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Low-Dose Targeted Complement Inhibition Protects against Renal Disease and Other Manifestations of Autoimmune Disease in MRL/*lpr* Mice[1]

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Complement appears to play a dual role in the progression of systemic lupus erythematosus, serving a beneficial role in enhancing immune complex clearance, while serving a pathogenic role in inducing local inflammation. To investigate these different roles of complement in a therapeutic setting, MRL/*lpr* mice were treated with the targeted murine C3 complement inhibitor, CR2-Crry, from 16 to 24 wk of age (after the development of proteinuria). The targeting moiety, CR2, binds to C3 breakdown products deposited at sites of complement activation and has the potential to provide complement inhibition locally without causing systemic inhibition. Administration of CR2-Crry i.v., at a dose of 0.25 mg once a week, was associated with a significant survival benefit, improved kidney function, and a significant reduction in glomerulonephritis and renal vasculitis. The presence of skin lesions and lung bronchiolar and vascular inflammation was also dramatically reduced by CR2-Crry treatment. CR2-Crry treatment also resulted in a significant reduction in autoantibody production, as measured by anti-dsDNA Ab levels, and did not cause an increase in circulating immune complex levels. These effects on autoimmunity and circulating immune complexes represent significant potential advantages over the use of Crry-Ig in MRL/*lpr* mice, a systemic counterpart of CR2-Crry. CR2-Crry localized preferentially to the kidneys in 16-wk MRL/*lpr* mice with a kidney-localized half-life of ~24 h. Thus, targeted complement inhibition at the C3 level is an effective treatment in murine lupus, even beginning after onset of disease. ***The Journal of Immunology,*** **2008, 180: 1231–1238.**

Complement plays an important role in the pathology of many autoimmune and inflammatory diseases and is involved in mediating immunopathology in systemic lupus erythematosus (SLE).[3] Complement activation is triggered by immune complexes that are either deposited or formed in situ within tissues. The kidney is a major site of immune complex deposition and complement activation, and about half of all lupus patients will develop kidney disease. Other disease manifestations such as arthritis, serositis, vasculitis, and pulmonary disease may have similar pathogenic mechanisms. The MRL/*lpr* mouse is a model of spontaneous SLE that develops many features of human SLE, such as autoantibodies, proliferative glomerulonephritis, pulmonary inflammation, skin disease, and vasculitis.

Complement inhibition is a potential therapeutic modality for autoimmune and inflammatory diseases. However, aside from considerations regarding the potential role of complement in the development of autoimmunity, complement appears to play a dual role in the progression of lupus, serving a beneficial role in the clearance of apoptotic cells and immune complexes, while serving a pathogenic role in inducing local inflammation. Indeed, although complement has been shown to play a key role in the development of renal disease in MRL/*lpr* mice, deficiency of C3 is not protective (1). Nevertheless, maintaining a high level of systemic complement inhibition in MRL/*lpr* mice with frequent doses of the C3 inhibitor Crry-Ig did protect against renal disease (2). There was, however, a significant increase in levels of circulating immune complexes in Crry-Ig-treated mice, and Crry-Ig had no effect on other features of autoimmune disease or on survival (2). The latter finding suggests that the mice died of a condition unrelated to kidney failure, possibly pulmonary inflammation which has been shown to be an important determinant of survival in MRL/*lpr* mice independent of glomerulonephritis (3).

There are potential risks associated with systemic complement inhibition, particularly whether prolonged inhibition is required, due to the important physiological roles of complement in host defense and immune homeostasis. These are particularly important considerations with regard to the therapy of lupus, since interfering with the catabolism of immune complexes and the clearance of apoptotic cells may exacerbate disease. Targeted complement inhibition has the potential to obviate the need to systemically inhibit complement, limit immunosuppression, limit disruption of immune homeostatic mechanisms, and increase efficacy by improving bioavailability. We have previously characterized a targeting strategy involving the linking of a complement inhibitor to the C3-binding region of complement receptor 2 (CR2). Natural ligands for the CR2-targeting moiety are iC3b, C3dg, and C3d, cell-bound cleavage fragments of C3 that are present at sites of complement activation (40–41). We have shown previously that a single dose of CR2-Crry, a targeted inhibitor of mouse complement, is highly effective in acute models of inflammation at doses

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[3] Abbreviations used in this paper: SLE, systemic lupus erythematosus; CR2, complement receptor 2; SUN, serum urea nitrogen.

that do not cause systemic inhibition of complement (4, 5). In this study, we investigate the efficacy of chronic use of this targeted complement inhibitor in the MRL/*lpr* mouse model of lupus characterizing its effects on disease, immune modulation, and immune complex clearance.

Materials and Methods

rCR2-Crry

The fusion protein CR2-Crry was produced and purified as described previously (4). In brief, a cDNA construct of the recombinant fusion protein was prepared by joining the mouse CR2 sequence encoding the four N-terminal short consensus repeat units (residues 1–257 of mature protein, National Center for Biotechnology Information (NCBI) GenBank accession number M35684) to sequences encoding extracellular regions of mouse Crry. The Crry sequence used encoded residues 1–319 of the mature protein (NCBI GenBank accession number NM013499). To join CR2 to Crry, a linking sequence encoding $(GGGGS)_2$ was used. The recombinant protein was expressed in NSO cells and purified by anti-Crry affinity chromatography as described (4).

Experimental protocol

Sixteen-week-old MRL/*lpr* mice (The Jackson Laboratory) were randomized into two groups: one group ($n = 10$) received 0.25 mg of CR2-Crry once a week from weeks 16 to 24 and the other group (control group, $n = 20$) received PBS. CR2-Crry and PBS were administered by i.v. tail vein injection. All work with mice was approved by the Medical University of South Carolina Animal Protocols Review Board and was performed in accordance with the National Institutes of Health *Guide for Care and Use of Laboratory Animals*.

Pathology

Kidneys were removed upon completion of the experiment and bisected longitudinally with one-half frozen for immunofluorescent analysis and the other half fixed in neutral-buffered formalin and processed to paraffin wax. Sections of paraffin-processed kidney were stained with H&E and periodic acid Schiff stain. The slides were interpreted in a blinded fashion by Dr. P. Ruiz (University of Miami School of Medicine, Miami, FL), grading the kidneys for glomerular inflammation, proliferation, crescent formation, and necrosis. Interstitial and tubular changes were also noted. Scores from 0 to 4 (where 0, is no damage and 4 severe) were assigned for each of these features (6). Lungs were inflated by intratracheal instillation of neutral-buffered formalin, processed to paraffin, sectioned, and stained with a H&E stain. Pulmonary infiltration was evaluated microscopically using the scoring system described previously (6). Briefly, the perivascular and peribronchiolar infiltrates were assessed semiquantitatively in >10 vessels per section and in >10 bronchioli by two independent observers blinded to the study group. Inflammation was scored from 0 to 3 with: 0, no inflammation; 1, less than three cell layers surrounding <50% of vessel or bronchioli; 2, three to six layers surrounding >50%; and, finally, 3, more than six layers.

Complement and Ig deposition

Immunofluorescent staining with fluorescein-conjugated anti-mouse IgM, IgG (Cappel Laboratories), and C3d (DakoCytomation) was performed on frozen kidney sections. C3d Ab was raised against human C3d, but the Ab cross-reacts with mouse C3, and binds to membrane-bound C3b and C3d (7, 8). The binding of Abs against C1q (Cedar Laboratories) and C9 (gifted by Dr. P. Morgan; Cardiff University, Cardiff, U.K.) was visualized by incubation with anti-rat Alexa 488 (Molecular Probes). Immunofluorescent-stained slides were read in a blinded fashion and graded 0–3+: 0, no staining; 1+, mild staining; 2+, moderate staining; and 3+, high staining. Renal C1q and C3 was further quantified by Western blot analysis of kidney tissue homogenates from animals in CR2-Crry-treated and PBS-treated (control) groups at 24 wk of age. Protein was extracted from kidney tissue by homogenization of kidneys in lysis buffer as previously described (9). Kidney samples containing equal protein concentrations were separated by electrophoresis on 4–15% SDS-PAGE gel under reducing conditions for C1q detection and nonreducing conditions for C3 detection. Western blot analysis was conducted using standard techniques (10) with primary Abs against mouse C1q (Cedarlane Laboratories) and C3 (MP Biomedicals) and HRP-conjugated secondary Abs (Calbiochem). Blots were developed using ECL (Bio-Rad) and quantification of Western blots was performed using Bio-Rad Molecular Imager Gel Doc. Results are presented as mean densities.

Assessment of dermatitis

MRL/*lpr* mice develop spontaneous skin lesions on the forehead, ears, and dorsum of the neck. The onset and incidence of macroscopic skin disease was recorded in treated (CR2-Crry) and untreated (PBS) mice. Mice were considered affected when there was evidence of skin ulceration, induration, scab formation, or alopecia at any of the aforementioned sites. To permit quantitative comparisons between the two groups, the severity of skin disease was scored using previously published methods. Briefly, animals were scored on a scale of 0–3: 0, no visible skin changes; 1, minimal hair loss with redness and a few scattered lesions; 2, redness, scabbing, and hair loss; and 3, ulcerations with an extensive area of involvement. Animals were observed by an individual blinded to the study group (F. Qiao).

Urine albumin excretion and serum urinary nitrogen levels

Mice were placed in metabolic cages for 24 h urine collection every 2 wk beginning at week 16. To prevent bacterial growth, antibiotics (ampicillin, gentamicin; Invitrogen Life Technologies) and chloramphenicol (Sigma-Aldrich) were added to collection tubes. Urinary albumin excretion was determined by ELISA using a standard curve of known concentrations of mouse albumin (Bethyl Laboratories) as previously described (2). Results are expressed as milligrams of albumin per milligram of creatinine per mouse per day. Urinary creatinine concentrations were detected with a Beckman Autoanalyzer (Beckman Coulter). Serum urea nitrogen (SUN) was determined for each mouse at week 16 and at the termination of the experiment at week 24. Blood was collected by retro-orbital bleed, and SUN was determined by using a Beckman Autoanalyzer (Beckman Coulter).

Measurement of anti-DNA Abs and circulating immune complexes

Anti-DNA Ab levels were measured by ELISA as previously described (11). Circulating immune complex levels were measured by a sandwich ELISA method as previously described (11), with serum samples from 24-wk-old MRL/*lpr* and BALB/c mice used as positive and negative controls, respectively.

Biodistribution and immunolocalization of CR2-Crry

Radiolabeling was conducted using ^{125}I (New England Nuclear) by the Iodo-Gen method (Pierce). A total of 5 mCi was used to label 100 μg of CR2-Crry protein. Free iodine was removed from the mixture after labeling by anion exchange resin. Iodine incorporation was in the 60–80% range. Radiolabeled protein was injected i.v. into 16-wk-old MRL/*lpr* mice and organs were harvested at 24, 48, and 72 h. Upon sacrifice ($n = 3$/group), blood was removed by cardiac puncture. Following blood removal, animals were perfused with PBS before removal of the heart, lung, liver, kidney, intestine, thymus, spleen, and skin. Tissues were rinsed in RPMI 1640 (Invitrogen Life Technologies) and counted with a Packard Instrument 5780 gamma counter at the ^{125}I-labeled window with appropriate corrections for count decay. To better assess the specific targeting of CR2-Crry and kinetic effects of CR2-Crry administration, 16-wk-old mice were administered a single dose 0.25 mg of CR2-Crry, and animals were sacrificed at 24, 72, and 7 days ($n = 3$/group). Kidneys were removed and snap-frozen in liquid nitrogen, and then sectioned for immunofluorescent localization of C3d, IgM, IgG, and CR2. The presence of C3d, IgM, IgG, and CR2 was assessed in sections from MRL/*lpr*-treated and untreated mice and observed in a blinded fashion. We also assessed the localization of C3d in relation to the binding of CR2 to ascertain spatial and temporal expression of the two proteins using a dual immunofluorescent staining technique. In brief, sections were coincubated with C3d-FITC (DakoCytomation) and anti-CR2 (gifted by M. Holers; University of Colorado, Denver, CO). The presence of CR2 was visualized with an anti-rat Alexa 555 (Molecular Probes). Sections were then washed and coverslipped with VectaMount (Vector Laboratories). Specificity of staining was confirmed by omission of primary Ab and by the use of isotype Ab controls.

Serum complement inhibition

Blood samples were taken from 16- and 24-wk-old CR2-Crry-treated and PBS-treated (control) MRL/*lpr* mice and serum was prepared to assay the effect of acute and chronic administration of the targeted complement inhibitor, CR2-Crry, on serum complement functions. Serum was taken from 16-wk-old MRL/*lpr* mice 24 h postadministration of CR2-Crry, and 7 days postadministration in 24-wk-old mice. The degree of serum complement inhibition was measured using a previously described method that uses flow cytometric analysis of C3 deposition on zymosan A particles (Sigma-Aldrich) (12).

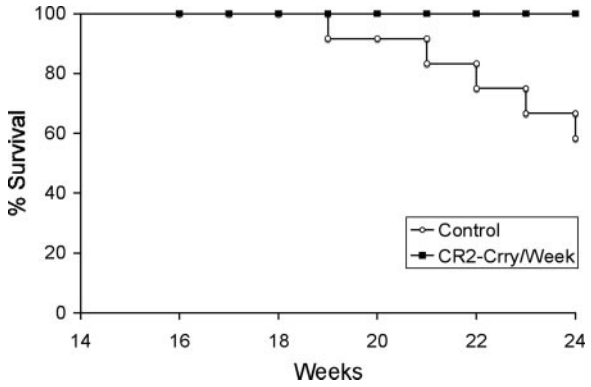

FIGURE 1. Survival curve for MRL/*lpr* mice treated with either CR2-Crry or PBS. Treatment was started at week 16. CR2-Crry treatment was associated with a significant survival benefit ($p = 0.03$, $n = 21$ for PBS-treated mice and $n = 10$ for CR2-Crry-treated mice).

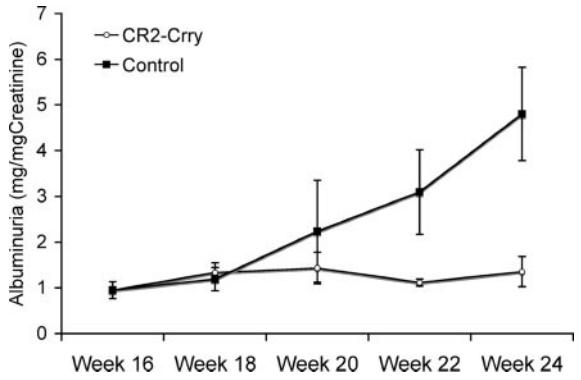

FIGURE 2. Urinary albumin excretion in MRL/*lpr* mice treated with CR2-Crry or PBS. Shown are cumulative biweekly albuminuria measurements from animals treated with inhibitor or PBS from 16 to 23 wk. Data are mean ± SD; $p = 0.04$ comparing animals treated with CR2-Crry to PBS controls.

Statistical analysis

All data are expressed as mean ± SD and were analyzed using SPSS version 15 software. When a single treatment group was compared with its control, two-sample *t* or Wilcoxon rank-sum tests were used for parametric and nonparametric data, respectively. Survival curves were analyzed by the nonparametric Kaplan-Meier method. For multiple comparisons, one-way ANOVA followed by Tukey's pairwise comparisons were used. A value of $p < 0.05$ was considered statistically significant.

Results

Survival

MRL/*lpr* mice were treated with either PBS or CR2-Crry once weekly from 16 to 24 wk of age, which was after the onset of renal disease. The low dose and dosing frequency (0.25 mg, once a week) was selected to investigate the effect and potential relative benefits of targeted complement inhibition and was determined via prior pharmacokinetic experiments previously published (4). CR2-Crry treatment significantly prolonged survival of MRL/*lpr* mice; at 24 wk, there was 40% mortality of control-treated mice whereas 100% of CR2-Crry-treated mice survived ($p = 0.03$) (Fig. 1).

Renal function

To determine the effect of CR2-Crry on renal function, we measured 24 h urinary albumin excretion beginning at week 16 and every 2 wk ending at week 24. In control-treated animals, there was a progressive increase in albuminuria from week 18 through to week 24 (Fig. 2). There was a significant reduction in albuminuria in CR2-Crry-treated animals when compared with controls ($p = 0.04$). At week 16, there was no significant difference in SUN levels between groups assigned for PBS or CR2-Crry treatment (17 ± 5.5 and 17 ± 6.5 mg/dl, respectively). At week 24, there was an increase in SUN in both groups, but SUN levels were significantly lower in the CR2-Crry-treated group compared with the PBS-treated group (28 ± 9 vs 37 ± 7 mg/dl, $p = 0.04$).

Renal pathology

Mice surviving to 24 wk of age were sacrificed and kidneys were removed for histopathological analysis. Histological scores for overall glomerular damage, crescent formation and necrosis, and incidence of vasculitis and interstitial inflammation are shown in Table I. Control-treated animals exhibited classical features of glomerular disease with diffuse glomerulonephritis including cellular proliferation, inflammation, expansion, and fibrocellular crescents. CR2-Crry treatment, however, resulted in a significant improvement in all pathological outcomes with the exception of interstitial inflammation ($p < 0.05$). CR2-Crry-treated mice had a marked decrease in glomerular features of disease with a reduction in mesangial expansion, glomerular inflammation, focal hypercellularity, and crescent formation. Furthermore, the frequency and intensity of arteritis, a constant feature of disease in MRL/*lpr* mice, was also markedly reduced in CR2-Crry-treated animals.

Renal deposition of complement and Ig

At week 24, glomerular deposition of the complement components C1q, C3d, and C9 was significantly reduced in mice treated with CR2-Crry (Table II and Fig. 3). Interestingly, there was also significantly less deposition of IgM and IgG in kidneys from CR2-Crry-treated mice indicating less deposition of immune complexes in the MRL/*lpr* mice (Table II and Fig. 3).

Further quantification of the presence of C1q and C3 was assessed in protein extracted from kidneys removed at the termination of the experiment, from both groups, by using semiquantitative Western blot analysis. The total concentration of C1q and C3 protein present, by densitometry, within the kidneys of CR2-Crry-treated animals was significantly reduced when compared with PBS-treated animals (Fig. 4, $p < 0.05$). Taken together, these analyses of total complement protein within the kidney support our findings with semiquantitative immunofluorescent analysis (Table II), and suggest that CR2-Crry treatment reduces kidney complement deposition.

Other pathological manifestations of autoimmune disease

Pulmonary inflammation in terms of vascular and bronchiole inflammation was determined. Fig. 5 shows representative histopathological images of lung pathology in both control and CR2-Crry-treated animals. The perivascular inflammatory infiltrate,

Table I. *Histopathological scoring of extent and severity of renal disease in treated MRL/*lpr *mice*[a]

Group	Glomerular Score	Interstitial Inflammation	Vasculitis (%)	Crescent/ Necrosis (%)
Control ($n = 12$)	12.1 ± 4.6	3 ± 0.6	100	50
CR2-Crry ($n = 9$)	7.6 ± 2.1	2.9 ± 1	66	33

[a] Scores for glomerular damage in 24-wk-old MRL/*lpr* mice treated with either CR2-Crry or PBS from weeks 16 to 23. Glomerular score is the sum of scores for glomerular inflammation, proliferation, crescent formation, and necrosis (each scored from 0 to 4). Interstitial inflammation was also scored 0–4, and incidence of vasculitis and crescent/necrosis was recorded as a percentage. Treatment with CR2-Crry was associated with a significant improvement in the glomerular score, crescent/necrosis, and a reduction in the incidence of vasculitis ($p < 0.02$). No significant difference was observed for interstitial inflammation ($p > 0.05$).

Table II. *Glomerular deposition of Igs and complement in treated MRL/*lpr *mice*[a]

Group	IgM	IgG	C1q	C3d	C9
Control ($n = 9$)	2.25 ± 0.48	2.34 ± 0.56	2.43 ± 0.52	2.90 ± 0.11	1.96 ± 0.56
CR2-Crry ($n = 6$)	1.87 ± 0.61	1.21 ± 0.60	1.59 ± 0.53	1.84 ± 0.52	0.46 ± 0.66

[a] Scores for immunofluorescence glomerular staining in 24-wk-old MRL/*lpr* mice treated with either CR2-Crry or PBS from wk 16 to 23. CR2-Crry treatment was associated with a significant reduction in all markers when compared to controls ($p < 0.03$).

marked by mononuclear cell infiltration, was significantly less in CR2-Crry-treated mice than in control animals (0.45 ± 0.45 vs 1.45 ± 0.45, $p < 0.05$) (Fig. 5, *A* and *B*). A similar pattern of reduced inflammation was seen when bronchiole inflammation was examined, with scores significantly reduced in CR2-Crry-treated animals (0.11 ± 0.1 vs 0.7 ± 01, $p < 0.05$) (Fig. 5, *C* and *D*).

Dermatitis in the scapular region and ear necrosis are considered consequences of small vessel vasculitis in MRL/*lpr* mice. We investigated both the incidence and the severity of skin lesions on a weekly basis. The prevalence of skin lesions was significantly different between the two groups; all animals in the control group (100%) developed skin lesions by week 24, compared with only 40% of animals in the CR2-Crry group (Fig. 6*A*). The severity of skin lesions affecting the ears and scapular region was also significantly reduced in CR2-Crry-treated mice compared with control animals, with scores of 0.9 ± 0.4 vs 1.8 ± 0.9, respectively ($p < 0.05$) (Fig. 6*B*).

Autoantibody and circulating immune complex analysis

Elevated serum anti-dsDNA Abs are a consistent feature of autoimmunity and disease in MRL/*lpr* mice and are associated with renal disease pathogenesis. As shown in Fig. 7, there was a progressive rise in circulating anti-dsDNA Abs in control MRL/*lpr* mice after week 16, but no significant increase of anti-dsDNA Abs in serum from CR2-Crry-treated mice. The difference between control and CR2-Crry-treated mice was significant from week 20 ($p < 0.01$) (Fig. 7).

Although immune complex deposition and complement activation induces local inflammation and injury, complement is also thought to serve a beneficial role in lupus by facilitating the clearance of circulating immune complexes. There was a progressive and gradual increase in the level of circulating immune complexes from week 16 in both control and CR2-Crry-treated animals with, surprisingly, no significant difference between the two groups (Fig. 8). In contrast to this finding, a previous study demonstrated that systemic complement inhibition in MRL/*lpr* mice with Crry-Ig resulted in markedly higher levels of circulating immune complexes compared with control-treated animals (2).

CR2-Crry targeting and complement inhibition

We determined previously that the circulatory half-life of CR2-Crry in BALB/c mice is 8.5 h, and that CR2-Crry has a minimal effect on serum complement activity 2.5 h after i.v. injection at the dose used here (4). We have also previously shown targeting of CR2-Crry to sites of complement activation in vivo (4), but the fact that a single weekly injection was protective in MRL/*lpr* mice suggests that CR2-Crry is retained at the target site for a prolonged period. We therefore performed a biodistribution study to investigate the half-life of CR2-Crry locally at target sites. ^{125}I-labeled CR2-Crry was administered to 16-wk-old MRL/*lpr* mice and the amount of radioactivity bound to various organs and tissues was determined at 24, 48, and 72 h after injection. At all time points analyzed, CR2-Crry localized preferentially to the kidneys (Fig. 9), a principal site of complement deposition in this model. There was

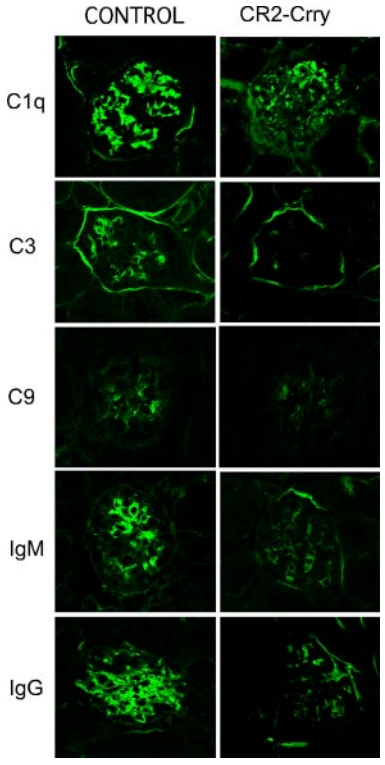

FIGURE 3. Ig and complement deposits in glomeruli of CR2-Crry and PBS-treated MRL/*lpr* mice. Representative immunofluorescence images showing C1q, C3, C9, IgM, and IgG deposition in 24-wk-old MRL/*lpr* mice treated with either PBS (control) or CR2-Crry.

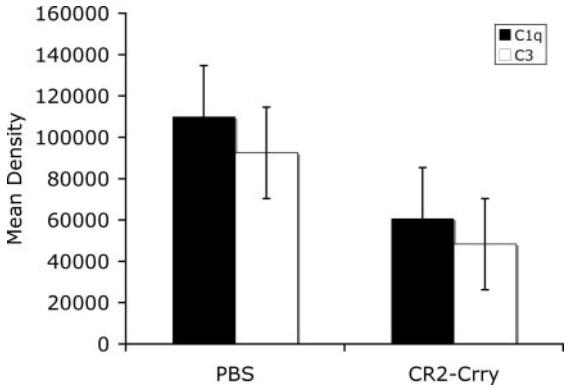

FIGURE 4. Semiquantitative Western blot analysis for total C1q and C3 protein concentration in kidneys from control and CR2-Crry-treated animals. There is a significant reduction in both C1q and C3 proteins in the kidneys of MRL/*lpr* mice treated with CR2-Crry when compared with controls. Results representative of $n = 3$ in each group with three replicates mean ± SEM ($p < 0.05$).

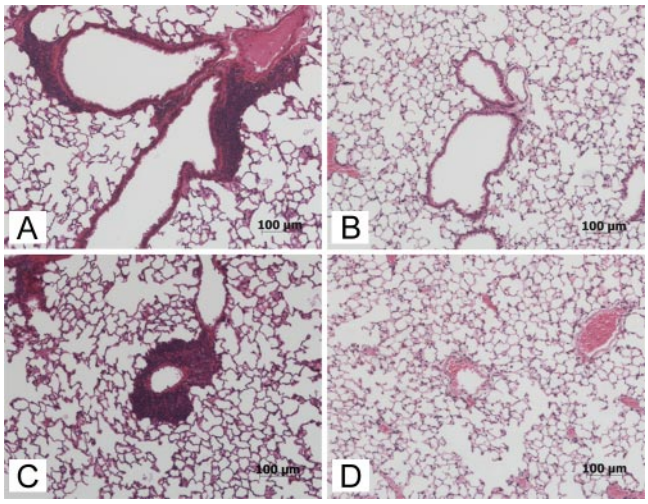

FIGURE 5. Images of lung sections from MRL/*lpr* mice treated with CR2-Crry or PBS. Shown are representative H&E-stained sections from 24-wk-old mice treated with PBS (*A* and *C*) or CR2-Crry (*B* and *D*). Lung from PBS-treated control mouse shows extensive perivascular (*A*) and peribronchial (*C*) inflammatory infiltrates which are composed of more than three layers of inflammatory cells. In contrast, CR2-Crry-treated lung shows only mild inflammation with only a few inflammatory cells surrounding lung vessels (*C*) and with inflammatory cells largely absent in peribronchial (*D*) sites. Representative images of $n = 10$ (control), $n = 7$ (CR2-Crry); scale denoted by scale bars in each image.

also a significant level of CR2-Crry binding in the liver, with similar relative levels bound as in the kidney at 48 and 72 h. This result is likely due to the liver being the major site of immune

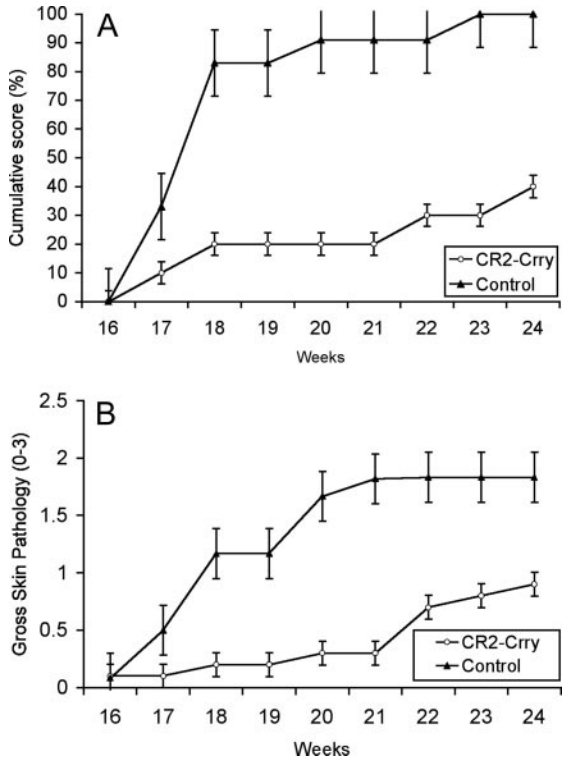

FIGURE 6. MRL/*lpr* mice are prone to the development of skin lesions, characterized by the development of ear necrosis and scapular dermatitis. Shown here (*A*) is the cumulative score and (*B*) gross pathology score for skin lesions seen in CR2-Crry and control animals. Note the significant reduction in skin lesions in CR2-Crry-treated animals ($p < 0.03$).

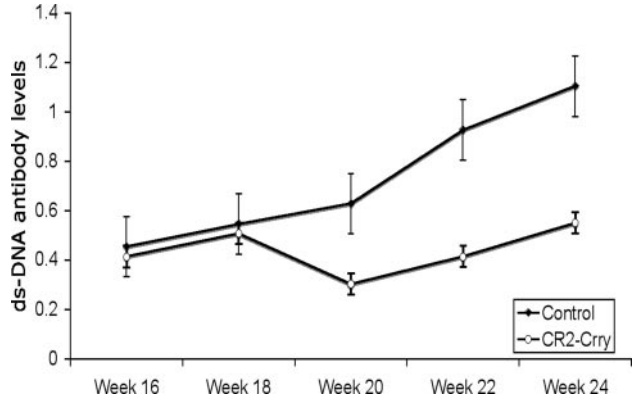

FIGURE 7. Serum anti-dsDNA Ab levels in MRL/*lpr* mice treated with CR2-Crry or PBS. Shown are Ab levels measured biweekly from animals treated with inhibitor or PBS from 16 to 23 wk. Mean ± SD (two-way ANOVA).

complex clearance, and CR2-Crry can be expected to bind to C3-opsonized immune complexes. CR2-Crry binding to circulating immune complexes may also account for the relatively long retention times of CR2-Crry in the blood in this model. With regard to the binding of CR2-Crry to the kidneys, the average microcurie per gram of tissue was 1.85, 0.78, and 0.32 at 24, 48, and 72 h, respectively, indicating a kidney-localized half-life of ~24 h. To assess further the tissue-binding characteristics of CR2-Crry and its spatial and temporal effects on C3 deposition, we performed a kinetic study. Sixteen-week-old MRL/*lpr* mice were administered a single dose of 0.25 mg of CR2-Crry and kidneys were harvested at 24 h, 72 h, and 7 days postadministration of inhibitor. To assess binding of CR2-Crry and its localization to C3d deposition, we used anti-CR2 and anti-C3d dual immunofluorescent staining and confocal microscopy. CR2-Crry was detected within the glomerular compartment and to a lesser extent in the renal tubules of treated animals at all time points, although the amount of CR2-Crry remaining bound at 7 days was significantly diminished (Fig. 10*H*). C3d staining in contrast was strong at 24 h, decreased at 72 h, and increased again at 7 days (Fig. 10, *A*, *D*, and *G*). The increased deposition of C3d at 7 days is likely due to the diminished concentration of CR2-Crry in the kidney at this time point after injection. CR2-Crry and C3d were colocalized within the kidney as indicated by similar distribution patterns and by dual immunofluorescence microscopy (Fig. 10, *C*, *F*, and *I*). Staining for IgM and IgG deposition was not significantly different across this series of samples, presumably due to the short nature of the

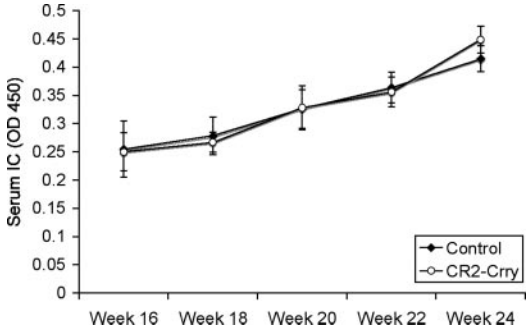

FIGURE 8. Serum immune complex levels in MRL/*lpr* mice treated with CR2-Crry or PBS. Shown are serum immune complex levels measured biweekly from animals treated with inhibitor or PBS from 16 to 24 wk. Mean ± SD.

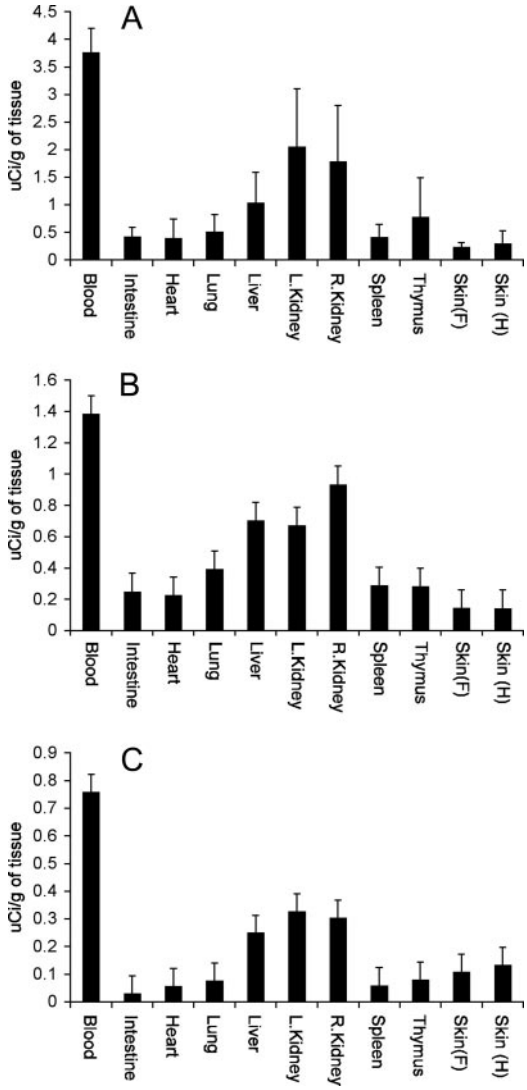

FIGURE 9. Biodistribution of CR2-Crry in 16-wk-old MRL/*lpr* mice following i.v. injection. Iodinated CR2-Crry was administered via tail vein injection and binding of ^{125}I-labeled CR2-Crry was quantified in major organs at 24 (*A*), 48 (*B*), and 72 h (*C*) postinjection. Mean ± SEM ($n = 3$).

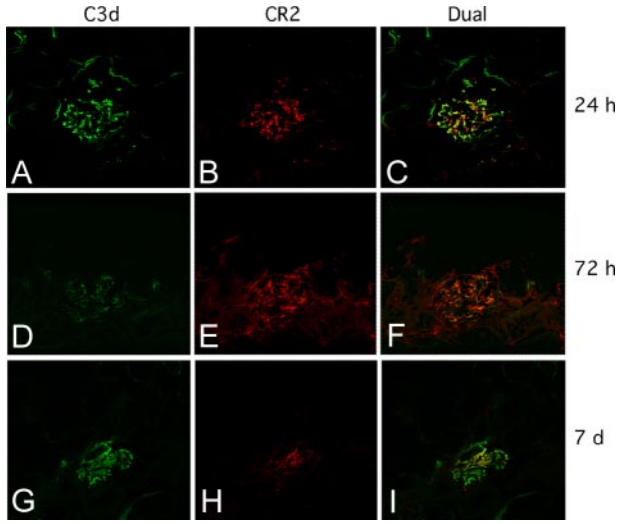

FIGURE 10. Renal localization of CR2-Crry and C3d. Shown is immunofluorescent staining for CR2, C3d, and colocalization of CR2 and C3d by confocal microscopy. A 0.25-mg dose of CR2-Crry was administered to 16-wk-old MRL/*lpr* mice and sections prepared from kidneys isolated at 24 h (*A–C*), 72 h (*D–F*), and 7 days (*G–I*) postadministration. Anti-C3d is demonstrated by green fluorescence, anti-CR2 by red fluorescence, and colocalization of both Ags by yellow/orange fluorescence. Representative images ($n = 3$ in all groups).

experiment and the animals only receiving a single dose of CR2-Crry (data not shown). Specificity of staining was confirmed by the absence of staining in untreated 16-wk-old MRL/*lpr* mice and in control sections on which the primary Ab was omitted.

Serum complement inhibition

Serum complement activity in MRL/*lpr* mice was assessed at 16 and 24 wk in CR2-Crry and control mice. Serum was collected 24 h postadministration in 16-wk-old mice and analyzed for the extent of complement inhibition. There was no significant reduction in complement activation between control and CR2-Crry-treated mice with control samples. Treatment with CR2-Crry in 16-wk-old animals was associated with an overall 12 ± 6.5% reduction in complement activation when compared with controls ($n = 6$ in all groups, $p < 0.05$). Furthermore, there was also no significant difference in serum complement activity in 24-wk-old mice who had received their last dose of CR2-Crry 7 days previously, with 24-wk-old animals exhibiting a 8 ± 6.5% reduction in serum complement activation ($n = 6$ in all groups, $p < 0.05$).

Discussion

There is a well-documented role of complement in the pathogenesis of immune complex renal disease, and inhibitors of the complement system represent an attractive potential therapy for immune complex-mediated diseases, including lupus. Systemic inhibitors of complement have demonstrated efficacy in partially preventing renal disease in murine models of lupus nephritis, providing proof of principle of this therapeutic approach. Herein, we report on the efficacy and potential advantages of a novel targeting strategy for inhibiting localized complement-mediated damage in the murine MRL/*lpr* model of lupus nephritis. Because the CR2-targeting strategy requires complement activation for efficient targeting (i.e., generation of C3 breakdown products that are CR2 ligands), we started treatment at 16 wk of age, after onset of autoantibody production and proteinuria in the test mice. This is a later time point than in previous studies investigating complement inhibition and is more clinically relevant. CR2-Crry treatment of MRL/*lpr* mice resulted in a number of significant differences in disease expression compared with controls including reduced renal disease, pulmonary disease, skin disease, vasculitis, autoantibody production, and survival. This efficacy was accomplished without impacting circulating immune complex levels, a key beneficial function of the complement cascade.

Crry is an inhibitor of both the classical and alternative complement pathways. A previous study demonstrated a therapeutic benefit of administering soluble Crry-Ig (3 mg) every other day to MRL/*lpr* mice from 12 wk of age (2). Crry-Ig treatment resulted in decreased proteinuria, maintenance of renal function, decreased glomerulosclerosis, decreased deposits of Ig and C3, and less skin disease (2). Despite these therapeutic outcomes, there was no statistically significant difference in survival and little improvement in glomerular pathologic disease. The lack of a more significant treatment benefit of Crry-Ig may be due to its effect on the systemic inhibition of immune complex clearance; Crry-Ig treatment resulted in markedly increased serum circulating ICs and increased serum C3 levels in the mice. Other differences between the previous use of systemic Crry-Ig and targeted CR2-Crry used in this

study are the start of treatment before disease (12 vs 16 wk, Crry-Ig vs CR2-Crry), frequency of administration (every other day vs weekly) and dose (3 vs 0.25 mg). Of further note, we previously demonstrated that systemic complement inhibition with Crry-Ig significantly enhanced susceptibility to infection in a mouse model of sepsis, whereas targeted CR2-Crry had no immunosuppressive effect (4).

In the current study, there was no change in circulating ICs associated with CR2-Crry treatment. There are a number of mechanisms by which immune complexes are believed to form in the glomerulus of lupus patients and in animal models of lupus. These include 1) deposition of circulating immune complexes; 2) DNA binding to charged glomerular molecules with subsequent binding of anti-DNA Abs; and 3) direct binding of autoantibodies to glomerular Ags such as laminin and heparan sulfate proteoglycan (reviewed in Ref. 13). Because CR2-Crry treatment resulted in reduced deposition of immunoreactants in the glomerulus, but did not impact circulating ICs, it would appear that the latter two mechanisms of immune complex deposition are most impacted by this therapy. The reason that circulating IC levels are unaffected in CR2-Crry-treated mice may be due to the fact that CR2-Crry does not interfere with complement-dependent IC clearance mechanisms, or it may be due to reduced IC formation because CR2-Crry treatment lowered autoantibody titers.

Biodistribution studies indicated that CR2-Crry had a half-life of ~24 h in the kidneys. Interestingly, this broadly corresponds to the reported half-life of C3d (the CR2-Crry ligand) in various rat models of complement-dependent glomerulonephritis (14). In this previous report (14), there was an ~30–60% reduction in C3d levels 24 h after the termination of complement activation, although C3d staining persisted for several days. In the current study, mice were treated once weekly with CR2-Crry, and a study was performed to better understand the kinetics of CR2-Crry binding and the deposition of complement and Abs. In the kinetic study, 16-wk-old mice were injected with a single therapeutic dose of CR2-Crry and kidney sections analyzed at 24 h, 72 h, and 7 days postinjection for CR2-Crry, C3d, IgM, and IgG. Our interpretation of the results are as follows: the initial presence of C3 (24 h) provides the targeting ligand for CR2-Crry. Binding of CR2-Crry results in complement inhibition and reduced C3 deposition at 72 h. By 7 days, there is little CR2-Crry remaining bound and activation of complement and C3 deposition is increased (at which time the next dose of CR2-Crry is administered in the therapeutic model). There was no significant difference in IgG or IgM deposition over the time period of the kinetic study. However, when analysis was done 7 days after the last injection of CR2-Crry in the therapeutic study (at 24 wk), there was significantly reduced C3, IgG, and IgM deposition compared with control-treated animals, and CR2-Crry was not detected. Together, the data indicate a cumulative effect of CR2-Crry treatment.

An unexpected finding in this study was the effect of CR2-Crry on anti-dsDNA Abs. Although the complement system plays a key role in amplifying T cell-mediated Ab responses, our prior analyses in C3, factor B, and factor D knockout MRL/*lpr* mice did not demonstrate any effect of complement component deficiency on autoantibody levels. Furthermore, neither transgenic expression of Crry in MRL/*lpr* mice (15), nor Crry-Ig treatment of MRL/*lpr* mice (2) had any effect on autoantibody levels. These previous results together with data presented here suggest that the CR2-mediated targeting of Crry may interfere with normal CR2 cell surface mediated B cell/T cell interactions and inhibit autoantibody production. Studies with CR2 knockout mice indicate that the lack of CR2, depending on background strain, leads to enhanced autoantibody production, implicating a role for CR2 in tolerance mechanisms (16). Dysfunctional CR2 has also been implicated in disease development in NZM2410 mice (17). Thus, a possibility is that increased levels of CR2 alone, or linked with a complement inhibitor, may enhance maintenance of tolerance leading to decreased autoantibody production. There are, however, other biologic functions of CR2 that may also impact disease and autoantibody expression. CR2 binds IFN-α (18, 19), and a recent study characterized the interaction of IFN-α with short consensus repeat domains 1 and 2 of CR2 (19), domains contained in the CR2-Crry construct. There is substantial data linking IFN-α with lupus disease pathogenesis in human and murine lupus in New Zealand Black mice (20–22), and the sequestering and clearance of IFN-α by CR2 could impact disease expression, including autoantibody expression in lupus (23). Against this hypothesis, however, is the recent finding that blocking IFN-α in MRL/*lpr* mice in fact worsened disease expression (24). CR2 has also been shown to bind DNA and could possibly clear circulating DNA and reduce autoantibody production by removing the inciting Ag. Use of DNase to decrease circulating DNA in mouse models of lupus has not proven to be efficacious, though knocking out DNase has induced lupus-like disease in normal mouse strains (25, 26). Further investigations are needed to define the precise mechanisms by which CR2-Crry impacts murine lupus disease expression.

This is the first report of the use of CR2-Crry to treat a chronic inflammatory complement-dependent disease. As noted, there were some significant differences in therapeutic outcome associated with targeted vs untargeted systemic complement inhibition. The survival benefit associated with CR2-Crry treatment may result from its more global effect on MRL/*lpr* disease. Although renal failure is a clear cause of death in MRL/*lpr* mice, progressive inflammatory pulmonary disease is also implicated in the mortality of these mice. The mice receiving CR2-Crry had significantly less pulmonary inflammatory disease than did the placebo-treated group. Significantly, lung disease was not impacted by factor B or D deficiency (27, 28). Also of interest, in the previous Crry-Ig study in which treatment did not result in any survival benefit, none of the Crry-Ig-treated mice had evidence of renal failure (2), raising the possibility that pulmonary disease was the cause of death. Thus, CR2 targeting appears to have a particularly prominent impact on inflammatory disease in the lung, be it secondary to lupus or due to remote organ injury as can occur in ischemia reperfusion injury. This global effect of CR2-targeted complement inhibition on disease may have important implications for the treatment of lupus in humans, which is typically systemic with multiple organ involvement.

The efficacy of CR2-Crry is a key benefit, but there are other advantages of this targeted approach. A significant advantage is the lack of effect on circulating IC levels, which is in contrast to the effect of the systemic inhibitor Crry-Ig, which markedly increased levels of circulating ICs. C3-deficient MRL/*lpr* mice have enhanced IC deposition in the kidneys and targeted, but not systemic, C3 inhibition allows the beneficial effect of complement system in the clearing of ICs to continue. A second advantage, as noted above, is that CR2-targeted complement inhibition appears to be minimally immune suppressive.

In conclusion, CR2-Crry given weekly to MRL/*lpr* mice resulted in significant improvement in autoantibody levels, renal disease, lung disease, and survival. The mechanisms for this improvement are likely multifactorial. Systemic complement inhibitors have demonstrated efficacy in improving renal disease in MRL/*lpr* mice (albeit at much higher doses and dosing frequency), but they have not demonstrated improved survival. The efficacy of a CR2-mediated targeting strategy of C3 inhibition and its apparent lack

of impact on protective complement functions make it an attractive approach for translation into human disease.

Disclosures

Drs. Gilkeson and Tomlinson are consultants for Taligen Therapeutics, a company developing complement inhibitors for therapeutic use.

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