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Overexpression of complement inhibitor Crry does not prevent cryoglobulin-associated membranoproliferative glomerulonephritis

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Overexpression of complement inhibitor Crry does not prevent cryoglobulin-associated membranoproliferative glomerulonephritis.

Background. Mice overexpressing thymic stromal lymphopoietin (TSLP) develop mixed cryoglobulinemia with renal disease closely resembling human cryoglobulin-associated membranoproliferative glomerulonephritis (MPGN), including glomerular deposits of immunoglobulins and complement. We assessed the effect of complement inhibition through overexpression of Crry (complement receptor-1 related gene/protein Y), which blocks the classic and alternative pathway of complement activation through inhibition of the C3 convertase, in cryoglobulinemia-associated immune complex glomerulonephritis.

Methods. TSLP transgenic mice were crossbred with animals overexpressing Crry. Mice were sacrificed after 50 days (females) or 120 days (males), and kidneys, blood, and urine were collected from seven mice of each experimental group (wild type, Crry transgenic, TSLP transgenic, and Crry/TSLP doubly transgenic).

Results. TSLP/Crry doubly transgenic animals demonstrated expected serum levels of Crry. Renal involvement, both in TSLP transgenic and TSLP/Crry doubly transgenic animals, was characterized by glomerular matrix expansion, macrophage influx, activation of mesangial cells, and deposition of immunoglobulins and complement. Overexpression of Crry did not result in significant improvement of renal pathology or laboratory findings. Expression of recombinant soluble Crry was confirmed by enzyme-linked immunosorbent assay (ELISA) in Crry transgenic animals. However, formation of the membrane attack complex C5b-9 as a marker of terminal active complement components and represented by glomerular C9 staining could not be inhibited in Crry transgenic TSLP mice.

Conclusion. These results indicate that overexpression of Crry was not sufficient to prevent renal injury in TSLP transgenic mice. We suggest that the inhibitory capacity of Crry may be overwhelmed by chronic complement activation. Further studies need to address the role of complement in cryoglobulinemic glomerulonephritis before therapeutic complement inhibition can be attempted.

Key words: complement, Crry, immune complexes, membranoproliferative glomerulonephritis (MPGN), cryoglobulins.

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Cryoglobulins are immunoglobulins (Ig) or immune complexes that precipitate in the cold and redissolve after rewarming [1]. The current classification of cryoglobulins distinguishes three types. Type I cryoglobulins consist of monoclonal immunoglobulins, and are usually associated with hematologic disorders. Mixed cryoglobulins, types II and III, are immune complexes composed of either a monoclonal IgM with rheumatoid factor activity that binds to polyclonal IgG (type II), or a mixture of polyclonal IgM and polyclonal IgG (type III) [1, 2]. Typical manifestations of cryoglobulinemia include vasculitis, arthralgia, skin purpura, and can include specific organs such as kidney, central nervous system, gut, and, rarely, the lung [3, 4]. About one third of patients develop renal disease that is histologically characterized by a membranoproliferative glomerulonephritis with distinctive features of macrophage influx, intracapillary deposits of cryoglobulin aggregates, microtubular appearance of glomerular immune deposits as revealed in ultrastructural studies in some cases, and at times vasculitis [5–7].

We have recently described a mouse model of cryoglobulinemic glomerulonephritis in which the overexpression of thymic stromal lymphopoietin (TSLP), a cytokine that promotes B-cell development, leads to the production of large amounts of type III cryoglobulins [8]. TSLP transgenic mice develop a systemic

inflammatory disease that involves the skin, lungs, liver, and spleen. Renal pathology closely resembles human cryoglobulin-associated membranoproliferative glomerulonephritis (MPGN). Glomeruli show subendothelial and mesangial immune deposits, increased glomerular matrix, glomerular macrophage influx, and occlusion of capillary loops by periodic-acid Schiff (PAS)-positive material that ultrastructurally is shown to be immune complexes with microtubular organization. Immunofluorescence displays large glomerular deposits of immunoglobulins (IgG, IgM, IgA) and the complement component C3.

Complement activation plays an important role in a variety of glomerular injuries, and its regulation is critical for the preservation of renal integrity [9, 10]. Although complement usually acts as a host defense mechanism, inappropriate activation of the complement cascade can lead to renal injury via the generation of chemotactic factors, and by insertion of the membrane attack complex C5b-9 (MAC) into the membranes of resident glomerular cells, which can then lead to cellular activation or injury with subsequent release of inflammatory mediators [9, 11]. To avoid these undesirable results of complement activation, nature exploits a variety of regulatory mechanisms that act on the formation of the C3/C5 convertase step of the complement cascade or on the assembly of the membrane attack complex [12]. The rodent Crry protein is a membrane-bound complement control factor that inhibits the cleavage of C3 into its active metabolites, and so blocks the downstream complement cascade of both the classic and the alternative pathway of complement activation [13–16]. Overexpression of Crry has been shown to protect mesangial cells from complement-mediated injury in vitro [17], and transgenic mice engineered to overexpress recombinant soluble Crry have been demonstrated to be protected against the antibody-induced glomerular injury that occurs in nephrotoxic serum nephritis (NSN) and genetic models of lupus-associated renal disease [18–20].

In this study we assessed the impact of chronic complement inhibition by Crry on the development of immune complex–mediated kidney disease in a rodent model of cryoglobulin-associated membranoproliferative glomerulonephritis.

METHODS

Animal study and experimental design

The experimental protocol for this study was reviewed and approved by the Animal Care Committee of the University of Washington in Seattle. Mice transgenic for TSLP have been previously described in detail [8]. Animals overexpressing the complement inhibitor Crry were crossbred with TSLP transgenic mice to generate doubly transgenic animals.

Both strains were backcrossed onto a C57BL/6 background. The animals were housed in a specific pathogen-free facility at the University of Washington with a fixed 12 hours light cycle, constant temperature of 25°C, and water and food ad libidum. At three weeks of age mice were weaned and genotyped as described previously [8, 18]. Experimental groups consisted of wild-type C57BL/6 mice, TSLP transgenic, Crry transgenic, and TSLP/Crry doubly transgenic animals, with 7 mice in each group. Female mice were sacrificed at 50 days of age, while males were kept for 120 days because of slower progression of the disease [8]. At the end of the study mice were anesthetized, blood was drawn by cardiac puncture, and organs were collected. Renal tissue was snap frozen in liquid nitrogen or stored in either 10% neutral buffered formalin, methyl Carnoy's solution (60% methanol, 30% chloroform, 10% acetic acid), or half-strength Karnovsky's solution (1% paraformaldehyde and 1.25% glutaraldehyde in 0.1 mol/L Na cacodylate buffer, pH 7.0) for later use.

Tissue preparation and histologic staining

Tissues fixed in formalin and methyl Carnoy's solution were embedded in paraffin using routine protocols. Paraffin-embedded materials were sectioned at 4 μm for routine staining with hematoxylin and eosin, PAS, and immunohistochemistry. Two-μm thick sections were used for periodic-acid methenamine silver stains (PAM). Immunofluorescence was performed on snap frozen kidneys, sectioned at 6 μm, and fixed in ice-cold acetone for 10 minutes.

Immunohistochemistry

For immunohistochemical staining, histologic sections of paraffin-embedded tissue were deparaffinized in xylene and rehydrated in graded ethanol. When required, antigen retrieval was performed by steam heating the tissue sections in Antigen Unmasking Solution (Vector Laboratories, Burlingame, CA, USA). Endogenous peroxidases were blocked in 3% hydrogen peroxide, and endogenous biotin was blocked using the Avidin/Biotin blocking kit (Vector Laboratories). The slides were then incubated with the primary antibody diluted in 1% bovine serum albumin (BSA) in phosphate-buffered saline solution (PBS) for one hour at room temperature. After that the sections were washed with PBS and immersed in the appropriate biotinylated secondary antibody, again diluted in PBS with 1% BSA. Signal amplification was performed using the ABC-Elite Reagent (Vector Laboratories). 3,3′-diaminobenzidine with nickel enhancement was used as chromogen, which results in a black color product. The slides were counterstained with methyl green, dehydrated, and then coverslipped.

A rat MAC-2 antibody (Cederlane; Ontario, Canada) was used to detect macrophages as described previously [21]. A mouse-adsorbed antirat antibody (Vector Laboratories) was utilized as secondary antibody. For the detection of mesangial cell activation and smooth muscle–like transformation, a α-smooth muscle actin antibody (clone 1A4; Dako, Carpenteria, CA, USA, as previously described [22]) was used. In this case, the secondary antibody was an anti-IgG_{2a} antibody (Pharmingen, San Diego, CA, USA). Cellular proliferation was assessed using a mouse monoclonal Ki67 antibody (previously described in [23]) (Pharmingen) with the Dako Animal Research kit. A goat polyclonal antihuman collagen IV antibody (Southern Biotechnology, Birmingham, CA, USA) was used for the staining of extracellular matrix [24]. A biotinylated rabbit antigoat antibody was applied as secondary antibody (Vector Laboratories).

For immunofluorescence, acetone-fixed sections were rehydrated in PBS and then incubated with fluorescein-conjugated antibodies against IgG, IgA, IgM, complement factor C3 (all from Cappel Pharmaceuticals, Aurora, OH, USA), or C9; antisera against rat C9, which cross-reacts with mouse complement factor 9, was generously provided by Paul Morgan, University of Wales College of Medicine, previously described in [25, 26]. Slides were coversliped with Vectashield mounting medium (Vector Laboratories) and viewed with a Zeiss fluorescence microscope (Thornwood, NY, USA).

Laboratory data

Urinary protein excretion was tested with urine test strips (Uristix; Bayer, Elkhart, IN, USA) and assessed semiquantitatively on a scale of 0 to 4. Blood urea nitrogen (BUN) was measured using a standard clinical chemistry analyzer (LX-20; Beckman Laboratories, Brea, CA, USA). Serum Crry levels were assessed by enzyme-linked immunosorbent assay (ELISA) as previously described [18]. Plasma C3 levels were determined by ELISA as described previously [27].

Analytical methods and statistics

Glomerular cellularity was assessed in tissue sections stained with hematoxylin and eosin. Fifteen random glomerular cross sections (gcs) were photographed with a digital camera (Olympus DP11; Olympus America, Melville, NY, USA). Pictures were imported into the Image Pro Plus Software (Media Cybernetics, Silver Spring, MD, USA) and glomerular cellularity and glomerular tuft area were quantified. The same method was used to quantify extracellular matrix in PAM-stained slides and in sections stained for collagen IV by immunohistochemistry. The number of Ki67-positive cells and MAC-2–expressing cells was assessed by counting positive cells in at least 40 random glomerular cross sections. Alpha-smooth muscle actin expression of mesangial cells was assessed semiquantitatively by scoring the amount of glomerular expression on a scale from 0 (no stain) to 4 (strong expression in the whole mesangial area) as described previously [28]. The fluorescence intensity of cryosections stained for immunoglobulins and complement was assessed in a similar way by describing the fluorescence intensity on a scale from 0 (negative) to 3 (strong staining) as previously described [8]. All morphometric analysis was performed by an examiner blinded for the origin of the sample.

Statistical analysis was performed using the InStat program (Version 3.0; Intuitive Software for Science, San Diego, CA, USA). Groups were compared using the nonparametric Kruskal-Wallis test and Dunn's multiple comparison test. All values are expressed as mean ± standard deviation. $P < 0.05$ was considered statistically significant.

Table 1. Kidney function in experimental animals

	Wild-type	TSLP transgenic	Crry transgenic	TSLP transgenic/ Crry transgenic
Females				
BUN *mg/mL*	23.2 ± 5.6	27.7 ± 7.3	23.0 ± 5.8	27.2 ± 4.0
Protein excretion	0.8 ± 0.4	0.8 ± 0.3	0.8 ± 0.6	0.8 ± 0.3
Males				
BUN *mg/mL*	23.2 ± 3.5	26.3 ± 3.5	28.0 ± 2.9	26.5 ± 3.6
Protein excretion	0.9 ± 0.2	1.3 ± 0.6	0.9 ± 0.3	1.3 ± 0.7

Data expressed as mean ± standard deviation. Proteinuria assessed by dipstick analysis: 1 = 30 mg/dL; 2 = 100 mg/dL. The results show no statistically significant differences between groups.

RESULTS

Kidney function remains unaffected by the disease and by complement inhibition

Excretory renal function was determined by measurement of BUN, and urinary protein excretion was assessed by dipstick tests. The four groups of animals tested did not demonstrate significant differences in renal function or proteinuria (Table 1).

Doubly transgenic Crry/TSLP transgenic mice develop increased glomerular matrix similar to TSLP transgenic mice

Female TSLP transgenic mice expressed more than twice the amount of extracellular matrix than wild-type animals when silver stained matrix in periodic-acid methenamine silver stained histologic sections was assessed morphometrically (315 ± 62 μm^2/gcs for wild-type mice vs. 737 ± 62 μm^2/gcs TSLP transgenic animals, $P < 0.01$) (Table 2 and Fig. 1). This result was congruent with the measurement of collagen IV, an important component of the extracellular matrix, in which TSLP transgenic mice showed an almost fourfold increase in

Table 2. Glomerular morphology

	Wild-type	TSLP transgenic	Crry transgenic	TSLP transgenic/ Crry transgenic
Females				
Cellularity *cells/gcs*	44 ± 6	45 ± 6	46 ± 8	50 ± 9
Cellularity *cells/area*	0.014 ± 0.0022	0.013 ± 0.0010	0.014 ± 0.0016	0.013 ± 0.0011
Matrix $\mu m^2/gcs$	315 ± 62	737 ± 165[b]	372 ± 111	724 ± 184[b]
Matrix % *of area*	11 ± 2	22 ± 5[b]	13 ± 3	22 ± 6[b]
Collagen IV $\mu m^2/gcs$	201 ± 27	740 ± 177c	210 ± 62	495 ± 41[c]
Collagen IV % *of area*	10 ± 2	25 ± 2[b]	9 ± 2	22 ± 2[c]
Glomerular size $\mu m^2/gcs$	3135 ± 283	3349 ± 385	3123 ± 528	3538 ± 347
Males				
Cellularity *cells/gcs*	39 ± 6	45 ± 3	40 ± 6	51 ± 8[a]
Cellularity *cells/area*	0.012 ± 0.0012	0.015 ± 0.0005	0.013 ± 0.0010	0.012 ± 0.0005
Matrix $\mu m^2/gcs$	470 ± 124	879 ± 233[a]	509 ± 91	1004 ± 267[b]
Matrix % *of area*	16 ± 2	24 ± 7[a]	16 ± 2	27 ± 4[b]
Collagen IV $\mu m^2/gcs$	240 ± 82	715 ± 124[a]	227 ± 29	780 ± 68[b]
Collagen IV % *of area*	9 ± 2	24 ± 4[a]	9 ± 1	24 ± 1[a]
Glomerular size $\mu m^2/gcs$	3312 ± 456	3731 ± 202	3181 ± 367	4012 ± 571

Morphometric analysis of glomerular cellularity, matrix, collagen IV, and glomerular size. Data is expressed as mean ± standard deviation.
Statistical significant differences are [a]$P < 0.05$, [b]$P < 0.01$, [c]$P < 0.001$ compared to wild-type control.

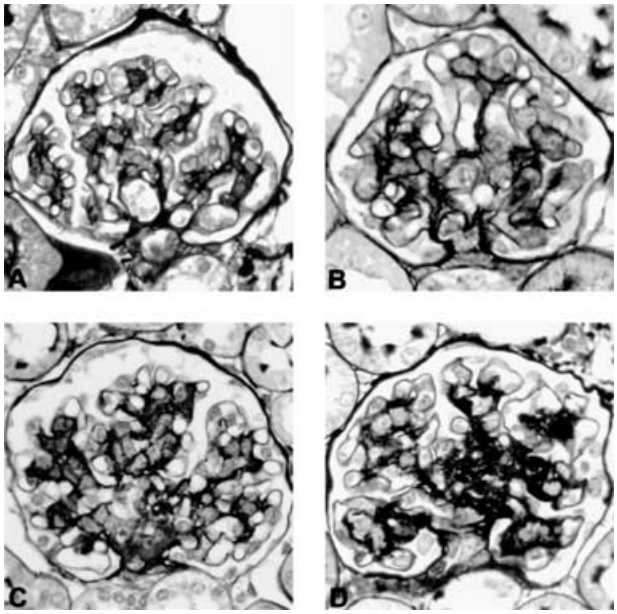

Fig. 1. Glomerular morphology. Glomerular morphology in individual experimental groups, periodic-acid methenamine silver stain. Normal glomerular architecture in (*A*) a wild-type mouse and (*B*) a Crry transgenic mouse. (*C*) Mesangial expansion and matrix accumulation in a thymic stromal lymphopoietin (TSLP) transgenic mouse, and (*D*) a doubly (TSLP and Crry) transgenic mouse, with a glomerulus showing increase in extracellular matrix.

area stained (740 ± 177 μm^2/gcs vs. 201 ± 27 μm^2/gcs in wild-type controls, $P < 0.01$). The results of the morphometric analysis of the staining of extracellular matrix in male TSLP animals at the age of 4 months matched that of the female TSLP group. Crry transgenic animals (males and females) showed no differences in the expression and staining pattern of glomerular extracellular matrix compared to wild-type controls (Fig. 1). Overexpression of the complement inhibitor Crry in TSLP transgenic mice failed to reduce extracellular matrix and glomerular collagen IV accumulation in both genders (Table 2 and Fig. 1).

Crry overexpression has no significant impact on glomerular cellularity, glomerular proliferation, and glomerular cell activation

Female mice of the different experimental groups showed no significant differences in glomerular cellularity at the age of 50 days. Male animals, kept in the study for 120 days, had comparable number of cells per glomerular cross section between wild-type, Crry overexpressing, and TSLP transgenic mice. Male doubly transgenic animals showed a slight increase in glomerular cellularity per glomerular cross section compared to Crry transgenic or wild-type mice ($P < 0.05$), but cellularity per glomerular area was not significantly different from other experimental groups (Table 2). Cellular proliferation rate, assessed as Ki67-positive cells per glomerular cross section, was not altered in mice overexpressing the complement inhibitor Crry, animals transgenic for TSLP that showed membranoproliferative glomerulonephritis, or doubly transgenic mice (data not shown).

Wild-type mice showed only occasional expression of α-smooth muscle actin in the glomeruli (Fig. 2). Comparable levels of expression were present in the glomeruli of Crry overexpressing mice. In contrast, mice that were transgenic for TSLP had elevated levels of glomerular α-smooth muscle actin expression (score of 1.3 ± 0.3/gcs for

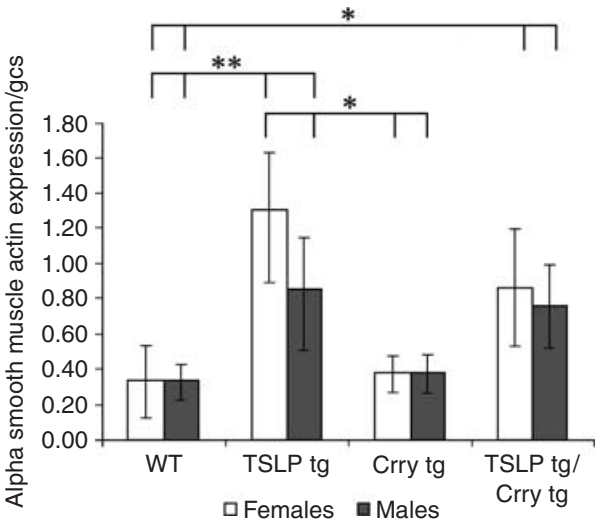

Fig. 2. α-smooth muscle actin expression. Semiquantitative assessment of glomerular α-smooth muscle actin expression. Graphs depict mean ± standard deviation, with statistical significant differences between groups marked as $^{*}P < 0.05$ and $^{**}P < 0.01$.

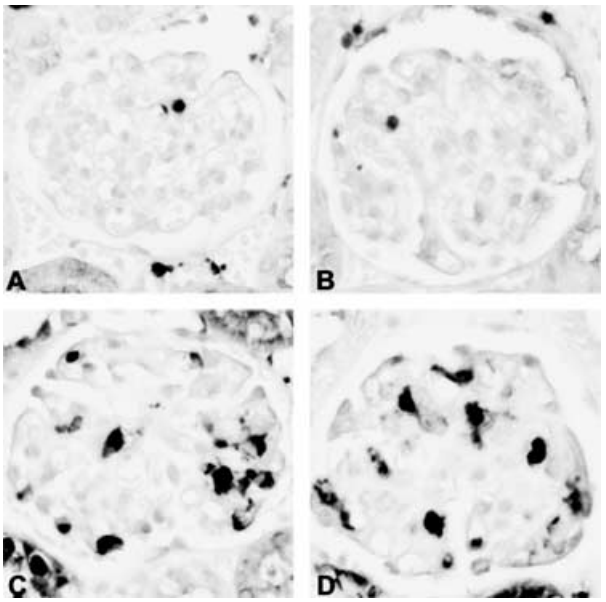

Fig. 3. Immunohistochemistry for macrophages. Macrophage immunohistochemistry using MAC-2 antibody, methyl green counterstain. Normal glomerulus of a (*A*) wild-type mouse and of a (*B*) Crry transgenic animal. (*C*) Mice transgenic for thymic stromal lymphopoietin (TSLP) have prominent glomerular macrophage influx, as do mice transgenic for TSLP and Crry, as illustrated in (*D*).

females and 0.9 ± 0.3/gcs for males vs. 0.3 ± 0.2/gcs and 0.3 ± 0.1/gcs for female and male control animals, respectively; $P < 0.01$). Overexpression of Crry did not reduce the level of mesangial activation in TSLP transgenic mice as assessed by the degree of α-smooth muscle actin expression. Expression patterns were similar in male and female mice.

No significant α-smooth muscle actin staining was noted in the tubulointerstitium in any of the experimental groups.

Complement inhibition has no effect on glomerular macrophage influx

Wild-type mice and animals transgenic for Crry had usually less then one glomerular macrophage per glomerular cross section (Fig. 3). TSLP transgenic mice displayed considerable macrophage influx, with 2.1 ± 0.7 MAC-2–positive cells/gcs for females ($P < 0.01$) and 1.9 ± 0.9 MAC-2–positive cells/gcs for males ($P < 0.01$). TSLP and Crry doubly transgenic mice did not show a significant reduction of macrophage influx (2.1 ± 0.7 MAC-2–positive cells/gcs for females and 1.7 ± 0.6 MAC-2–positive cells/gcs for males).

TSLP/Crry doubly transgenic animals express Crry

Elevated plasma levels of the recombinant soluble Crry protein were confirmed in 5 male mice of each experimental group by ELISA. Due to the limited amount of plasma available from each mouse and the consistency with which the Crry transgene is expressed in Crry transgenic animals of both sexes [18], this assay was only performed in male animals, which yield more blood during necropsy due to the later age at sacrifice. TSLP/Crry doubly transgenic animals had the highest plasma levels of soluble recombinant Crry that did exceed that of Crry transgenic mice sixfold (6.1 ± 1.7 μg/mL vs. 0.9 ± 0.3 μg/mL in TSLP/Crry and Crry transgenic animals, respectively, $P < 0.001$). No detectable Crry levels were present in TSLP transgenic mice and wild-type control animals.

Overexpression of Crry has no effect on plasma C3 levels and glomerular complement or immunoglobulin deposition

Plasma complement factor C3 was measured to further clarify the role of complement in this model of TSLP-induced cryoglobulin-associated membranoproliferative glomerulonephritis. Approximately 60% of patients with cryoglobulinemia show decreased levels of early complement factors like C3 in their serum. However, the degree of hypocomplementemia correlates poorly with the cryocrit levels or the presence of clinical symptoms [4]. TSLP transgenic mice did not demonstrate a significant decrease of the complement factor C3 as determined by measurement of plasma C3 levels (Fig. 4). The introduction of the Crry transgene itself did not influence the amount of plasma C3 in control animals, and also left plasma C3 levels unaffected in TSLP transgenic mice.

Wild-type control mice had only minimal glomerular deposition of immunoglobulins (IgG, IgM, and IgA) (Fig. 5), in accord with previous findings. In contrast, mice

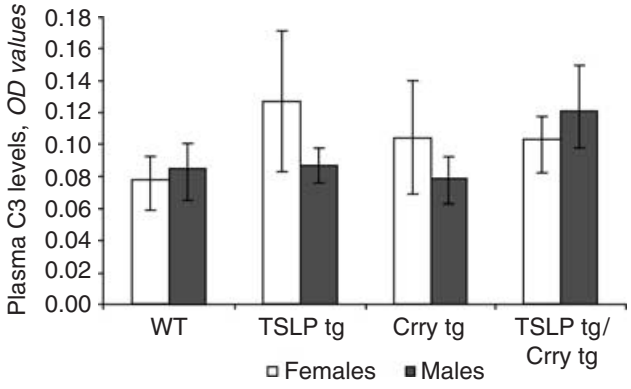

Fig. 4. Plasma C3 levels. Plasma levels of C3 were measured by enzyme-linked immunosorbent assay (ELISA) in female and male animals. Data is presented as mean ± standard deviation. No statistically significant differences were observed.

transgenic for TSLP showed strong glomerular staining for immunoglobulins. As expected, Crry overexpressing animals had no or only small glomerular depositions of immunoglobulins. Overexpression of Crry in TSLP transgenic animals did not lead to a reduction in glomerular deposits of immunoglobulins.

Immunofluorescence staining for C3 and C4 was performed to assess the role of early components of the complement cascade in the pathogenesis of cryoglobulin-associated MPGN in TSLP transgenic mice. Wild-type and Crry transgenic animals showed only minimal glomerular deposition of C3. TSLP transgenic mice and TSLP/Crry doubly transgenic animals had significantly stronger glomerular staining for C3. In contrast, glomerular deposition of C4 was consistently high even in wild-type or Crry transgenic animals, and did not increase in TSLP transgenic mice. It was not affected, either, by the overexpression of Crry in these animals (Fig. 5).

In addition, the glomerular deposition of the complement factor C9 was assessed to directly measure mediators of glomerular damage. C9 is generally believed to be a good representative for the presence of the membrane attack complex C5b-9 (MAC), a complex of later factors in the complement cascade that is thought to be a direct mediator of complement induced cellular injury by inserting into the cellular membranes and leading to lytic or sublytic damage. Wild-type and Crry transgenic mice showed only negligible amounts of glomerular C9 deposition while TSLP transgenic mice had increased glomerular deposition of C9, demonstrating that the formation of the MAC may be involved in the induction of cryoglobulin-associated renal injury (Fig. 5). However, the expression of the Crry transgene was not sufficient to reduce glomerular C9 deposition, indicating that the complement inhibitory action of Crry may be overwhelmed by the high levels of circulating immune complexes in TSLP transgenic mice.

DISCUSSION

Activation of the complement system plays an important role in the initiation and maintenance of a variety of glomerular injuries. Glomerular damage in cryoglobulin-associated membranoproliferative glomerulonephritis in humans and rodents is conferred by large numbers of circulating immunoglobulins consisting of monoclonal or polyclonal IgG and IgM, which deposit in subendothelial locations within the capillary walls or aggregate in capillary lumina [8]. Complement activation by these immune complexes is thought to contribute to the pathogenesis of the disease because patients with cryoglobulin-associated membranoproliferative glomerulonephritis typically show low serum levels of complement C4 and CH50, as well as normal to low levels of C3, indicating activation of mainly the classic complement pathway [29]. Renal biopsies of affected individuals also show large glomerular deposits of immunoglobulins together with complement components [4, 8, 29]. We recently described a mouse model of cryoglobulinemic glomerulonephritis in TSLP transgenic mice, which demonstrated a reproducible renal lesion characterized by glomerular deposition of immune complexes and complement component C3, glomerular infiltration by monocytes/macrophages, and increased glomerular matrix formation. We hypothesized that activation of complement by immune complexes was a key amplification step in this form of glomerular injury. In this scenario, complement activation should lead to the recruitment of monocytes/macrophages, activation and proliferation of glomerular cells, and, as disease progression ensues, increased matrix formation. To test this role of complement in TSLP transgenic mice, we used a genetically engineered mouse strain to inhibit complement activation.

Natural inhibition of undesired complement activation in mammals is regulated by circulating plasma and membrane-bound complement inhibitors that either block the assembly of the membrane attack complex or act at the level of C3/C5 convertases, thus inhibiting the classic and alternative pathway similarly. Crry, a membrane-bound complement inhibitor, represents the rodent functional analog for human decay accelerating factor (DAF) and membrane cofactor protein (MCP) and blocks complement activation at the level of the C3 convertase [16, 30]. Its physiologic importance in regulating complement activation in renal disease models in vivo has been demonstrated by the use of anti-Crry antibodies, which lead to the suppression of intrinsic Crry and result in tubulointerstitial injury in healthy control rats [31]. Inhibition of Crry by anti-Crry antibodies has also been shown to aggravate renal injury in the anti-Thy 1 nephritis model of rats where it resulted in more severe mesangiolysis, more prominent cellular infiltration, and increased complement deposition in glomeruli of affected

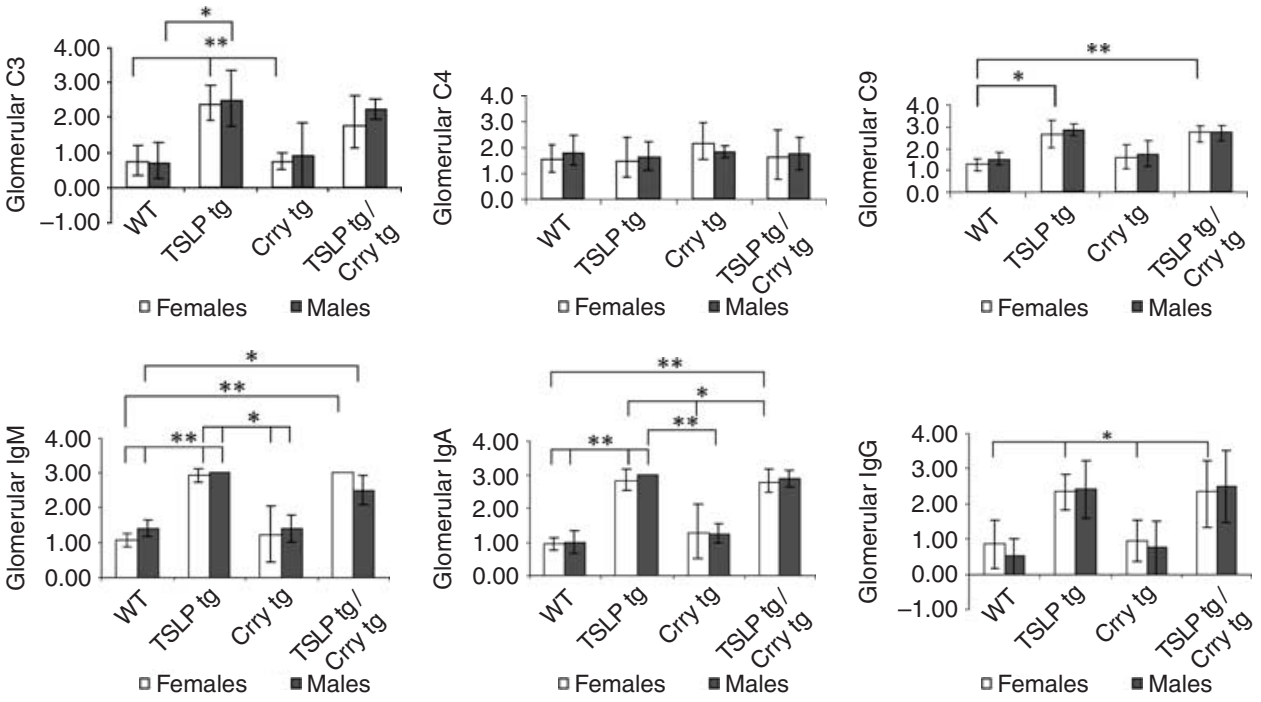

Fig. 5. Immunoglobulins and complement. Semiquantitative assessment of glomerular immunoglobulin and complement deposition. Graphs show mean ± standard deviation, with statistical significant differences between groups expressed as $^{*}P < 0.05$ and $^{**}P < 0.01$.

animals [32]. The reduction of Crry expression via antisense oligodeoxynucleotides augmented tubulointerstitial injury in puromycin-aminonucleoside nephritis [33].

A soluble recombinant form of Crry with an extended half-life was created by fusing the Crry protein to mouse IgG1 hinge, CH2, and CH3 domains. The complement-inhibitory activity of this construct has been demonstrated in complement activation assays in vitro, as well as in nephrotoxic serum nephritis in vivo, where it was shown to diminish renal injury [34]. A transgenic mouse strain has been developed that overexpresses soluble recombinant Crry to achieve continuous complement pathway blockade [18]. Previous studies with these animals have been encouraging by showing that transgenic mice were protected against several forms of renal injury, including nephrotoxic serum nephritis, antibody-induced renal failure, and renal function impairment in lupus-associated kidney disease [18–20]. These mice were used in our study to test whether the benefits of Crry overexpression could be extended to an established model of immune complex–mediated glomerulonephritis. A surprising finding of this study is that inhibition of complement activation by high levels of circulating recombinant soluble Crry in TSLP transgenic mice with cryoglobulin-associated membranoproliferative glomerulonephritis fails to prevent or significantly ameliorate the injury ensuing from immune complex and complement deposition. Doubly transgenic mice did not show a decrease in mesangial cell activation, glomerular matrix accumulation, or glomerular macrophage influx. Circulating levels of the complement component C3 and glomerular deposition of immunoglobulins and complement C3 were not influenced by elevated circulating levels and local expression of Crry. Similarly, glomerular deposition of C9, an established marker for membrane attack complex (MAC, C5b-9) formation, which indicates terminal complement activation, was high in TSLP transgenic mice and was not reduced in Crry/TSLP doubly transgenic animals, pointing to a conclusion that complement inhibition may not have been complete.

High levels of circulating Crry in mice singly transgenic for this molecule have been described, and we confirmed such levels were also present in our doubly transgenic mice [18]. The functional efficacy of recombinant soluble Crry from serum and urine of the Crry transgenic mice has been previously demonstrated to block complement activation in vitro [18]. However, we do not know whether a further increase of complement inhibitor levels, such as by adding treatment of the doubly transgenic animals with Crry-Ig, would have altered the disease. Interestingly, doubly transgenic mice had significantly higher plasma levels of the recombinant protein than mice transgenic for Crry alone. It seems unlikely that this is the result of impaired excretion of the protein into the urine because cryoglobulinemic glomerulonephritis usually does not lead to a significant decline in renal function. It could be speculated that Crry forms complexes with circulating immune complexes, which then inhibits its

excretion, leading to higher plasma levels, but the actual basis for the observed increase in levels is not known.

Three possible explanations for the failure of Crry to protect TSLP mice from glomerulonephritis deserve consideration. The first of these is the strong and chronic stimulation of the complement system by high levels of serum cryoglobulins that might have overwhelmed the inhibitory capacity of Crry. This was directly evaluated by measurements of circulating C3, and by assessment of the terminal complement component C9, a significant part of the membrane attack complex C5b-C9, which should be inhibited or reduced if Crry overexpression would lead to sufficient complement inhibition. Circulating levels of C3 revealed no significant differences between any of the experimental groups. This finding indicates the chronic immune complex deposition occurring in TSLP mice did not cause a process of increased complement consumption that could not be counterbalanced by the overexpression of Crry. Glomerular C9 deposition was increased in TSLP transgenic mice, but did not show a reduction in TSLP/Crry doubly transgenic mice, pointing to a conclusion that complement inhibition by Crry may not have been enough to inhibit the formation of complement effector products like the membrane attack complex that leads to subsequent cellular injury. With this in mind, it can be noted that in other studies of the efficacy of Crry in models of renal injury [e.g., nephrotoxic serum nephritis (NSN) and antibody-induced renal failure], the protective effect that was demonstrated involved a rather short and time-limited stimulus to the complement system. Renal morphology and function in these models was not assessed longer than 30 hours after initiation of disease. At that 30-hour time point a difference in C3 deposition between transgenic and wild-type animals in NSN was already lost, indicating that the protective capacity of Crry diminished over time. Such may have been the case in our long-term model. A study of anti-GBM glomerulonephritis suggests that the role of complement in the acute phase of injury might differ from its function in more chronic disease processes. Deficiency of C3 in mice with anti-GBM glomerulonephritis led to reduced injury in the early heterologous phase of the disease while C3−/− animals had heavier proteinuria and more severe uremia in the late autologous phase after 14 days [35]. The only study that used complement inhibition in a model of chronic kidney injury involving complement activation was performed in MRL/lpr lupus mice, which develop a proliferative glomerulonephritis [19]. Overexpression of Crry in these mice led to an improvement in renal function and a survival benefit along with a reduction in glomerular C3 deposition. However, clinical benefits were disassociated from morphologic manifestations of the disease and renal pathology. The extent of glomerular immunoglobulin deposition, the amount of glomerulosclerosis, the number of glomerular crescents, and the glomerulonephritis scores were unaltered in lupus mice with and without complement inhibition. This is consistent with the data obtained in our study.

A second reason for the relative lack of efficacy of complement inhibition by Crry might be that the degree of complement inhibition may have been incomplete and may have been insufficient to significantly alter the disease. Measures of complement activity performed in this study were serum levels of C3 and renal deposition of C3, C4, and C9, as determined by immunofluorescence microscopy. We sought to expand our measures of complement activation, but the low hemolytic activity of mouse serum precluded utilizing a conventional assay of CH50, and values for animals from all experimental groups were under or at the lower detection limit. We therefore cannot confirm that overexpression of Crry had no effect on complement activation, but the comparable levels of C3 in plasma of wild-type control animals, TSLP mice, and doubly transgenic mice are a strong indication that serum complement activation was largely unaffected by the documented overexpression of the transgene. A persistent conundrum is that Crry transgenic animals can be protected from immune complex–mediated disease in some injury models, despite an inability to demonstrate an effect on complement activation in the serum. Such observations suggest complement could still be an important mediator of the immune injury in these animals, but the mechanisms of this mediation are not readily apparent and do not readily conform to traditional paradigms of systemic complement activation and amplification of injury, but instead are related to local effects of complement inhibition. In any case, our findings suggest that the beneficial effects of Crry inhibition on renal immune injury, whether or not an effect on systemic complement activation can be demonstrated, may be animal strain and model system–dependent and were not effective in this model of cryoglobulinemic glomerulonephritis.

A third explanation, which challenges the usual paradigm by which complement activation amplifies immune complex–mediated injury, is that complement may have a protective role in the pathophysiology of the disease. In this scenario, the beneficial effects of complement in promoting the disposal of immune complexes override the effects of amplification of inflammatory cascades. The validity of this paradigm is supported by studies performed in rats with chronic serum sickness glomerulonephritis, in which the depletion of plasma complement led to a virtual cessation of glomerular immune complex removal [36]. In humans with lupus, it is known that naturally occurring deficiencies of complement components increase the susceptibility to this immune complex–mediated disease, supporting the idea that complement may be protective in some forms of glomerulonephritis. Our study is inconclusive with regard to this last possibility because we were unable to demonstrate alterations in

circulating C3 levels or complement deposition with Crry overexpression.

A final consideration comes from data from other forms of immune complex–mediated diseases, such as the Arthus reaction or passive immune complex alveolitis, which have indicated a prominent role for mediation of disease by Fc receptors, rather than complement activation, by demonstrating that tissue injury is unchanged in complement-deficient mice, while Fcγ receptor–deficient animals were protected from the disease [37, 38]. Lending credence to this possibility, we have preliminary data that one such receptor, the FcγRIIb, is an important mediator of glomerular injury in the TSLP model, and that deletion of this receptor causes significant aggravation of the glomerulonephritis [39].

CONCLUSION

Complement inhibition by overexpression of recombinant soluble Crry in a mouse model of chronic glomerulonephritis secondary to immune complex deposition failed to demonstrate significant improvement in morphologic parameters of injury, despite demonstration of increased serum levels of Crry, most likely because complement activation was too strong and overwhelmed the inhibitory capacity of Crry, or because complement inhibition by Crry was incomplete. This study indicates the importance of defining which glomerular lesions are driven by complement activation and might therefore benefit from therapeutic complement inhibition. An immune complex deposition process with local activation of complement is not sufficient to define such a lesion.

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