria it leads to lesser amounts of complement proteins available for activation on tubular cells.

The glomerulus is an effective barrier preventing the passage of protein into the nearly 170 liters of ultrafiltrate formed daily in humans. This restriction occurs in a size-selective manner. Because almost all proteins of the complement activation cascade are large (molecular mass, \(\sim 200\) kDa), few of these proteins will appear in this ultrafiltrate and reach tubular lumina under normal circumstances. As such, the apical aspects of tubules are not endowed with significant quantities of proteins that regulate the cleavage of C3/C5 or formation of the MAC (4, 5, 41). Consistent with this, complement can be spontaneously activated on tubular cells in vitro (42). In any disease in which the glomerular barrier to protein passage is disrupted, complement proteins can appear in the tubular fluid and lead to complement activation. This is supported by circumstantial findings in humans showing complement activation products on tubules (43) and in the urine of proteinuric patients (44–46). Such complement activation can occur in any condition accompanied by proteinuria, including immunological diseases as well as those not considered to have an immunological basis, such as the common disease diabetic nephropathy. Studies in experimental rodents have been very consistent in showing a complement dependence of progressive tubulointerstitial damage in proteinuric conditions. Deficiency of C3 and C4 (47), complement depletion (48), and systemic complement inhibition (49) result in significantly reduced tubulointerstitial injury, whereas down-regulating a complement inhibitor on proximal tubules promotes injury (50). The fact that C6-deficient rats with experimental nephrotic syndrome were protected from tubulointerstitial injury implicates formation of the MAC as being pathogenic (51, 52); this is consistent with the findings in our study in which targeted delivery of CD59 ameliorated disease. Hence, preventing complement activation in any kidney disease in which proteinuria occurs has the potential to prevent or delay the development of progressive renal insufficiency and end stage renal disease (53), and the current data establish the benefits of targeted complement inhibition with regard to both efficacy and safety.

![Figure 10](http://www.jimmunol.org/Downloaded from http://www.jimmunol.org/)