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DAF/Crry double deficiency in mice exacerbates nephrotoxic serum-induced proteinuria despite markedly reduced systemic complement activity

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Abstract

Decay-accelerating factor (DAF) and complement receptor 1-related gene/protein y (Crry) are two membrane-anchored complement regulatory proteins in rodent. Although both proteins are broadly distributed and exert complement regulation at the same steps of the complement cascade, DAF knockout mice are viable whereas Crry knockout mice die *in utero* as a result of maternal complement attack. The latter outcome has prevented the dissection of overlapping functions of DAF and Crry in adult mouse tissues *in vivo*. By crossing female DAF$^{-/-}$/Crry$^{-/-}$/C3$^{-/-}$ mice with male DAF$^{-/-}$/Crry$^{+/-}$/C3$^{+/-}$ mice, we circumvented maternal complement attack during fetal development and generated viable DAF$^{-/-}$/Crry$^{-/-}$/C3$^{+/-}$ mice to address the consequence of DAF/Crry double deficiency. DAF$^{-/-}$/Crry$^{-/-}$/C3$^{+/-}$ mice were born at the expected frequency and survived to adulthood. However, they were found to have greatly reduced systemic complement activity due, at least in part, to spontaneous C3 activation and consumption. Plasma C3 proteins in DAF$^{-/-}$/Crry$^{-/-}$/C3$^{+/-}$ mice were 30% of that of wild-type mice, and serum complement activity, as assessed by zymosan and immune complex C3 opsonization assays, was 90% reduced in DAF$^{-/-}$/Crry$^{-/-}$/C3$^{+/-}$ mice. Remarkably, despite greatly reduced systemic complement activity, DAF$^{-/-}$/Crry$^{-/-}$/C3$^{+/-}$ mice developed more severe proteinuria after induction of nephrotoxic serum nephritis as compared with DAF$^{-/-}$/Crry$^{+/-}$/C3$^{+/-}$ and DAF$^{-/-}$/Crry$^{-/-}$/C3$^{-/-}$ littermate controls. The results highlight the critical and overlapping role of Crry and DAF *in vivo* in preventing complement activation and tissue injury.

Keywords: DAF; CD55; Crry; Complement; Inflammation; Glomerulonephritis; Anti-GBM

1. Introduction

Host cells are protected from homologous complement attack by several membrane inhibitors of complement (Kim and Song, 2006; Miwa and Song, 2001). In humans, this includes decay-accelerating factor (DAF) (Lublin and Atkinson, 1989), complement receptor 1 (Krych et al., 1999), membrane cofactor protein (MCP) (Liszewski et al., 1991) and CD59 (Takizawa et al., 1992). DAF, CR1 and MCP regulate complement activation at the C3 and C5 steps, while CD59 prevents the formation of the membrane attack complex at the terminal step (Kim and Song, 2006; Miwa and Song, 2001). In the mouse, two DAF genes, referred to as daf-1 and daf-2, have been identified. Daf-1, encoding a GPI-anchored protein with broad tissue distribution, is considered to be the murine ortholog of human DAF (Miwa and Song, 2001; Song et al., 1996; Spicer et al., 1995). Daf-2, on the other hand, encodes primarily a transmembrane protein, which is restricted to the testis in its expression (Miwa and Song, 2001; Miwa et al., 2001; Song et al., 1996; Spicer et al., 1995). Besides having two DAF genes, the mouse also differs from human in having a rodent-specific membrane inhibitor, complement receptor 1-related gene/protein y (Crry) (Holers et al.,

Abbreviations: DAF, decay accelerating factor; Crry, complement receptor 1-related gene/protein y; MCP, membrane cofactor protein; GVBS^{++}, gelatin-veronal buffered saline; FACS, fluorescence-activated cell sorter

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1992; Kim and Song, 2006; Li et al., 1993; Miwa and Song, 2001). Crry has both MCP and DAF activities and, like daf-1, is widely expressed in various mouse tissues (Holers et al., 1992; Li et al., 1993; Miwa and Song, 2001). Given the limited expression of murine MCP, it is assumed that Crry acts as a functional homolog of MCP in extra-testicular tissues in the mouse (Holers et al., 1992; Kim and Song, 2006; Li et al., 1993; Miwa and Song, 2001).

Despite having similar patterns of tissue distribution and sites of complement inhibition, daf-1 and Crry gene knockout mice had very different phenotypes (Sun et al., 1999; Xu et al., 2000). Daf-1 deficient mice ($DAF^{-/-}$) were viable, but they displayed increased sensitivity to complement-mediated injury in several inflammatory disease models (Miwa et al., 2002a; Sogabe et al., 2001; Sun et al., 1999; Yamada et al., 2004). By contrast, Crry gene knockout was embryonically lethal (Xu et al., 2000). The lethal phenotype of $Crry^{-/-}$ mice could be rescued by maternal C3 deficiency (Xu et al., 2000), and immunohistochemical staining showed that Crry but not DAF was expressed on the developing mouse embryos (Miwa et al., 2001; Xu et al., 2000). This led to the hypothesis that $Crry^{-/-}$ embryos were susceptible to complement attack because DAF was not available to compensate for the lack of Crry in early development (Miwa et al., 2001; Xu et al., 2000). Arguing against this hypothesis, however, was the subsequent finding that $Crry^{-/-}/C3^{-/-}$ mouse erythrocytes were susceptible to complement attack despite having DAF expression (Miwa et al., 2002b; Molina et al., 2002). Further characterization of erythrocytes that are singly or doubly deficient in Crry and DAF revealed that, although Crry was more critical than DAF in regulating alternative pathway complement activation, Crry and DAF were equally active and could compensate for each other during classical pathway complement activation on red blood cells (Miwa et al., 2002b; Molina et al., 2002).

Though many previous studies have demonstrated the protective role of DAF or Crry in models of complement-mediated inflammatory injury (Quigg et al., 1998a,b; Sogabe et al., 2001; Song, 2004; Yamada et al., 2004), the potential redundancy between DAF and Crry in regulating classical pathway complement activation in these settings has not been investigated. We generated viable daf-1 and Crry double knockout mice ($DAF^{-/-}/Crry^{-/-}$) that retained one copy of the C3 gene by crossing female $DAF^{-/-}/Crry^{-/-}/C3^{-/-}$ mice with male $DAF^{-/-}/Crry^{+/-}/C3^{+/-}$mice. This experimental strategy circumvented the problem of Crry-null embryos' susceptibility to maternal complement attack and provided us a mouse model to assess the consequences of daf-1 and Crry double deficiency in a complement-dependent disease process. The objectives of our study are to determine if daf-1/Crry double knockout mice are viable and if they are, whether they would be more susceptible than daf-1 deficient mice to nephrotoxic serum nephritis.

2. Materials and methods

Mice and breeding experiments. Experiments with mice were conducted by following established guidelines for animal care and all protocols were approved by the appropriate institutional committees. The generation of $DAF^{-/-}$, $Crry^{-/-}/C3^{-/-}$ and $DAF^{-/-}/Crry^{-/-}/C3^{-/-}$ mice, all on a mixed C57BL/6 and 129J background, were described previously (Molina et al., 2002; Sun et al., 1999; Xu et al., 2000). Initial breeding experiments were carried out between these mice and/or C57BL/6 wild-type mice and were aimed at generating the following pairs of breeders: female $Crry^{-/-}/C3^{-/-}$ × male $Crry^{+/-}/C3^{+/-}$ mice, female $DAF^{-/-}/Crry^{-/-}/C3^{-/-}$ × male $DAF^{-/-}/Crry^{+/-}/C3^{+/-}$ mice (Table 1). To generate male $Crry^{+/-}/C3^{+/-}$ breeders, female $Crry^{-/-}/C3^{-/-}$ mice were crossed with wild-type mice. To generate male $DAF^{-/-}/Crry^{+/-}/C3^{+/-}$ breeders, female $DAF^{-/-}/Crry^{-/-}/C3^{-/-}$ mice were crossed with $DAF^{-/-}$ mice.

Table 1
Generation of $DAF^{-/-}/Crry^{-/-}/C3^{+/-}$ mice

Male × female			
$DAF^{+/+}/Crry^{+/-}/C3^{+/-}$ × $DAF^{+/+}/Crry^{-/-}/C3^{-/-}$ (group 1)			
$DAF^{-/-}/Crry^{+/-}/C3^{+/-}$ × $DAF^{-/-}/Crry^{-/-}/C3^{-/-}$ (group 2)			
Genotype of pups		Number of pups (percentage of total)	
Crry	C3	$DAF^{+/+}$ (group 1)	$DAF^{-/-}$ (group 2)
−/−	+/−	23 (28%)	44 (26.2%)
+/−	+/−	23 (28%)	46 (27.4%)
−/−	−/−	23 (28%)	35 (20.8%)
+/−	−/−	13 (16%)	43 (25.6%)
		$DAF^{+/+}$ (group 1)	$DAF^{-/-}$ (group 2)
Total pup number		82	168
Total litter number		12	29
Average litter size		6.8	5.8

In the breeding strategy listed in Table 1, the genotype of the daf-1 gene is either wild-type ($DAF^{+/+}$, group 1) or knockout ($DAF^{-/-}$, group 2), whereas the genotypes of the Crry and C3 genes are either heterozygous (+/−) or knockout (−/−). These breeding pairs produced four different types of mice as littermates. Genotypes of mice were determined by a combination of PCR analysis of tail DNA and fluorescence-activated cell sorter (FACS) analysis of erythrocyte DAF or Crry expression. The C3 allele was typed with the following primers, 5′-GATCCCCAGAGCTAATG-3′, 5′-TCGTCCTGCAGTTCATTCAG-3′ and 5′-AGGGACCA-GCCCAGGTTCAG-3′. To genotype DAF and Crry, mouse erythrocytes, obtained by tail vein bleeding, were stained with polyclonal rabbit antibodies specific for mouse DAF or Crry (Dr. Michael Holers, University of Colorado Health Sciences Center, Denver). After washing several times with FACS buffer (PBS solution containing 0.1% bovine serum albumin, 0.1% sodium azide), cells were stained with Phycoerythrin (PE)-conjugated goat anti-rabbit IgG (Sigma, St. Louis, MO). Cells were analyzed by FACScan (Becton Dickinson, San Jose, CA).

C3 deposition assay. Assays of antibody-induced C3 deposition on cells were performed as described previously (Molina et al., 2002). Briefly, erythrocytes (1×10^6 cells in 200 μl PBS) from $DAF^{-/-}/Crry^{-/-}/C3^{-/-}$ mice were opsonized with a mouse anti-mouse erythrocyte monoclonal antibody 34-3C, 50 μg/ml (Clynes and Ravetch, 1995) (gifted from Dr. Raphael Clynes, Columbia, New York, NY). Antibody-opsonized cells

were incubated with mouse serum in gelatin-veronal buffered saline ($GVBS^{++}$) at 37 °C for 30 min. Cells were washed in FACS buffer, then incubated with a FITC-conjugated goat anti-mouse C3 antibody (ICN Aurora, OH), and analyzed by FACS for C3 deposition. To measure alternative pathway complement activity, mouse sera were incubated with zymosan particles at 37 °C for 60 min (in a total volume of 100 μl, made by mixing serum and zymosan in GVBS containing 2 mM $MgCl_2$ and 10 mM EGTA, final concentration of zymosan: 2.5 mg/ml). The zymosan particles were then washed in FACS buffer, incubated with FITC-conjugated goat anti-mouse C3 antibody, and analyzed by FACS for C3 deposition.

Assessment of erythrocyte survival in vivo. To determine the viability of complement regulator-deficient erythrocytes *in vivo*, cells (from 150 μl blood) from $DAF^{-/-}/Crry^{-/-}/C3^{-/-}$ mice were labeled *ex vivo* with biotin as previously described (Miwa et al., 2002b; Molina et al., 2002) and introduced into various host mice via the tail vein. Blood samples were collected at 5 min after erythrocyte infusion, and then at various indicated time points thereafter. Collected erythrocytes were stained with *R*-(PE)-conjugated streptavidin (Molecular probes), and the percentage of biotinylated cells was determined at each time point.

ELISA for plasma C3 quantification. Microtiter plates were coated with 50 μl (2.3 μg/ml in PBS pH 7.4) of rabbit anti-rat Fc antibody (ICN Pharmaceuticals, Inc., Ohio) for 2 h. The wells were saturated with 1% BSA in PBS (blocking buffer) followed by incubation with rat anti-mouse C3 mAb 2–16 (1:160 dilution of hybridoma supernatant in blocking buffer). The mAb 2–16 was generated in house and has been published elsewhere (Mastellos et al., 2004). Mouse plasma, serially diluted in blocking buffer, was then added and incubated for 1 h. Subsequently, polyclonal goat anti-mouse C3 horse radish peroxidase-conjugated antibody (3.2 μg/ml in blocking buffer, ICN Pharmaceuticals, Inc., Ohio) was added and incubated for an additional 1 h. The bound antibody was detected by addition of the substrate solution (0.05% 2,2′-azino-di-[3 ethylbenzthiazoline sulfonic acid] [ABTS; Roche, Indianapolis, IN], 0.1% H_2O_2 in 0.1 M Nacitrate buffer pH 4.2). All incubations were carried out at room temperature, and the wells were washed with PBS containing Tween 20 (0.05%) between each incubation step.

Western blot for the detection of intact and activated C3 in the plasma. Mouse plasma (20 μl, diluted 1:70 in H_2O) was mixed with 5 μl reducing sample dye and loaded onto a 7.5% SDS-polyacrylamide gel. The proteins were transferred to a PVDF membrane (Polyscreen, Perkin-Elmer Life Sciences, Boston, MA). The membrane was blocked with 10% milk in PBS and then incubated for 1 h with a polyclonal rabbit anti-mouse C3c antibody (generated in house, 1:5000 dilution in blocking buffer). Bound polyclonal antibodies were detected by incubation for 30 min with a horseradish peroxidase-conjugated goat anti-rabbit IgG antibody (0.25 μg/ml in blocking buffer, Bio-Rad, Richmond, CA). The luminescent reaction was performed using the ECL kit (Amersham Pharmacia Biotech, Piscataway, NJ). All incubations were carried out at room temperature, and the membrane was washed with PBS containing Tween 20 (0.05%) between incubation steps.

Induction of nephrotoxic serum (NTS) nephritis. Two models of nephrotoxic serum-induced nephritis were employed. Nephrotoxic serum was prepared by immunizing rabbit or sheep with purified mouse glomerular basement membrane fraction (Sogabe et al., 2001). Accelerated NTS nephritis was induced as previously described (Sogabe et al., 2001). Briefly, 8-week-old male mice were preimmunized (i.p.) with rabbit IgG (Sigma) in complete Freund's adjuvant (CFA, Sigma) (0.5 mg IgG per 20 g body weight, mixed with an equal volume of CFA). Five days later, rabbit NTS (0.05 ml per 20 g body weight, diluted with five parts of saline) was injected through the tail vein. Urine samples were collected in metabolic cages before disease induction (day 0) and at days 3, 5 and 7 after NTS injection. Mice were killed at day 7 and the kidneys were harvested for pathologic analysis. In the non-accelerated NTS nephritis model, 8-week-old male mice were injected with NTS through the tail vein (0.1 ml of sheep NTS, diluted 1:1 in pyrogen-free saline). Urine samples were collected in metabolic cages at days 2 and 5 after NTS injection.

Urinary albumin and creatinine detection. Urinary albumin concentration was determined by a mouse albumin ELISA quantification kit (Bethyl laboratories Inc., Montogomery, TX). Urinary creatinine concentration was measured by a colorimetric microplate assay kit (Oxford Biomedical Research, Oxford, MI).

Histology. At day 5 or day 7 after NTS injection, mice were sacrificed, and kidneys were collected and fixed in methyl Carnoy's solution overnight and then embedded in paraffin. Kidneys were sectioned to 4-μm thickness and stained with H&E and periodic acid-Schiff (PAS). Renal pathology was assessed by a single investigator (M.P.M.) without knowledge of genotype or manipulation of the mice. Kidneys were graded for severity of disease in three areas (vascular, interstitial, and glomerular) in a range of 0 (for no pathology evident) to 4+ (most severe pathology, end stage disease) (Chan et al., 1999a,b), and cumulative scores were calculated.

Immunofluorescence. Mouse kidneys were frozen in OCT and sectioned to 4 μm. After fixing the tissue to positively charged slides (Fisher Scientific, Newark, DE), the slides were washed three times with PBS, fixed for 10 min with ether/ethanol, and for 20 min with 95% ethanol. They were then rewashed three times in PBS. To check the deposition of C3, mouse or sheep IgG, the slides were incubated with FITC-conjugated goat anti-mouse C3 or anti-mouse or sheep IgG (Cappel, ICN Pharmaceuticals, Aurora, OH). After a final wash step, the slides were mounted in a medium (Aquamount; Fisher Scientific), dried at 4 °C and viewed on the following day. C3 staining was graded on a 1–4 scale.

3. Results

$DAF^{-/-}/Crry^{-/-}/C3^{+/-}$ mice were born at the expected frequency and were viable. We previously found that by using female $Crry^{-/-}/C3^{-/-}$ mice as breeders, viable $Crry^{-/-}/C3^{-/-}$ mice could be produced and thrive to adulthood without overt pathology (unpublished observation). We reasoned that survival of adult $Crry^{-/-}/C3^{-/-}$ mice might be due to compensation of Crry by the daf-1 gene, which co-expresses with

Crry on virtually all adult mouse tissues, but not on developing embryos (Li et al., 1993; Miwa et al., 2001; Song et al., 1996). Therefore, the first question that was to be addressed was whether DAF$^{-/-}$/Crry$^{-/-}$/C3$^{+/-}$ mice, produced by using female mice deficient in C3 to circumvent maternal complement attack, were viable. To this end, we set up two breeding experiments in order to compare the effect on progeny viability of single Crry gene deficiency versus daf-1/Crry double gene deficiency (Table 1). To our surprise, we found that in both breeding experiments, all four genotypes were produced at the expected Medelian ratio (Table 1). Thus, like Crry$^{-/-}$/C3$^{+/-}$ mice, DAF$^{-/-}$/Crry$^{-/-}$/C3$^{+/-}$ mice were born at the expected frequency, and they survived to adulthood with no overt abnormalities (Table 1).

DAF$^{-/-}$/Crry$^{-/-}$/C3$^{+/-}$ mice were unable to eliminate DAF/Crry-deficient erythrocytes. In a previous study, we demonstrated that DAF$^{-/-}$/Crry$^{-/-}$/C3$^{-/-}$ mouse erythrocytes were susceptible to rapid complement-mediated elimination when transfused into complement-sufficient wild-type mice (Molina et al., 2002). Thus, the viability of DAF$^{-/-}$/Crry$^{-/-}$/C3$^{+/-}$ mice was perplexing, since at least their erythrocytes, deficient in both DAF and Crry, should be susceptible to attack by their own complement. To determine if the erythrocytes of DAF$^{-/-}$/Crry$^{-/-}$/C3$^{+/-}$ mice had acquired other complement resistant mechanisms so that they became refractory to complement attack, we transfused erythrocytes from DAF$^{-/-}$/Crry$^{-/-}$/C3$^{+/-}$ or DAF$^{-/-}$/Crry$^{-/-}$/C3$^{-/-}$ mice into wild-type recipients and compared their survival. Fig. 1A shows that both types of cells were rapidly eliminated from the wild-type recipient mice, establishing that erythrocytes of DAF$^{-/-}$/Crry$^{-/-}$/C3$^{+/-}$ mice were no different from that of DAF$^{-/-}$/Crry$^{-/-}$/C3$^{-/-}$ mice in terms of susceptibility to complement attack.

To test the possibility that erythrocytes in DAF$^{-/-}$/Crry$^{-/-}$/C3$^{+/-}$ mice were attacked *in vivo* by their own complement but the animals managed to thrive by increasing hematopoiesis as a compensatory response, we collected erythrocytes from DAF$^{-/-}$/Crry$^{-/-}$/C3$^{+/-}$ mice, and after labeling with a tracer *ex vivo*, re-introduced them back into the same mice to monitor their turnover. Fig. 1B shows that, unlike their rapid elimination in wild-type recipients (Fig. 1A), DAF$^{-/-}$/Crry$^{-/-}$/C3$^{+/-}$ erythrocytes were not subjected to accelerated turnover in DAF$^{-/-}$/Crry$^{-/-}$/C3$^{+/-}$ mice. This result suggested that alternative pathway complement activity in DAF$^{-/-}$/Crry$^{-/-}$/C3$^{+/-}$ mice was not sufficient to eliminate the otherwise susceptible DAF/Crry-deficient erythrocytes.

Because DAF$^{-/-}$/Crry$^{-/-}$/C3$^{+/-}$ mice had only one copy of the C3 gene, we wondered if there might be a gene dosage effect such that the concentration of plasma C3 in DAF$^{-/-}$/Crry$^{-/-}$/C3$^{+/-}$ mice was lower, potentially explaining the inability of these mice to eliminate DAF/Crry-deficient erythrocytes. To test this possibility, we adoptively transferred DAF$^{-/-}$/Crry$^{-/-}$/C3$^{-/-}$ erythrocytes into regular C3$^{+/-}$ and wild-type mice and followed their survival. Fig. 1C shows that, although the kinetics of elimination was somewhat slower in C3$^{+/-}$ mice than in wild-type mice, DAF/Crry-deficient erythrocytes could be quantitatively eliminated in mice with only one copy of the C3 gene.

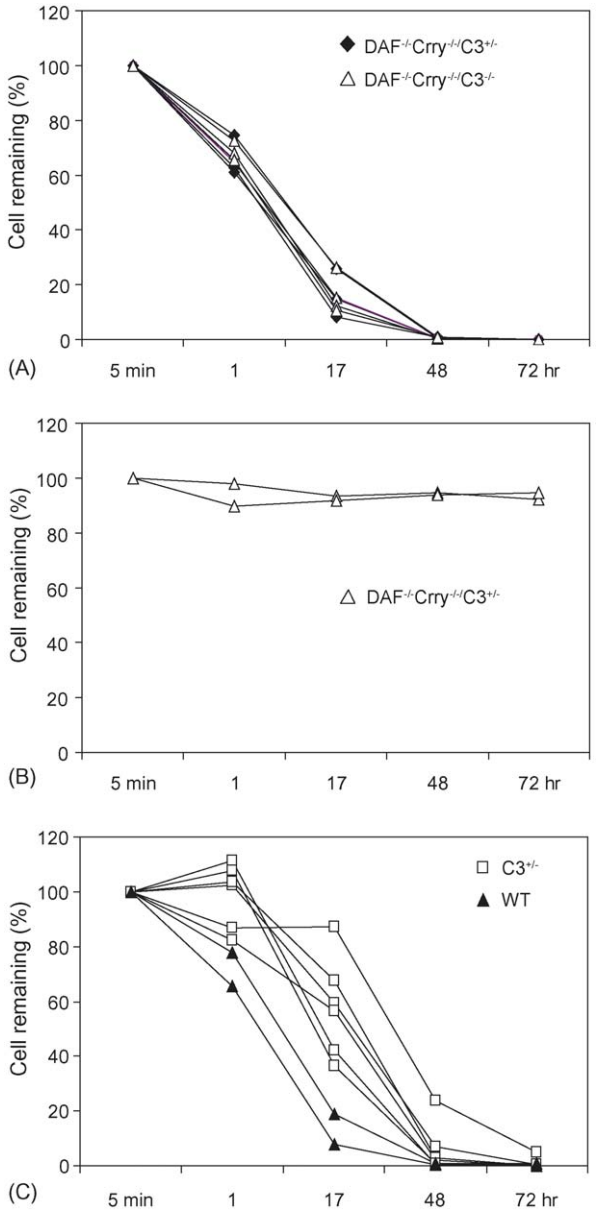

Fig. 1. Assessment of erythrocyte sensitivity to complement attack in DAF$^{-/-}$/Crry$^{-/-}$/C3$^{+/-}$ mice. Erythrocytes were labeled *ex vivo* and transfused into recipient mice. The percentage of labeled erythrocytes in blood samples collected from the recipients at various time points was normalized to that at 5 min. (A) Comparison of survival of DAF$^{-/-}$/Crry$^{-/-}$/C3$^{+/-}$ ($n = 4$) and DAF$^{-/-}$/Crry$^{-/-}$/C3$^{-/-}$ ($n = 3$) mouse erythrocytes after transfusion into wild-type mouse recipients. Both types of cells were susceptible to complement attack. (B) DAF$^{-/-}$/Crry$^{-/-}$/C3$^{+/-}$ ($n = 2$) erythrocytes did not undergo rapid elimination in DAF$^{-/-}$/Crry$^{-/-}$/C3$^{+/-}$ mice. Erythrocytes were harvested from DAF$^{-/-}$/Crry$^{-/-}$/C3$^{+/-}$ mice, labeled *ex vivo* and infused back to the same donors. (C) One copy of the C3 gene in the recipient mice is sufficient to eliminate DAF/Crry deficient erythrocytes. DAF$^{-/-}$/Crry$^{-/-}$/C3$^{-/-}$ mouse erythrocytes were transfused into wild-type (WT, $n = 2$) or regular C3 heterozygous mice (C3$^{+/-}$, $n = 6$).

DAF$^{-/-}$/Crry$^{-/-}$/C3$^{+/-}$ mice had reduced serum complement activity in zymosan and immune complex opsonization assays. To confirm that DAF$^{-/-}$/Crry$^{-/-}$/C3$^{+/-}$ mice

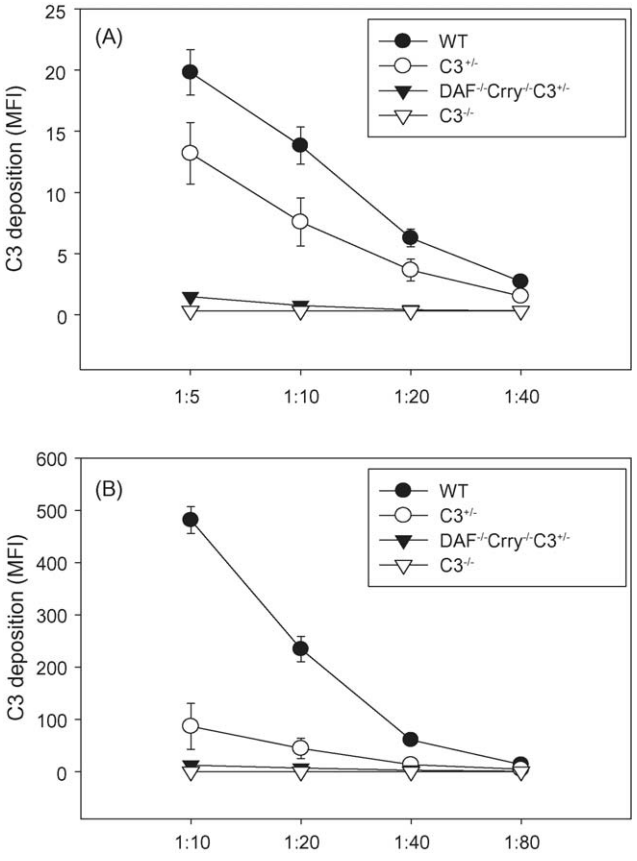

Fig. 2. Assessment of serum complement activity in DAF$^{-/-}$/Crry$^{-/-}$/C3$^{+/-}$ mice. (A) Comparison of alternative pathway complement activity in the sera of wild-type (WT, $n=6$), regular C3 heterozygous (C3$^{+/-}$, $n=6$), C3 knockout (C3$^{-/-}$, $n=6$) and DAF$^{-/-}$/Crry$^{-/-}$/C3$^{+/-}$ ($n=6$) mice. C3 deposition on zymosan particles was measured by FACS and expressed as mean fluorescence intensity (MFI). (B) Comparison of classical pathway complement activity in the sera of wild-type (WT, $n=8$), regular C3 heterozygous (C3$^{+/-}$, $n=9$), C3 knockout (C3$^{-/-}$, $n=8$) and DAF$^{-/-}$/Crry$^{-/-}$/C3$^{+/-}$ ($n=8$) mice. C3 deposition on antibody-opsonized DAF$^{-/-}$/Crry$^{-/-}$/C3$^{-/-}$ mouse erythrocytes was measured by FACS and expressed as MFI. *X*-axis depicts serum dilution factors.

had reduced serum complement activity, we performed complement activation assays of sera from WT, C3$^{+/-}$, DAF$^{-/-}$/Crry$^{-/-}$/C3$^{+/-}$ and DAF$^{-/-}$/Crry$^{-/-}$/C3$^{-/-}$ mice, using C3 opsonization of zymosan particles as a measurement of alternative pathway complement activity and C3 opsonization of Ig-coated DAF$^{-/-}$/Crry$^{-/-}$/C3$^{-/-}$ mouse erythrocytes as a measurement of classical pathway complement activity. Fig. 2A shows that, at 1:5 to 1:40 dilutions, alternative pathway complement activity in normal C3$^{+/-}$ mouse serum was 50–60% of that of wild-type mouse serum. In contrast, alternative pathway complement activity in DAF$^{-/-}$/Crry$^{-/-}$/C3$^{+/-}$ mouse serum was less than 10% of that of wild-type mouse serum. A similar degree of reduction in classical pathway complement activity was observed with DAF$^{-/-}$/Crry$^{-/-}$/C3$^{+/-}$ mouse serum (Fig. 2B). Of interest, under the serum dilution range tested, classical pathway complement activity in normal C3$^{+/-}$ mouse serum was also disproportionately reduced to less than 25% of that of wild-type mouse serum (Fig. 2B).

Spontaneous C3 activation occurred in DAF$^{-/-}$/Crry$^{-/-}$/C3$^{+/-}$ mice. To determine the mechanism of reduced serum

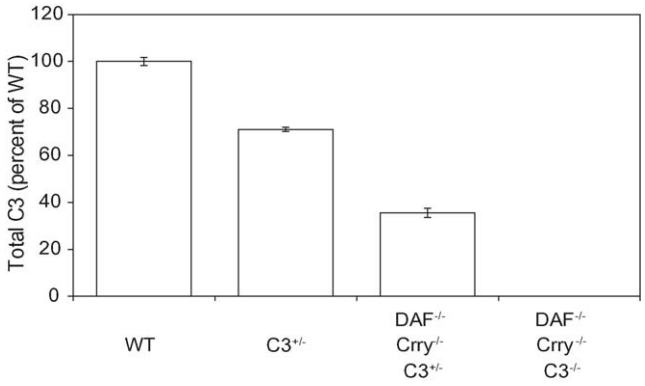

Fig. 3. ELISA quantification of total plasma C3 proteins. Plasma samples were collected from wild-type (WT, $n=4$), regular C3 heterozygous (C3$^{+/-}$, $n=6$), DAF$^{-/-}$/Crry$^{-/-}$/C3$^{+/-}$ ($n=4$) and DAF$^{-/-}$/Crry$^{-/-}$/C3$^{-/-}$ ($n=4$) mice and total C3 proteins quantitated by sandwich ELISA. This assay detected intact as well as activated C3.

complement activity in DAF$^{-/-}$/Crry$^{-/-}$/C3$^{+/-}$ mice, we examined their plasma C3 levels by ELISA and Western blot analysis. Fig. 3 shows that normal C3$^{+/-}$ mice had about 70% of the wild-type mouse plasma C3 level whereas in DAF$^{-/-}$/Crry$^{-/-}$/C3$^{+/-}$ mice, the level was decreased to 30%. Because the ELISA assay we used detected intact as well as activated C3 (Mastellos et al., 2004), the actual level of intact and functional C3 was likely to be even lower in DAF$^{-/-}$/Crry$^{-/-}$/C3$^{+/-}$ mice. The reduction in plasma C3 was confirmed by Western blot analysis (Fig. 4). In two separate experiments, DAF$^{-/-}$/Crry$^{-/-}$/C3$^{+/-}$ mice were shown to have lower amounts of intact C3 and relatively higher amounts of activated C3 than DAF$^{-/-}$/Crry$^{+/-}$/C3$^{+/-}$ littermate controls (Fig. 4). These data established that C3 is spontaneously activated and consumed in DAF$^{-/-}$/Crry$^{-/-}$/C3$^{+/-}$ mice.

DAF$^{-/-}$/Crry$^{-/-}$/C3$^{+/-}$ mice were more susceptible to nephrotoxic serum-induced proteinuria. Because DAF$^{-/-}$/Crry$^{-/-}$/C3$^{+/-}$ mice still retained some functional complement activity, we evaluated their susceptibility to NTS nephritis, a model of glomerulonephritis known to implicate complement-dependent inflammatory injury (Adler and Couser, 1985; Couser et al., 1985). We employed two experimental protocols for this study. In the first experiment, mice were challenged with 100 μl sheep NTS, and urine albumin secretion was evaluated at days 2 and 5. In this experiment, DAF$^{-/-}$/Crry$^{+/-}$/C3$^{+/-}$ mice and DAF$^{-/-}$/Crry$^{-/-}$/C3$^{-/-}$ mice were used as littermate controls. Fig. 5A shows that, despite greatly reduced systemic complement activity, DAF$^{-/-}$/Crry$^{-/-}$/C3$^{+/-}$ mice had increased albuminuria at days 2 and 5 as compared with Crry-sufficient DAF$^{-/-}$/Crry$^{+/-}$/C3$^{+/-}$ mice or complement-deficient DAF$^{-/-}$/Crry$^{-/-}$/C3$^{-/-}$ mice.

In the second experiment, we used an accelerated NTS nephritis model wherein mice were preimmunized with rabbit IgG and glomerulonephritis was induced 5 days later by NTS injection. At day 3 after disease induction, DAF$^{-/-}$/Crry$^{-/-}$/C3$^{+/-}$ mice likewise had more severe albuminuria than their DAF$^{-/-}$/Crry$^{+/-}$/C3$^{+/-}$ littermate controls (Fig. 5B). However, proteinuria was similar in the two groups at days 5 and 7 (Fig. 5B), suggesting that additional complement-

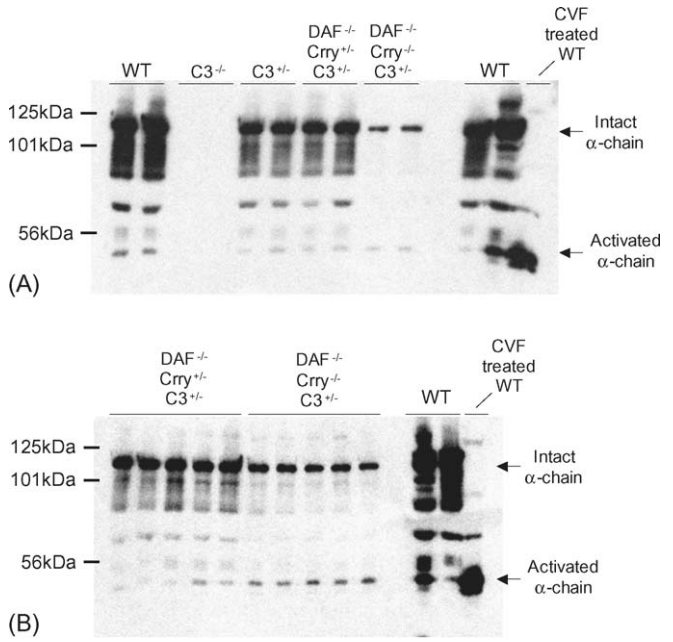

Fig. 4. Western blot analysis of mouse plasma C3 proteins. Two experiments were performed. In the experiment shown in panel (A), plasma samples from four wild-type (WT) and two each of C3 knockout ($C3^{-/-}$), C3 heterozygous ($C3^{+/-}$), $DAF^{-/-}/Crry^{+/-}/C3^{+/-}$ and $DAF^{-/-}/Crry^{-/-}/C3^{+/-}$ mice were analyzed. A sample of WT mouse serum treated with cobra venom factor (CVF) was used as a control for activated plasma C3 (far right lane). Note that the level of intact C3 α-chain in $DAF^{-/-}/Crry^{-/-}/C3^{+/-}$ mice was significantly lower than that of $C3^{-/-}$ or $DAF^{-/-}/Crry^{+/-}/C3^{+/-}$ mice. In experiment shown in panel (B), the plasma levels of intact and activated C3 were compared in five additional $DAF^{-/-}/Crry^{+/-}/C3^{+/-}$ and $DAF^{-/-}/Crry^{-/-}/C3^{+/-}$ mice. This again demonstrated that the level of intact C3 α-chain was lower and that of activated C3 α-chain was higher in $DAF^{-/-}/Crry^{-/-}/C3^{+/-}$ mice.

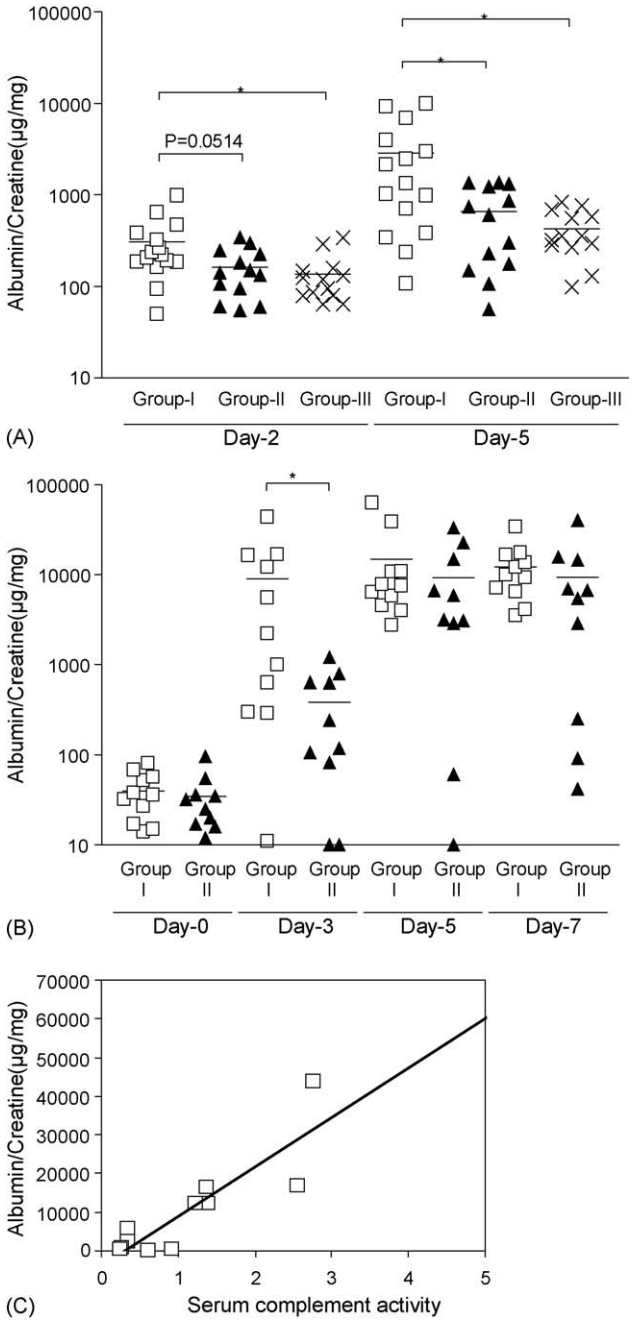

Fig. 5. $DAF^{-/-}/Crry^{-/-}/C3^{+/-}$ mice developed more severe proteinuria in models of nephrotoxic serum nephritis despite diminished plasma complement activity. (A) Scatter plot of urinary albumin excretion in mice subjected to the passive model of nephrotoxic serum nephritis with a single injection of sheep anti-mouse GBM. Group I: $DAF^{-/-}/Crry^{-/-}/C3^{+/-}$ ($n = 15$); group II: $DAF^{-/-}/Crry^{+/-}/C3^{+/-}$ ($n = 13$); group III: $DAF^{-/-}/Crry^{-/-}/C3^{-/-}$ ($n = 13$). Statistical significance ($P < 0.05$, Student's t-test) is indicated by an asterisk (*). (B) Scatter plot of urinary albumin excretion in mice subjected to the accelerated model of nephrotoxic serum nephritis with rabbit IgG immunization followed by rabbit anti-mouse GBM injection. Group I: $DAF^{-/-}/Crry^{-/-}/C3^{+/-}$ ($n = 11$), group II, $DAF^{-/-}/Crry^{+/-}/C3^{+/-}$ ($n = 10$). Statistical significance ($P < 0.05$, Student's t-test, indicated as an asterisk (*)) was observed only at day 3. (C) Level of urinary albumin excretion at day 3 in $DAF^{-/-}/Crry^{-/-}/C3^{+/-}$ mice was correlated with serum complement activity. Serum samples were collected at day 7 when mice were sacrificed for terminal pathology analysis. Complement activity was measured by zymosan C3 deposition assays using 1:5 diluted plasma and is expressed in arbitrary unit of mean fluorescence intensity.

independent pathways may become dominant at later stages of disease development. It is notable that the level of albuminuria in $DAF^{-/-}/Crry^{-/-}/C3^{+/-}$ mice at day 3 was correlated with their serum complement activity (Fig. 5C). Although serum complement activity was measured after disease induction at day 7, the result likely reflected preexisting complement activity levels before anti-GBM injection since, unlike systemic autoimmune diseases such as lupus (Walport, 2001a,b), localized immune complex formation in the glomeruli has not been known to depress systemic complement activity (Adler and Couser, 1985). Despite exacerbated proteinuria in $DAF^{-/-}/Crry^{-/-}/C3^{+/-}$ mice, however, we detected no significant difference in the degree of glomerular injury or C3 deposition when terminally harvested kidneys were analyzed morphologically under light or fluorescence microscopy (data not shown).

4. Discussion

DAF and Crry are two well-characterized membrane complement regulators in rodent. Both proteins exert their inhibitory activity at C3 and C5 convertases and both have similar tissue expression patterns in adult animals. These features suggest that the two proteins may play redundant roles *in vivo*. Sur-

prisingly, however, targeted disruption of the daf-1 gene, the murine homolog of human DAF, produced no overt phenotypes whereas Crry gene disruption resulted in embryonic lethality (Sun et al., 1999; Xu et al., 2000). The lethal phenotype of the Crry knockout mouse could be rescued by C3 deficiency, proving that complement-dependent injury was the cause of fetal demise (Mao et al., 2003; Xu et al., 2000). Immunohistochemical staining revealed that Crry is expressed on developing mouse embryos whereas DAF is not (Miwa et al., 2001; Xu et al., 2000). Thus, DAF was not available on the developing embryos to compensate for the lack of Crry and this may explain why Crry-deficient embryos were susceptible to complement attack. Consistent with this hypothesis, when Crry$^{-/-}$/C3$^{-/-}$ mice were used as female breeders to circumvent maternal complement attack, viable Crry$^{-/-}$/C3$^{+/-}$ mice were produced, grew to adulthood and thrived (H.M. unpublished results). An objective of this study was to test the hypothesis that Crry$^{-/-}$/C3$^{+/-}$ mice were able to survive and thrive because DAF compensated for the lack of Crry post-embryonic development.

Result of the breeding experiment did not support our initial hypothesis. It showed that DAF$^{-/-}$/Crry$^{-/-}$/C3$^{+/-}$ mice could also be produced at the expected frequency and survived normally (Table 1). This finding was perplexing at first since we had previously shown that DAF/Crry-deficient erythrocytes (from Crry$^{-/-}$/DAF$^{-/-}$/C3$^{-/-}$ mice) were susceptible to complement attack when transfused into C3-sufficient mice (Miwa et al., 2002b; Molina et al., 2002). To explain why erythrocytes in DAF$^{-/-}$/Crry$^{-/-}$/C3$^{+/-}$ mice were not attacked by their own complement to threaten their survival, we performed erythrocyte transfusion experiments using DAF$^{-/-}$/Crry$^{-/-}$/C3$^{+/-}$ mice as donors or recipients. These experiments established that erythrocytes of DAF$^{-/-}$/Crry$^{-/-}$/C3$^{+/-}$ mice were similarly susceptible to complement attack when transfused into wild-type recipients. On the other hand, DAF$^{-/-}$/Crry$^{-/-}$/C3$^{+/-}$ mice were shown to have impaired complement activity and were unable to eliminate DAF/Crry-deficient erythrocytes. This presumably explained why they were able to survive in the wake of DAF/Crry double deficiency.

By ELISA assay and Western blot analysis, we found that reduced serum complement activity in DAF$^{-/-}$/Crry$^{-/-}$/C3$^{+/-}$ mice was at least in part attributable to spontaneous C3 activation and consumption. Total plasma C3 proteins (intact and activated) were significantly lower in DAF$^{-/-}$/Crry$^{-/-}$/C3$^{+/-}$ mice than in heterozygous C3 knockout mice. Western blot analysis of plasma samples showed that there was less intact C3 and more activated C3 α-chain fragment in DAF$^{-/-}$/Crry$^{-/-}$/C3$^{+/-}$ mice than in DAF$^{-/-}$/Crry$^{+/-}$/C3$^{+/-}$ littermate controls. Because such spontaneous C3 activation is not observed in DAF$^{-/-}$ mice, it must have occurred in DAF$^{-/-}$/Crry$^{-/-}$/C3$^{+/-}$ mice as a result of Crry deficiency rather than DAF deficiency. Indeed, we observed a similar phenomenon in Crry$^{-/-}$/C3$^{+/-}$ mice where factor B, another component of the alternative pathway, was also partially depleted (H.M. unpublished result). Although we did not examine plasma factor B level in DAF$^{-/-}$/Crry$^{-/-}$/C3$^{+/-}$ mice, it is likely that it is also abnormally consumed by increased alternative pathway complement activation.

Despite greatly reduced plasma complement activity, DAF$^{-/-}$/Crry$^{-/-}$/C3$^{+/-}$ mice developed more severe proteinuria than DAF$^{-/-}$/Crry$^{+/-}$/C3$^{+/-}$ littermate controls in two different models of nephrotoxic serum-induced nephritis. We did not detect significant difference between the two groups of mice in their glomerular C3 deposition or injury scores under fluorescence or light microscopy. It is possible that, given the considerable variation in disease severity within each genotype, the fluorescence/light microscopic and semi-quantitative method we used was not sensitive enough to reveal a differences in these parameters. Another possibility is that the time points at which we assessed these two parameters were not optimal (terminal pathology analysis was performed at day 5 or day 7 of disease induction). Indeed, in the accelerated model of NTS nephritis, increased proteinuria in DAF$^{-/-}$/Crry$^{-/-}$/C3$^{+/-}$ mice was obvious only at an early stage of the disease induction (day 3). At later time points, proteinuria was similar in DAF$^{-/-}$/Crry$^{-/-}$/C3$^{+/-}$ and DAF$^{-/-}$/Crry$^{+/-}$/C3$^{+/-}$ mice, possibly reflecting a more dominant role of complement-independent immune mechanisms such as Fc receptor-mediated injury (Clynes et al., 1998). Nevertheless, there is little doubt that increased proteinuria in DAF$^{-/-}$/Crry$^{-/-}$/C3$^{+/-}$ mice was mediated by complement since proteinuria was significantly lower in the complement-deficient DAF$^{-/-}$/Crry$^{-/-}$/C3$^{-/-}$ littermate controls in the passive nephritis model. Furthermore, severity of proteinuria in DAF$^{-/-}$/Crry$^{-/-}$/C3$^{+/-}$ mice at day 3 of the accelerated nephritis model was clearly correlated with serum complement activity (Fig. 5C).

The finding that DAF$^{-/-}$/Crry$^{-/-}$/C3$^{+/-}$ mice were more sensitive than DAF$^{-/-}$/Crry$^{+/-}$/C3$^{+/-}$ mice in the nephotoxic serum nephritis model suggests that DAF and Crry together must play a critical role normally in protecting glomerular tissues from classical pathway complement-mediated inflammatory injury. Previous studies of Crry and/or DAF deficient mouse erythrocytes indicated that, while Crry but not DAF is indispensable for preventing alternative pathway complement activation, both proteins are equally active on red blood cells in inhibiting classical pathway complement activation and their role in the latter setting was redundant and overlapping (Miwa et al., 2002b; Molina et al., 2002). It is likely that a similarly redundant role of Crry and DAF in classical pathway complement regulation is operative in the kidney. Thus, deficiency of either DAF or Crry may cause only moderate sensitivity to classical pathway complement-mediated injury (Miwa et al., 2002a,b; Molina et al., 2002; Sogabe et al., 2001; Yamada et al., 2004), whereas DAF and Crry double deficiency leaves a total void in the ability of glomerular cells to inhibit classical pathway complement activation. This may explain why DAF$^{-/-}$/Crry$^{-/-}$/C3$^{+/-}$ mice developed more severe nephrotoxic serum-induced proteinuia than DAF$^{-/-}$/Crry$^{+/-}$/C3$^{+/-}$ mice, despite having markedly reduced total plasma complement activity.

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