The Journal of Immunology

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J Immunol 2006; 177:5558-5566; ;
doi: 10.4049/jimmunol.177.8.5558
http://www.jimmunol.org/content/177/8/5558

The Journal of Immunology is published twice each month by
The American Association of Immunologists, Inc.,
1451 Rockville Pike, Suite 650, Rockville, MD 20852

Print ISSN: 0022-1767 Online ISSN: 1550-6606.

Retrovirus-Mediated Over-Expression of Decay-Accelerating Factor Rescues Crry-Deficient Erythrocytes from Acute Alternative Pathway Complement Attack[1]

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Decay-accelerating factor (DAF) and complement receptor 1-related gene/protein y (Crry) are two membrane-bound complement regulators on murine erythrocytes that inhibit C3/C5 convertases. Previously, we found that Crry- but not DAF-deficient erythrocytes were susceptible to alternative pathway complement-mediated elimination in vivo. To determine whether it is a unique activity or a higher level expression of Crry makes it indispensable on murine erythrocytes, we over-expressed DAF on Crry-deficient ($Crry^{-/-}$) erythrocytes by retroviral vector-mediated DAF gene transduction of bone marrow stem cells. DAF retrovirus-transduced erythrocytes expressed 846 ± 127 DAF molecules/cell (DAF^{high}) compared with 249 ± 94 DAF molecules/cell (DAF^{low}) and 774 ± 135 Crry molecules/cell on control mouse erythrocytes. DAF^{high}-$Crry^{-/-}$ erythrocytes were significantly more resistant than either DAF^{low}-$Crry^{-/-}$, $DAF^{-/-}$ -$Crry^{+/+}$ or wild-type erythrocytes to classical pathway complement-mediated C3 deposition in vitro. Furthermore, increased DAF expression rescued $Crry^{-/-}$ erythrocytes from acute alternative pathway complement attack in vivo. Notably, long term monitoring revealed that DAF^{high}-$Crry^{-/-}$ erythrocytes were still more susceptible than wild-type erythrocytes to complement-mediated elimination as they had a shorter half-life in complement-sufficient mice but survived equally well in complement-deficient mice. These results suggest that both a high level expression and a more potent anti-alternative pathway complement activity of Crry contributed to its indispensable role on murine erythrocytes. Additionally, they demonstrate the feasibility of using stem cell gene therapy to correct membrane complement regulator deficiency on blood cells in vivo. *The Journal of Immunology,* **2006, 177: 5558–5566.**

Complement, a form of the innate immune system, is spontaneously activated by susceptible surfaces such as bacteria or yeast cell walls via the alternative pathway (1). Host tissues avoid complement activation and the ensuing inflammation and cytolysis by expressing a number of complement inhibitory proteins on their cell surface (2, 3). In humans, these proteins include decay-accelerating factor (DAF),[3] membrane cofactor protein (MCP), complement receptor 1 (CR1) and CD59. DAF inhibits complement activation by preventing the formation and accelerating the decay of C3 and C5 convertases (4, 5). MCP acts as a cofactor for factor I-mediated degradation of surface-bound C3b and C4b (6), and CD59 is an inhibitor of the membrane attack complex (7, 8). CR1 regulates complement activation by exerting both MCP and DAF activities (9).

Proteins homologous to human DAF, CD59, and MCP have also been identified in the rodent (2), though their genetic composition and tissue distribution do not always match those of their human counterparts (2, 10). In particular, the mouse has two DAF (daf-1, daf-2) (11–14) and two CD59 (cd59a, cd59b) genes (15, 16), with daf-1 and cd59a representing the true murine homologue of human DAF and CD59, respectively. Also, unlike human MCP, which is widely expressed (6, 17), mouse MCP is expressed only in the testis (18). Finally, a rodent-specific complement regulator termed Crry (complement receptor 1-related gene/protein y) exists and is ubiquitously expressed in various mouse tissues (19, 20). Like human CR1, Crry has both MCP and DAF activities (10, 19, 21).

Daf-1 and Crry gene knockout mouse studies have revealed remarkable differences in their in vivo biology. Although no gross abnormalities were discernible in daf-1 knockout mice under normal conditions (22), Crry knockout resulted in embryonic lethality that was preventable by C3 deficiency (23). Immunohistochemical staining showed that Crry but not DAF was present on trophoblasts of the developing mouse embryos (23–25), which offered a potential explanation for the critical role played by Crry on developing mouse embryos. However, in subsequent erythrocyte transfusion experiments, Crry-deficient mouse erythrocytes, despite expressing DAF on their surface, were found to be also susceptible to alternative pathway complement attack (26, 27). In the latter experiments, Crry- but not DAF- or CD59-deficient mouse erythrocytes were spontaneously eliminated by alternative pathway complement from the systemic circulation (26, 27).

Two possibilities may explain why Crry but not DAF is indispensable on mouse erythrocytes: DAF may be expressed at a much lower level than Crry on these cells or Crry, possessing both MCP and DAF activities, is a much more active regulator of the alternative pathway complement activation. To distinguish these two possibilities, we have enumerated DAF and Crry molecules expressed on normal mouse erythrocytes. We also over-expressed DAF on Crry-deficient mouse erythrocytes by retroviral vector-mediated DAF gene transfer of bone marrow (BM) stem cells to see if a higher level DAF expression could fully compensate for

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Received for publication January 26, 2006. Accepted for publication July 24, 2006.

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[1] This work was supported by National Institutes of Health Grants AI-44970, AI-49344, and AI-63288 and by National Multiple Sclerosis Society Grant RG3671-A-1.3671-A-1 (to W.-C.S.).

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[3] Abbreviations used in this paper: DAF, decay-accelerating factor; Crry, complement receptor 1-related gene/protein y; MCP, membrane cofactor protein; $GVBS^{++}$, gelatin-Veronal buffered saline; MFI, mean fluorescent intensity.

0022-1767/06/$02.00

the lack of Crry. Our results show that increased expression of DAF on Crry-deficient erythrocytes was sufficient to prevent acute complement-mediated elimination in vivo. Notably, however, DAF^{high}-$Crry^{-/-}$ erythrocytes were still more susceptible than wild-type or $DAF^{-/-}$-$Crry^{+/+}$ erythrocytes to alternative pathway complement-mediated elimination when studied over an extended period of time. We conclude that Crry is more active than DAF as an alternative pathway complement regulator but tissue resistance to spontaneous complement attack is also determined by the level of DAF expression.

Materials and Methods

Mice

The generation of daf-1 and C3 double-deficient ($DAF^{-/-}$/$C3^{-/-}$) and Crry and C3 double-deficient ($Crry^{-/-}$/$C3^{-/-}$) mice were described previously (22–24, 26, 27). $C3^{-/-}$ mice backcrossed to C57BL/6 background for six generations were obtained from The Jackson Laboratory and were backcrossed in-house for five more generations. Factor B knockout mice were kindly provided by Dr. John Lambris (University of Pennsylvania) (28). Six- to eight-week-old congenic C57BL/6 wild-type mice (CD45.1, CD45.2) were obtained from The Jackson Laboratory. Unless specified, male mice were used as recipients in all transfusion experiments. Mice were maintained under specific pathogen-free conditions at the University of Pennsylvania. All animal experiments were approved by the Institutional Animal Care and Use Committees of the University of Pennsylvania.

Generation of recombinant retroviruses containing DAF gene

The mouse GPI-DAF (daf-1) cDNA (nucleotide 1 to 1334) was excised from the pCDNA3 vector (11, 25) and subcloned at *Eco*RI sites into MigR1, a bicistronic retroviral vector carrying a GFP marker downstream of encephalomyocarditis virus internal ribosomal entry site in tandem with the transgene (29, 30). The MigR1 retroviral vector was provided by Dr. W. S. Pear (University of Pennsylvania, Philadelphia, PA) (30). Infectious viral particles encoding DAF and GFP were produced in human embryonic kidney 293t cells (HEK 293t) by $CaPO_4$-mediated cotransfection (Profection) of MigR1-DAF with pHZT123 and pCGP, two separate plasmids that encode viral structural genes necessary for encapsidating viral RNAs (29, 30). The viral particles were harvested from cell culture medium at 24- and 36-h posttransfection and then used to infect NIH-3T3 cells at 1/20 dilution to verify the titer of the viral stock. Both HEK 293t and NIH-3T3 cells were cultured in DMEM (Invitrogen Life Technologies), supplemented with 10% heat-inactivated FBS (JRH Biosciences), 100 U/ml streptomycin (Invitrogen Life Technologies), 100 U/ml penicillin (Invitrogen Life Technologies), and 2 mmol/L L-glutamine (Invitrogen Life Technologies). Viral stocks were kept at −80°C until use.

Retroviral transduction of BM cells

On day 1, 200 mg/kg 5-fluorouracil (American Pharmaceutical Partners) was injected (i.p) into donor mice to stimulate proliferation of BM stem cells (29). On day 5, BM cells from femurs and tibias of 8-wk-old male $Crry^{-/-}$/$C3^{-/-}$ mice were isolated by flushing with culture medium using a 3-ml syringe (BD Biosciences). The cells were cultured at a density of 1×10^6 cells/ml in a 6-well plate in DMEM (Invitrogen Life Technologies) containing 10% heat-inactivated FBS (Invitrogen Life Technologies), 6 U/ml recombinant mouse IL-3 (IL-3; PeproTech), 10,000 U/ml recombinant mouse IL-6 (PeproTech), 5 U/ml recombinant mouse stem cell factor (PeproTech), 4 μg/ml polybrene (Sigma-Aldrich), 100 U/ml streptomycin (Invitrogen Life Technologies), 100 U/ml penicillin (Invitrogen Life Technologies), and 2 mmol/L L-glutamine (Invitrogen Life Technologies). The following day, 4×10^6 cells were added to a well in a 6-well plate containing 3 ml of BM culture medium and 1 ml of retroviral supernatant (equivalent to 1/4 dilution of the viral stock). BM cells were then transduced by spinoculation at 2500 rpm, 37°C, for 90 min. After spinoculation, the cells were returned to the incubator for continuing culture. Twenty-four hours later, a second round of spinoculation was performed and the BM cells were again returned to the incubator for culture. After 16 h, 1×10^6 cells were injected into the tail vein of each lethally irradiated (950R) syngeneic recipient mouse.

Three months after the BM transfer, the recipient mice were completely reconstituted with the retrovirus-transduced donor mouse BM cells. FACS analysis at that time revealed that approximately 40% of peripheral erythrocytes were positive for GFP as a marker transgene (Fig. 1*D*). To increase the percentage of erythrocytes positive for the transgene, we used the reconstituted mice as donors and performed a second round of BM transfer. BM cells were harvested from the reconstituted mice as before and GFP-positive BM cells were sorted by FACS (Vantage cell sorter; BD Biosciences). Approximate 2×10^6 GFP-positive BM cells were then injected into each irradiated (950R) syngeneic recipient mouse via the tail vein as before.

Antibodies

The following Abs were purchased from BD Pharmingen: biotin-conjugated rat anti-mouse Crry mAb, purified rat anti-mouse Crry mAb, PE-conjugated streptavidin, PE-conjugated hamster anti-mouse DAF (CD55) mAb, and allophycocyanin-conjugated streptavidin. Polyclonal goat IgG fraction to anti-mouse complement C3 and HRP goat anti-mouse complement C3 were obtained from MP Biomedicals, Inc. PE-conjugated mouse anti-rat IgG2a mAb was purchased from Southern Biotechnology.

Biotinylation of goat anti-mouse C3 Abs was performed as described (31, 32). In brief, 20 mg of Abs (4 mg/ml) were dialyzed twice in 1 liter of 0.1 M sodium borate buffer (pH 8.8) for 6 h. After dialysis, 10 mg of Abs (4 mg/ml) were incubated with 2.5 mg of *N*-hydroxysuccinimide biotin (2.5 mg/ml in dimethyl formamide) (Sigma-Aldrich) at room temperature for 4 h. Next, the Abs were incubated with 200 μl of 1 M NH_4Cl at room temperature for 10 min. Biotin-conjugated Abs were then dialyzed twice in 1 liter of Dulbecco's PBS (Invitrogen Life Technologies) for 6 h. Biotin-conjugated Abs were stored at 4°C in 0.05% of sodium azide (Sigma-Aldrich).

FACS analysis

Peripheral blood cells were analyzed by gating erythrocytes in forward and side scattering and BM cells were analyzed by gating live cells (33). To detect Crry expression, cells were stained with biotin-conjugated anti-mouse Crry mAb, followed by PE-conjugated streptavidin or allophycocyanin-conjugated streptavidin. To detect DAF expression, cells were stained directly with PE-conjugated anti-mouse DAF (CD55) mAb. Cells were washed three times after each Ab staining and were resuspended at a concentration of 2×10^7 cells/ml before FACS analysis. All staining, washing, and resuspension were conducted in FACS buffer (0.1% of albumin and sodium azide in PBS). All incubations with Abs were performed at 4°C for 1 h. Samples were processed on a FACSCalibur flow cytometer (BD Biosciences). FACS data were acquired with the CellQuest software (BD Biosciences) and analyzed with the FlowJo software (Tree Star).

Enumeration of DAF and Crry by flow cytometric analysis

Using a standard curve generated by a set of four precalibrated fluorescent beads, QuantiBRITE beads (BD Biosciences) (34, 35), we enumerated the number of DAF and Crry molecules on mouse erythrocytes based on the mean fluorescent intensity (MFI) of DAF and Crry staining with PE-labeled primary or secondary mAbs. Maximum MFIs of DAF and Crry staining on erythrocytes were derived from saturation binding curves using multiple concentrations of PE-conjugated Abs. To calculate the number of DAF and Crry molecules, fluorescence/protein conjugation ratios (F/P molar ratios) of the PE-conjugated primary or secondary Abs were first determined by measuring their absorbance at 566 nm and 280 nm, assuming an extinction coefficiency of $A_{566} = 8.2$ for a 1 mg/ml solution of PE-conjugated Ab with a 1:1 ratio between PE and IgG (36).

To obtain anti-DAF saturation binding curve, the cells were stained with various dilutions of PE-conjugated anti-mouse DAF (CD55) mAb, which was predetermined to have a PE/IgG conjugation ratio of 1.36. To obtain anti-Crry saturation binding curve, the cells were first saturated with a purified rat anti-mouse Crry mAb (1/10 dilution), washed three times with FACS buffer, and then stained with various dilutions of PE-conjugated mouse anti-rat IgG2a mAb which was pre-determined to have a PE/IgG conjugation ratio of 2.22. MFIs (adjusted with PE-conjugation ratio) were plotted against various dilutions of PE-conjugated Abs to generate saturation binding curves, which were used to calculate maximum MFI (MFI_{max}) values by nonlinear regression one-site binding analysis by the GraphPad 3.0 software (GraphPad). Using QuantiBRITE-derived standard curve generated by the QuantiQuest software (BD Biosciences), the MFI_{max} values were converted into number of molecules per cell (34, 35).

Immunofluorescence

For confocal microscopy, peripheral erythrocytes were washed three times with PBS and then stained with PE-conjugated anti-mouse DAF (CD55) mAb (1:30). After fixing with 1% paraformaldehyde for 10 min at 4°C, the cells were washed three times with PBS before mounting on glass coverslips. Immunofluorescence was analyzed with a Zeiss LSM 510 confocal microscope with a ×63 oil immersion objective. Confocal images were arranged using Zeiss LSM Image Browser software (version 3,5,0,376).

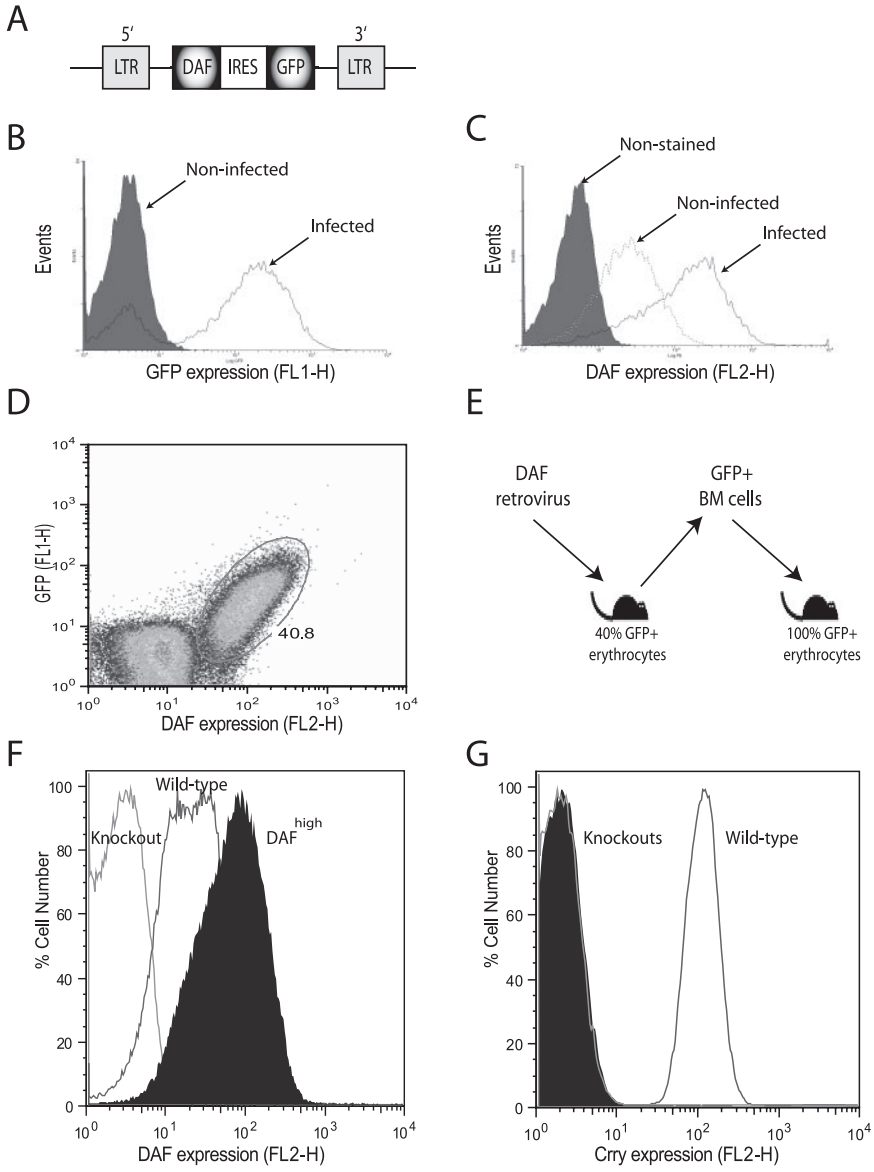


FIGURE 1. Retroviral vector-mediated DAF over-expression on Crry-deficient erythrocytes. *A*, Schematic structure of the retroviral vector (MigR1-DAF) used to transduce NIH-3T3 cells and murine BM stem cells. *B*, The majority of NIH-3T3 cells infected with MigR1-DAF-expressed GFP. *C*, Staining with a DAF-specific mAb shows that DAF expression is markedly increased on MigR1-DAF-infected NIH-3T3 cells compared with non-infected cells (representing endogenous level of DAF expression). Non-stained NIH-3T3 cells were used as a control to indicate the specificity of the Ab. *D*, FACS analysis showing that 40% of the erythrocytes were GFP positive and DAF^{high} at the end of the first round of transgenic BM reconstitution. *E*, Schema for the derivation of BM chimera mice that had 100% GFP-positive DAF^{high} erythrocytes. *F*, Histogram analysis of DAF expression on DAF^{high} Crry$^{-/-}$/C3$^{-/-}$ erythrocytes (filled area) in comparison with that of wild-type (representing endogenous DAF level) and daf-1$^{-/-}$ (knockout) mouse erythrocytes. *G*, Histogram analysis of Crry expression on DAF^{high} Crry$^{-/-}$/C3$^{-/-}$ erythrocytes (knockout, filled area) in comparison with that of wild-type mouse erythrocytes.

C3 deposition assays

Classical pathway complement activation on mouse erythrocytes was assayed as previously described with some modifications (27). In brief, wild-type, DAF$^{-/-}$/C3$^{-/-}$, or Crry$^{-/-}$/C3$^{-/-}$ mouse erythrocytes were mixed with DAF retrovirus-transduced Crry$^{-/-}$/C3$^{-/-}$ mouse erythrocytes in a 1:1 ratio (total 2×10^6 cells in 200 μl of PBS). The latter type of cells were distinguishable by GFP expression and their inclusion in each assay ensured that comparison with other cells for C3 deposition was made directly under identical experimental conditions. Cells were opsonized with a monoclonal mouse IgG2a erythrocyte autoantibody 34-3C (50 μg/ml). The hybridoma for 34-3C, originally derived from the autoimmune NZB mice (37, 38), was kindly provided by Dr. Raphael Clynes (The Rockefeller University). The 34-3C Ab was purified by ammonium sulfate precipitation of concentrated tissue culture supernatant followed by protein A-G affinity chromatography. Ab-opsonized cells were incubated with diluted factor B-deficient mouse serum (1.25, 2.5, and 5%) in gelatin-veronal buffered saline (GVBS^{++}) (Sigma-Aldrich) at 37°C for 30 min. Cells were washed three times in PBS and then stained with biotinylated anti-mouse C3 Abs, followed by PE-conjugated streptavidin. C3 deposition on GFP-positive (DAF^{high} Crry$^{-/-}$/C3$^{-/-}$) and -negative erythrocytes was determined by FACS analysis. The MFI of GFP-negative erythrocytes was normalized against that of GFP-positive erythrocytes, which was set as 100 arbitrary units.

Alternative pathway complement activation on wild-type, DAF$^{-/-}$/C3$^{-/-}$, Crry$^{-/-}$/C3$^{-/-}$, and DAF retrovirus-transduced Crry$^{-/-}$/C3$^{-/-}$ mouse erythrocytes was assayed in a similar way as described above, except that the cells were not opsonized with Ab and were treated with wild-type mouse serum (prepared in-house from 129J/C57BL/6 mice, used at 25, 50, or 100%) in GVBS^{++} buffer containing 5 mM $MgCl_2$-EGTA.

Measurement of serum alternative pathway complement activity

Zymosan particles (Sigma-Aldrich) were first suspended at 1% in 0.15 M NaCl and then placed in a boiling water bath for 1 h. After centrifugation, the pellets were resuspended in saline solution (50 mg/ml) (39). For measurement of serum alternative pathway complement activity, mouse serum samples were mixed with the prepared zymosan particles (250 μg) in 100 μl of GVBS^{++} buffer containing 5 mM $MgCl_2$-EGTA and incubated at 37°C for 60 min. After incubation, the zymosan particles were washed three times in PBS and then stained with biotinylated anti-mouse C3 Abs, followed by PE-conjugated streptavidin. C3 deposition on zymosan particles was determined by FACS and expressed as MFI.

Assessment of erythrocyte survival in vivo

To determine the survival of transfused erythrocytes in vivo, peripheral blood cells (from 150 μl of blood) of donor mice were labeled ex vivo either with CFSE (Molecular Probes) in 1 ml of PBS containing 5 μM CFSE (33) or with biotin as previously described (26). The labeled cells were then introduced into wild-type, C3$^{-/-}$, or various BM chimera mice via the tail vein. Blood samples were collected at 5 min after erythrocyte infusion and at various time points thereafter as indicated. Collected erythrocytes were either analyzed directly by FACS for CFSE labeling or stained with PE-conjugated streptavidin before analysis, and the percentage of the labeled erythrocytes was determined at each time point.

Generation of BM chimera mice

C57BL/6 mice (CD45.1 allotype, 6- to 8-wk-old) were lethally irradiated (1 × 950 rad), anesthetized, and infused through the tail vein with BM cells (1 × 10^7/mouse) from wild-type (CD45.2) or $C3^{-/-}$ mice. Additionally, C57BL/6 wild-type mice (CD45.2 allotype, 6- to 8-wk-old) were lethally irradiated (1 × 950 rad), anesthetized, and infused with BM cells (1 × 10^7/mouse) from DAF retrovirus-transduced $Crry^{-/-}/C3^{-/-}$ mice or $Crry^{-/-}/C3^{-/-}$ mice. Chimera mice were studied after 9- to 10 wk of BM transplantation.

Measurement of plasma C3 levels

Plasma C3 level was measured by ELISA using HRP goat anti-mouse C3. In brief, the plasma sample was serially diluted in PBS buffer (pH 7.5) and then incubated on a 96-well microplate for 1 h at room temperature. After washing three times with PBS (0.05% Tween 20, pH 7.5), the plate was incubated with HRP goat anti-mouse complement C3 (3.2 μg/ml) for 1 h at room temperature. The bound Ab was detected by the addition of substrate solution (0.05% ABTS (Roche), 0.1% H_2O_2 in 0.1 M sodium citrate buffer (pH 4.2)).

Results

We previously showed that, when transferred into wild-type mice, Crry-deficient erythrocytes (from $Crry^{-/-}/C3^{-/-}$ mice) were spontaneously eliminated by alternative pathway complement (26, 27). This happened despite the fact that DAF was expressed on these cells. Conversely, DAF-deficient erythrocytes (from daf-$1^{-/-}/C3^{-/-}$ mice) did not meet the same fate when transferred into wild-type mice (26, 27). To determine whether the apparent "irrelevance" of DAF on mouse erythrocytes was due to its low level expression, we used retroviral vector-mediated gene transduction of BM stem cells to over-express DAF on mouse erythrocytes. The murine DAF cDNA was cloned into MigR1, a bicistronic retroviral vector containing GFP as a reporter gene (Fig. 1*A*). Recombinant MigR1-DAF viruses were produced in HEK 293t cells, and when tested on NIH-3T3 cells, were shown to have high transduction efficiency. The majority of MigR1-DAF infected NIH-3T3 cells became GFP positive (Fig. 1*B*) and had markedly increased DAF expression (Fig. 1*C*). The MigR1-DAF virus was then used to infect BM stem cells isolated from $Crry^{-/-}/C3^{-/-}$ donor mice. DAF gene-transduced BM cells were adoptively transferred to lethally irradiated $Crry^{-/-}/C3^{-/-}$ recipient mice. After the hemopoietic system of the recipient mice was completely reconstituted with the donor BM cells (2–3 mo), we analyzed peripheral erythrocytes for their expression of GFP and DAF. Fig. 1*D* shows that ~40% of the erythrocytes in the chimeric mice were positive for GFP and/or had a higher level of DAF expression. To increase the percentage of transgene-positive erythrocytes in the mice, we performed a second round BM transfer. GFP-positive BM cells from the chimeric mice generated in the initial experiment were sorted by FACS and used to reconstitute new lethally irradiated $Crry^{-/-}/C3^{-/-}$ recipient mice (Fig. 1*E*). These steps generated transgenic $Crry^{-/-}/C3^{-/-}$ mice that had their erythrocytes uniformly positive for GFP (data not shown) and expressing a higher level of DAF (Fig. 1*F*). As expected, no Crry staining was detected on the erythrocytes of these mice (Fig. 1*G*). Over-expression of DAF on $Crry^{-/-}/C3^{-/-}$ mouse erythrocytes was also confirmed by confocal immunofluorescence microscopy (Fig. 2). Both constitutively expressed and over-expressed DAF appeared to distribute evenly on the cell membrane (Fig. 2, *A–D*).

We then estimated by flow cytometry the number of molecules of Crry, endogenously expressed DAF and over-expressed DAF on the mouse erythrocytes. First, fluorescence/protein ratios (F/P molar ratios) in the primary and secondary Abs used for DAF and Crry staining were determined by measuring the absorbance at 566 and 280 nm, respectively. Next, the MFI_{max} (F/P molar ratio adjusted) for each Ag was calculated from monophasic saturation binding curves generated with a wide range of Ab dilutions (Fig. 3, *A* and *B*). The background levels of fluorescence in all dilutions were identical (data not shown). Finally, the number of Ag molecules on each type of erythrocytes was calculated against a standard curve obtained from precalibrated PE-labeled fluorescent beads (Fig. 3*C*). Using this assay, the average copy number of endogenously expressed DAF on wild-type and $Crry^{-/-}/C3^{-/-}$ mouse erythrocyte was calculated to be 249 ± 94 and 252 ± 64 per cell, respectively (Fig. 3*D*). The average copy number of DAF on the erythrocytes of two-round BM reconstituted transgenic $Crry^{-/-}/C3^{-/-}$ mice was 846 ± 127 per cell (Fig. 3*D*). The average copy number of Crry on wild-type and $DAF^{-/-}/C3^{-/-}$ erythrocytes was 774 ± 135 and 734 ± 101 per cell, respectively (Fig. 3*D*). Thus, the number of over-expressed DAF molecules on $Crry^{-/-}/C3^{-/-}$ mouse erythrocytes was comparable to that of Crry molecules on wild-type mice.

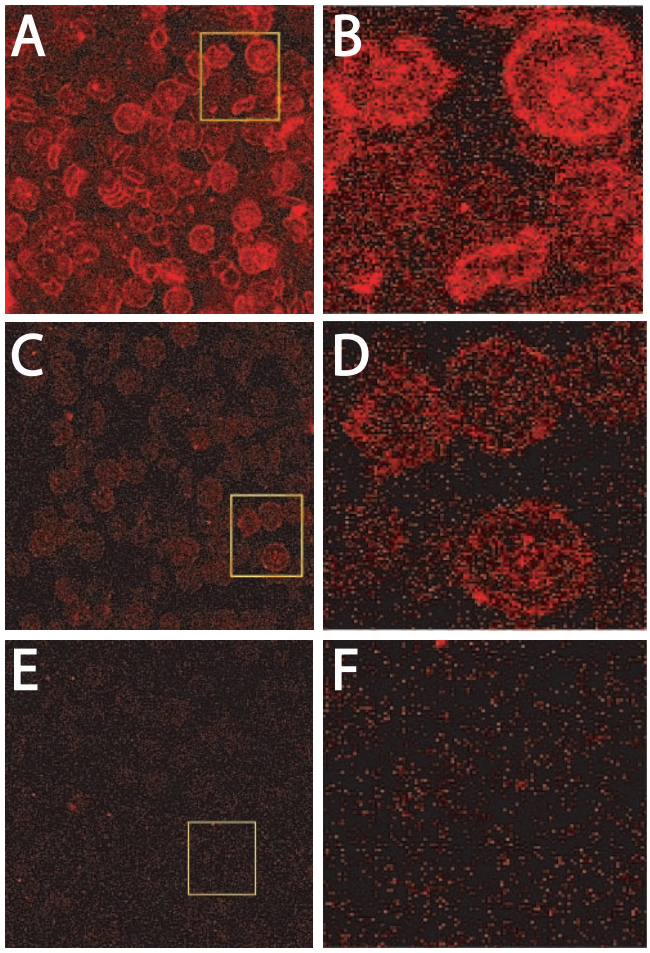
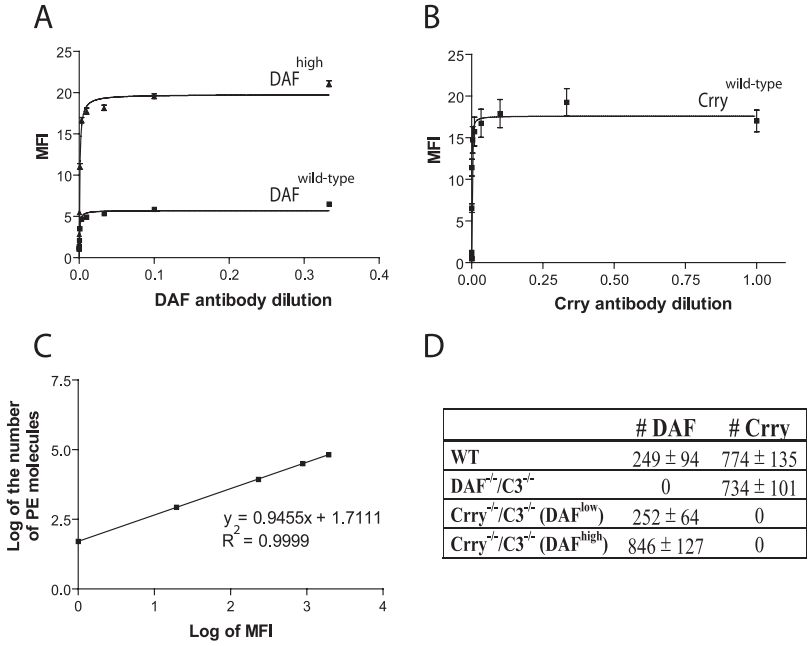


FIGURE 2. Confocal immunofluorescence microscopy of DAF expression on erythrocytes. *A* and *B*, Erythrocytes from two-round BM reconstituted DAF transgenic mice (DAF^{high} $Crry^{-/-}/C3^{-/-}$). *C* and *D*, Erythrocytes from wild-type mice (DAF^{low}). *E* and *F*, Erythrocytes from $DAF^{-/-}$ were used as a negative control. *B*, *D*, and *F* (×600) are magnifications of the boxed areas in *A*, *C*, and *E* (×189).

To confirm that the over-expressed DAF on $Crry^{-/-}/C3^{-/-}$ mouse erythrocyte was functional, we evaluated the sensitivity of DAF^{high} $Crry^{-/-}/C3^{-/-}$ mouse erythrocytes to classical pathway complement activation in vitro using factor B-deficient serum. Fig. 4*A* shows that DAF^{high} $Crry^{-/-}/C3^{-/-}$ mouse erythrocytes were significantly more resistant than DAF^{low} $Crry^{-/-}/C3^{-/-}$, $DAF^{-/-}/C3^{-/-}$ or wild-type mouse erythrocytes to classical pathway complement activation. Two conclusions can be drawn from this experiment. First, the over-expressed DAF was functional (compare column 1 and 3 in



	# DAF	# Crry
WT	249 ± 94	774 ± 135
DAF$^{-/-}$/C3$^{-/-}$	0	734 ± 101
Crry$^{-/-}$/C3$^{-/-}$ (DAF^{low})	252 ± 64	0
Crry$^{-/-}$/C3$^{-/-}$ (DAF^{high})	846 ± 127	0

FIGURE 3. Enumeration of DAF and Crry on mouse erythrocytes. *A*, Saturation binding curves of anti-DAF mAb on wild-type and DAF^{high} Crry$^{-/-}$/C3$^{-/-}$ erythrocytes (n = 6 mice for wild-type erythrocytes, MFI_{max} ± SD = 5.3 ± 0.2766, R^2 = 0.9727; n = 3 mice for DAF^{high} Crry$^{-/-}$/C3$^{-/-}$ erythrocytes, MFI_{max} ± SD = 19.34 ± 0.4163, R^2 = 0.9899. Duplicate assays for each mouse were performed). *B*, Saturation binding curve of anti-Crry mAb on wild-type erythrocytes (n = 4 mice, duplicate assays for each mouse. MFI_{max} ± SD = 17.61 ± 0.441, R^2 = 0.9703). *C*, Standard curve generated by precalibrated PE-labeled beads showing the relationship between the number of fluorescent molecules and the MFI in logarithmic scale. *D*, Calculated expression levels of DAF and Crry on various mouse erythrocytes.

Fig. 4*A*). Second, DAF is a significantly more potent inhibitor of classical pathway complement than Crry (compare column 1, 2, and 3 in Fig. 4*A*). In a separate experiment, we measured the sensitivity of DAF^{high} Crry$^{-/-}$/C3$^{-/-}$, DAF^{low} Crry$^{-/-}$/C3$^{-/-}$, DAF$^{-/-}$/C3$^{-/-}$ and wild-type mouse erythrocytes to alternative pathway complement activation in vitro. Fig. 4*B* shows that DAF^{high} Crry$^{-/-}$/C3$^{-/-}$ mouse erythrocytes were significantly more resistant than DAF^{low} Crry$^{-/-}$/C3$^{-/-}$, but less resistant than DAF$^{-/-}$/C3$^{-/-}$ or wild-type, mouse erythrocytes to alternative pathway complement attack. This experiment showed that the over-expressed DAF was also functional as an alternative pathway complement inhibitor (compare column 1 and 3 in Fig. 4*B*). Furthermore, it suggested that Crry is a more potent alternative pathway complement inhibitor than DAF (compare column 1 and 2 in Fig. 4*B*).

Next, we asked whether over-expression of DAF on Crry$^{-/-}$/C3$^{-/-}$ erythrocytes would rescue the otherwise susceptible Crry$^{-/-}$/C3$^{-/-}$ cells from complement-mediated destruction in vivo (26, 27). To test this possibility, we harvested erythrocytes from DAF retrovirus-transduced Crry$^{-/-}$/C3$^{-/-}$ mice and transfused them into complement-sufficient wild-type mice to study their susceptibility to complement attack. In the first experiment, erythrocytes were harvested from partially reconstituted DAF transgenic Crry$^{-/-}$/C3$^{-/-}$ mice that had approximately 60% DAF^{high} and 40% DAF^{low} erythrocytes. These cells were labeled ex vivo with CFSE and then transfused into wild-type mice. As shown in Fig. 5*A*, the transfused erythrocytes were CFSE positive and clearly distinguishable from the endogenous erythrocyte population. At 5 min after transfusion, the DAF^{low} (designated by X) and DAF^{high} (designated by Y) cells were both present (Fig. 5*A*). As expected, the DAF^{low} population of Crry$^{-/-}$/C3$^{-/-}$ erythrocytes was rapidly eliminated from the circulation and became undetectable by 48–72 h (Fig. 5). In contrast, the vast majority of DAF^{high} Crry$^{-/-}$/C3$^{-/-}$ erythrocytes survived up to 72 h (Fig. 5). Thus, over-expression of DAF effectively rescued Crry-deficient erythrocytes from acute complement-mediated elimination.

To determine whether DAF over-expression rendered Crry$^{-/-}$/C3$^{-/-}$ erythrocytes completely resistant to complement attack in vivo or simply delayed their elimination, we performed a second experiment wherein we compared the survival of DAF^{low} Crry$^{-/-}$/C3$^{-/-}$, DAF^{high} Crry$^{-/-}$/C3$^{-/-}$, DAF$^{-/-}$/C3$^{-/-}$, and wild-type mouse erythrocytes over a period of 7 days. Erythrocytes from Crry$^{-/-}$/C3$^{-/-}$, DAF transgenic Crry$^{-/-}$/C3$^{-/-}$ (two round reconstituted, 100% GFP positive), DAF$^{-/-}$/C3$^{-/-}$ and wild-type mice were harvested, labeled ex vivo with CFSE, and then transfused into wild-type recipient mice. As shown in Fig. 6*A*, we confirmed that, unlike DAF^{low} Crry$^{-/-}$/C3$^{-/-}$ erythrocytes, which disappeared before 96 h, DAF^{high} Crry$^{-/-}$/C3$^{-/-}$ erythrocytes were resistant to rapid elimination. Interestingly, beginning at 24 h, a difference in survival between DAF^{high} Crry$^{-/-}$/C3$^{-/-}$ and wild-type or DAF$^{-/-}$/C3$^{-/-}$ mouse erythrocytes was clearly noticed (Fig. 6*A*). This difference became more pronounced with time, and by day 7, there were approximately 50% more wild-type or DAF$^{-/-}$/C3$^{-/-}$ cells than DAF^{high} Crry$^{-/-}$/C3$^{-/-}$ cells remaining in the circulation (Fig. 6*A*). When the same experiment was repeated with C3-deficient mice as recipients, all four types of cells were found to be resistant to elimination (Fig. 6*B*). Thus, both the acute elimination of DAF^{low} Crry$^{-/-}$/C3$^{-/-}$ erythrocytes and the accelerated elimination of DAF^{high} Crry$^{-/-}$/C3$^{-/-}$ erythrocytes over an extended period of time were complement mediated.

Given the improved survival of transfused DAF^{high} Crry$^{-/-}$/C3$^{-/-}$ erythrocytes in wild-type mice, we sought to determine whether DAF retrovirus-transduced BM stem cells could successfully engraft in wild-type mice and give rise to viable erythrocytes. We therefore adoptively transferred BM cells from DAF^{high} Crry$^{-/-}$/C3$^{-/-}$ mice to lethally irradiated wild-type mice and monitored the survival of the recipients and their erythrocyte phenotype. As a control experiment, we also transplanted Crry$^{-/-}$/C3$^{-/-}$ BM cells to lethally irradiated wild-type mice. We found that lethally irradiated wild-type mice receiving DAF^{high} Crry$^{-/-}$/C3$^{-/-}$ mouse BM cells survived normally (3/3) and, as expected, their erythrocytes time dependently exhibited the phenotype of the donor mouse cells (GFP positive and Crry negative) (Fig. 7*A*). Unexpectedly, a similar outcome was observed in the control BM transplantation experiment, i.e., lethally irradiated wild-type mice receiving Crry$^{-/-}$/C3$^{-/-}$ mouse BM cells also survived (3/3) and their erythrocytes displayed the expected donor mouse erythrocyte phenotype (Crry negative) (Fig. 7*B*).

Since both types of BM donor mice were deficient in C3, we hypothesized that C3-deficient phagocytes (e.g., splenic macrophages) derived from the engrafted donor stem cells might be

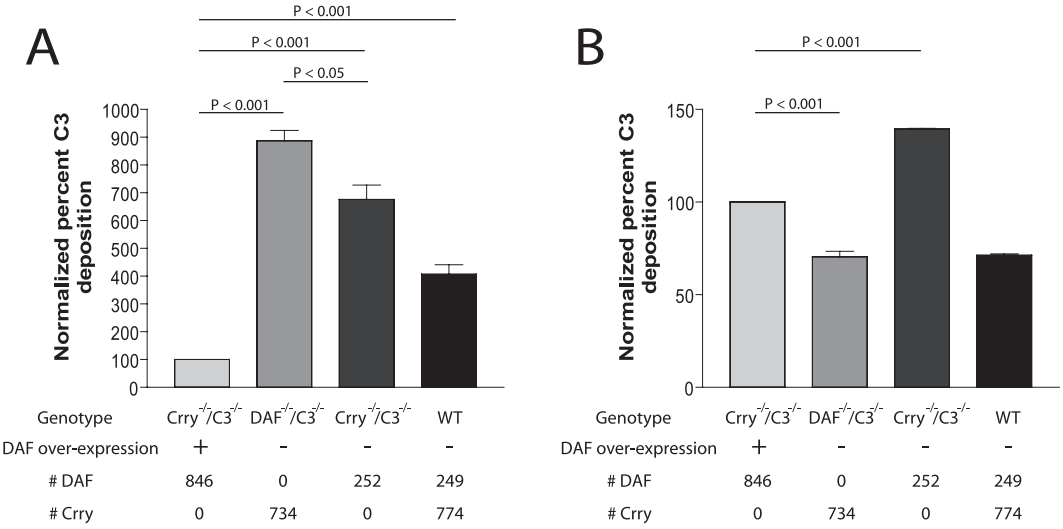


FIGURE 4. Sensitivity of various mouse erythrocytes to classical and alternative pathway complement activation. *A*, In vitro classical pathway complement deposition assay. Erythrocytes were opsonized with 34-3C (50 μg/ml) and then treated with factor B-deficient mouse serum (1:20, 1:40, or 1:80) ($n = 3$ mice for each group). Results shown are from experiments with 1/40 serum dilution. Similar results were obtained with 1/20 or 1/80 serum dilution. *B*, In vitro alternative pathway complement deposition assay. Erythrocytes were incubated with wild-type mouse serum (1/4, ½, or undiluted) in the presence of $MgCl_2$-EGTA. Results shown are from experiments with 1/2 diluted serum ($n = 3$ mice for each group). Similar results were obtained with 1/4 or undiluted serum. Triplicate assays for each mouse were performed in all C3 deposition experiments. *p* values refer to Student *t* test.

defective in their ability to eliminate complement-opsonized Crry$^{-/-}$/C3$^{-/-}$ erythrocytes. We also considered the possibility that the irradiation procedure might somehow impair the extravascular hemolysis mechanism of the BM chimera mice. Both scenarios would explain why Crry$^{-/-}$/C3$^{-/-}$ → wild-type BM chimeras were able to survive. To test these possibilities, we adoptively transferred C3$^{-/-}$ and wild-type BM cells to irradiated congenic wild-type recipient mice. After 8 wk and confirming that donor BM cells had successfully engrafted in the hosts (data not shown), we transfused Crry$^{-/-}$/C3$^{-/-}$ erythrocytes into these BM chimeras to determine their susceptibility to extravascular hemolysis. Fig. 7*C* shows that Crry$^{-/-}$/C3$^{-/-}$ erythrocytes were rapidly eliminated in both types of BM chimeras. Thus, neither C3 deficiency in phagocytes nor the irradiation procedure could possibly explain the unexpected survival of Crry$^{-/-}$/C3$^{-/-}$ → wild-type BM chimeras.

To confirm that the complement-dependent extravascular hemolysis mechanism was impaired in Crry$^{-/-}$/C3$^{-/-}$ → wild-type BM chimeras, we transfused labeled Crry$^{-/-}$/C3$^{-/-}$ erythrocytes into these mice and monitored the survival of the transfused cells. Fig. 7*D* shows that the transfused Crry$^{-/-}$/C3$^{-/-}$ erythrocytes were resistant to rapid elimination in Crry$^{-/-}$/C3$^{-/-}$ → wild-type BM chimeras. In contrast, when the same cells were transfused into DAF^{high} Crry$^{-/-}$/C3$^{-/-}$ → wild-type BM chimeras, they were rapidly eliminated (Fig. 7*D*). Thus, although both Crry$^{-/-}$/C3$^{-/-}$ → wild-type BM chimeras and DAF^{high} Crry$^{-/-}$/C3$^{-/-}$ → wild-type BM chimeras were able to survive, the underlying mechanisms were likely to be different. The former mice had impaired complement-dependent extravascular hemolysis mechanism, whereas survival of the later mice was most likely attributable to increased complement resistance of their erythrocytes.

To explain the impaired complement-dependent extravascular hemolysis mechanism in Crry$^{-/-}$/C3$^{-/-}$ → wild-type BM chimeras, we measured their plasma C3 levels and serum alternative pathway complement activity using ELISA and zymosan C3 deposition assays, respectively. Fig. 7, *E* and *F*, shows that plasma C3 and serum alternative pathway complement activity in DAF^{high} Crry$^{-/-}$/C3$^{-/-}$ → wild-type BM chimeras were similar to that of wild-type mice but were significantly reduced in Crry$^{-/-}$/C3$^{-/-}$ → wild-type BM chimeras.

Discussion

Crry and DAF are two functionally overlapping and ubiquitously expressed membrane regulators of C3 activation in rodent (2). Despite their many similarities, Crry and DAF appeared to have dramatically different in vivo biological functions. Crry gene knockout in the mouse was embryonically lethal (23, 24), whereas daf-1 gene knockout caused no gross abnormalities (22). Furthermore, despite the presence of DAF, Crry-deficient mouse erythrocytes were susceptible to rapid alternative pathway complement-mediated elimination in vivo (26, 27). The purpose of the present study was to determine whether the difference in functional significance between DAF and Crry on mouse erythrocytes was attributable to different expression levels of the two proteins or to a difference in their intrinsic activity as an alternative pathway complement regulator.

By quantitative flow cytometry analysis, we determined that three times more Crry molecules are normally expressed on mouse erythrocytes than DAF. Through retroviral vector-mediated DAF gene transduction of BM stem cells, we over-expressed DAF on Crry$^{-/-}$/C3$^{-/-}$ mouse erythrocytes to a level that was comparable to that of Crry (Fig. 3). Both the constitutive Crry expression level (774 ± 135 molecules/cell) and the over-expressed DAF level (846 ± 127 molecules/cells) were comparable to the reported number of CR1 molecules on human erythrocytes (estimated at 200-1000 molecules/cell) (40, 41), but they were substantially lower than the estimated number of DAF molecules on human erythrocytes (3000 molecules/cell) (4).

The over-expressed DAF molecules on Crry$^{-/-}$/C3$^{-/-}$ mouse erythrocytes, like the endogenously expressed DAF, were evenly distributed on the cell membrane and were fully functional. Thus, DAF^{high} Crry$^{-/-}$/C3$^{-/-}$ erythrocytes were dramatically more resistant than DAF^{low} Crry$^{-/-}$/C3$^{-/-}$ cells to classical pathway complement activation. It is also notable that both DAF^{low} Crry$^{-/-}$/C3$^{-/-}$ cells (with 249 ± 94 DAF molecules/cell) and DAF^{high} Crry$^{-/-}$/C3$^{-/-}$ cells (with 846 ± 127 DAF molecules/cell) were significantly more resistant than DAF$^{-/-}$/C3$^{-/-}$ cells (with 774 ± 135 Crry molecules/cell) to classical pathway complement attack (Fig. 4*A*). This result clearly established that DAF is much more potent than Crry as a classical pathway complement regulator. Since part of

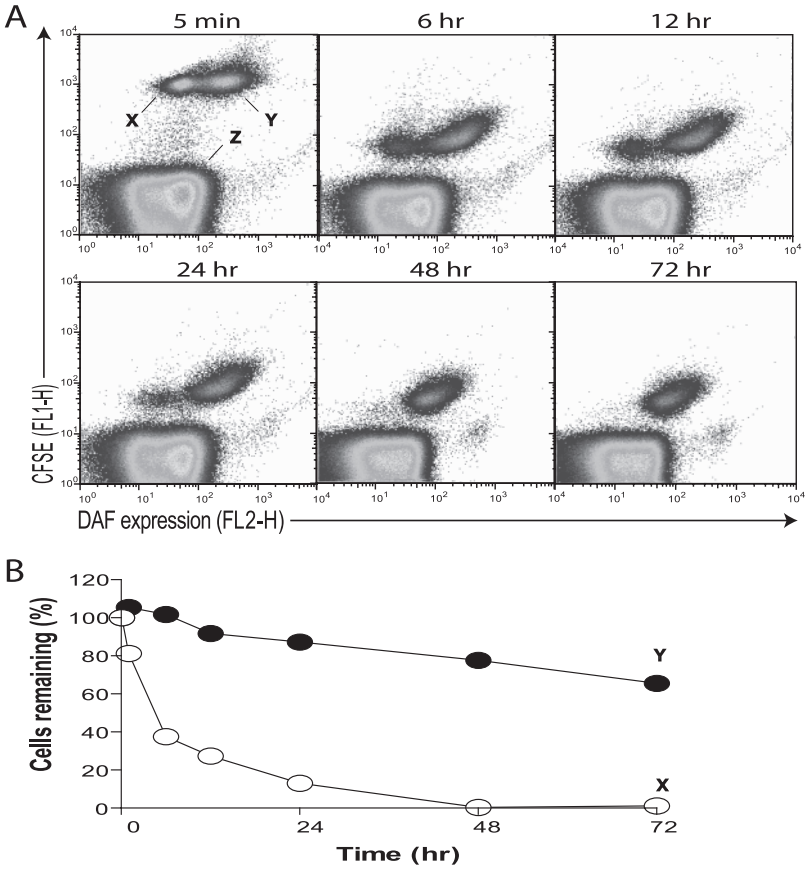


FIGURE 5. Over-expression of DAF protects Crry-deficient erythrocytes from acute complement-mediated elimination. *A*, DAF^{low} Crry$^{-/-}$ erythrocytes (population X) were eliminated within 48 h when introduced into wild-type mice. In contrast, the vast majority of DAF^{high} Crry$^{-/-}$ erythrocytes (population Y) survived up to 72 h after transfusion. Mixtures of DAF^{high} Crry$^{-/-}$ and DAF^{low}Crry$^{-/-}$ erythrocytes were harvested from partially reconstituted donor mice after the second round BM transfer procedure. Erythrocytes were labeled with CFSE before transfusion to distinguish them from the endogenous erythrocytes (population Z). Blood samples were collected from the tail vein at various time points for FACS analysis after staining with anti-DAF. *B*, Plot of elimination kinetics of DAF^{high} Crry$^{-/-}$ and DAF^{low} Crry$^{-/-}$ erythrocytes in *A*. The percentage of cells remaining at each time point was normalized against the percentage of the same population of cells at 5 min after transfusion.

the regulatory activity of Crry is also exerted by its membrane cofactor activity, it can be concluded that the decay-accelerating activity of DAF on the classical pathway C3 convertase, C4bC2a, is far greater than that of Crry.

Conversely, our data suggested that Crry is a more potent inhibitor than DAF of the alternative pathway C3 convertase. Thus, DAF$^{-/-}$/C3$^{-/-}$ erythrocytes (with 774 ± 135 Crry molecules/cell) incurred less C3 deposition than DAF^{high} Crry$^{-/-}$/C3$^{-/-}$ cells (with 846 ± 127 DAF molecules/cell) in alternative pathway complement activation assays in vitro (Fig. 4*B*). Moreover, DAF$^{-/-}$/C3$^{-/-}$ erythrocytes had the same half-life as wild-type cells in vivo, whereas DAF^{high} Crry$^{-/-}$/C3$^{-/-}$ erythrocytes were susceptible to complement-dependent accelerated turnover (Fig. 6). Nevertheless, unlike DAF^{low} Crry$^{-/-}$/C3$^{-/-}$ erythrocytes, DAF^{high} Crry$^{-/-}$/C3$^{-/-}$ erythrocytes were resistant to acute complement destruction and had markedly improved survival in complement-sufficient host (Fig. 6), suggesting that tissue sensitivity to alternative pathway complement attack could also be determined by the level of DAF expression.

Our conclusion that Crry is a more potent alternative pathway complement inhibitor than DAF is supported by result of a previous study of recombinant soluble DAF and Crry proteins (42). Two possibilities may explain the difference between DAF and Crry in this regard. Crry may have a higher decay-accelerating activity than DAF for the alternative pathway C3 convertase, C3bBb, or that its additional MCP activity contributed to the higher anti-alternative pathway complement activity. Although our data did not distinguish these two possibilities, previous studies have shown that the decay-accelerating activity of Crry toward the alternative pathway C3 convertase C3bBb was considerably lower than its membrane cofactor activity (43), suggesting that the MCP activity of Crry may be principally responsible for its strong alternative pathway complement regulating activity.

We showed that DAF^{high} Crry$^{-/-}$/C3$^{-/-}$ BM cells transplanted into C3-sufficient recipients could engraft successfully and supported the production of normal BM chimera animals with viable erythrocytes. Unexpectedly, DAF^{low} Crry$^{-/-}$/C3$^{-/-}$ mouse BM cells, when transplanted into complement-sufficient hosts, also

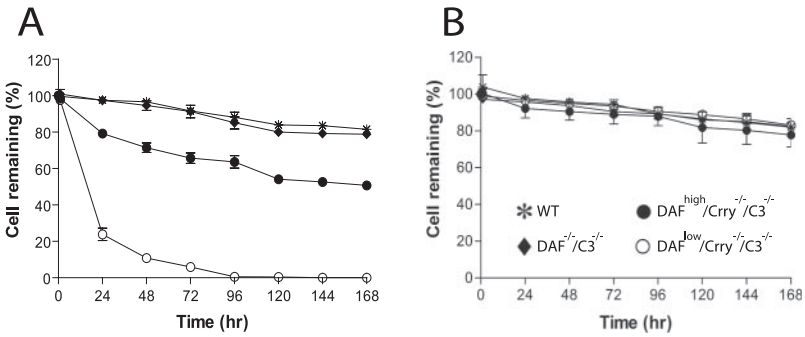


FIGURE 6. Long term survival of DAF^{high} Crry$^{-/-}$ erythrocytes in complement-sufficient or complement-deficient recipients. *A*, Compared with wild-type (∗, n = 3) or DAF$^{-/-}$/C3$^{-/-}$ erythrocytes (◆, n = 3), DAF^{high} Crry$^{-/-}$/C3$^{-/-}$ erythrocytes (●, n = 3) displayed accelerated elimination in wild-type (C3-sufficient) recipients when monitored over a 1-wk period. As expected, DAF^{low} Crry$^{-/-}$/C3$^{-/-}$ erythrocytes (○, n = 3) cells were eliminated acutely, disappearing almost completely by 72 h. *B*, No difference in survival was observed between the four types of erythrocytes when they were transfused into C3-deficient recipients.

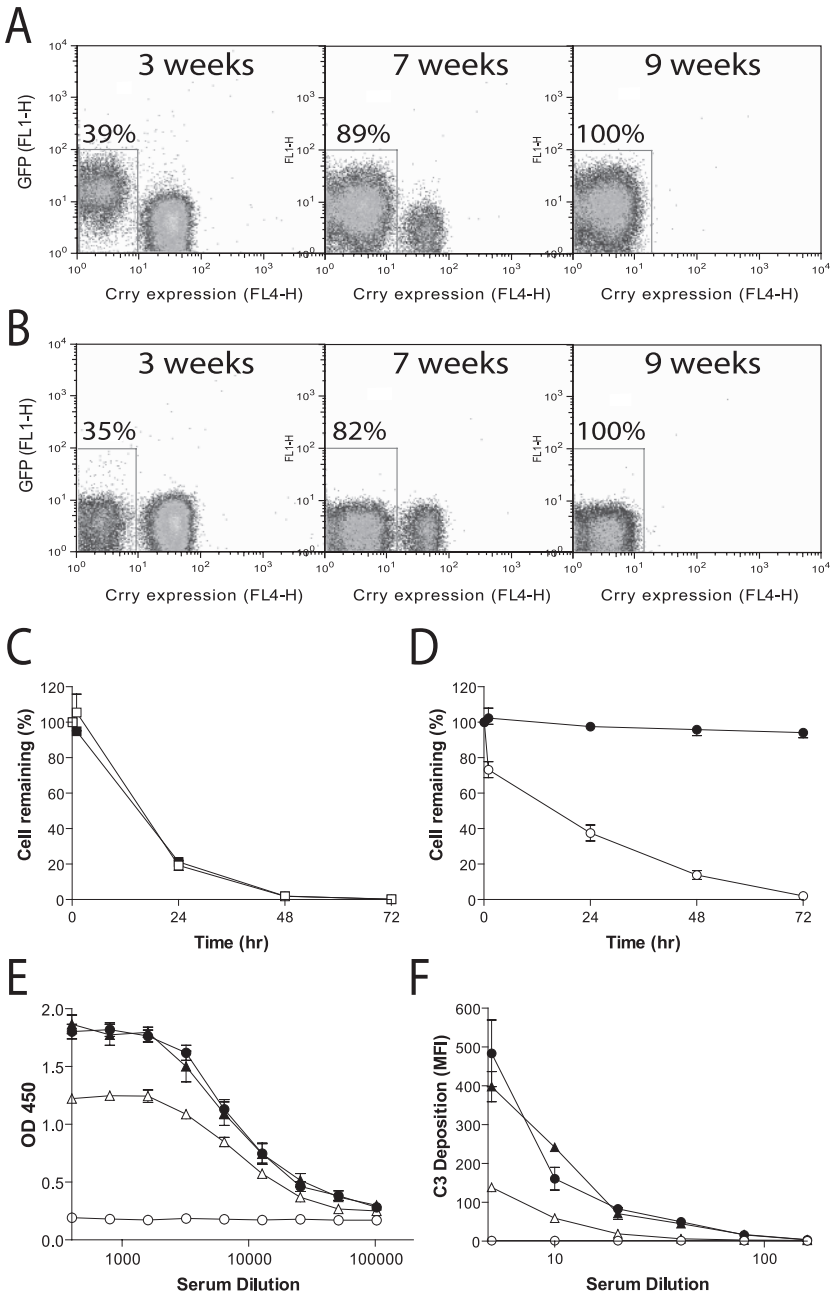


FIGURE 7. Characterization of Crry$^{-/-}$/C3$^{-/-}$ → wild-type BM chimeras. *A*, Engraftment of DAF^{high} Crry$^{-/-}$/C3$^{-/-}$ BM cells in irradiated wild-type host. Erythrocytes were analyzed at 3, 7, and 9 wk after BM transfer for donor mouse phenotype (GFP positive and Crry negative). *B*, Engraftment of DAF^{low} Crry$^{-/-}$/C3$^{-/-}$ BM cells in irradiated wild-type host. Erythrocytes were analyzed at 3, 7, and 9 wk after BM transfer for donor mouse phenotype (Crry negative). *C*, Rapid elimination of Crry-deficient erythrocytes in C3$^{-/-}$ → wild-type BM chimeras (■, $n = 4$) and wild-type → wild-type BM chimeras (□, $n = 3$). *D*, Crry-deficient erythrocytes were rapidly eliminated in DAF^{high} Crry$^{-/-}$/C3$^{-/-}$ → wild-type BM chimeras (○, $n = 3$) but survived normally in DAF^{low} Crry$^{-/-}$/C3$^{-/-}$ → wild-type BM chimeras (●, $n = 3$). *E*, ELISA of plasma C3 levels in wild-type mice (●, $n = 3$), Crry$^{-/-}$/C3$^{-/-}$ mice (○, $n = 3$), DAF^{high} Crry$^{-/-}$/C3$^{-/-}$ → wild-type BM chimeras (▲, $n = 3$), DAF^{low} Crry$^{-/-}$/C3$^{-/-}$ → wild-type BM chimeras (△, $n = 3$). *F*, Serum alternative pathway complement activity as assessed by C3 deposition on zymosan particles. Symbol designations are the same as shown in *E*. All values are expressed as mean ± SE.

supported the production of viable BM chimera mice. However, the latter chimera mice had reduced serum alternative pathway complement activity due, at least in part, to decreased plasma C3 levels (Fig. 7). Thus, the survival of DAF^{high} Crry$^{-/-}$/C3$^{-/-}$ → wild-type BM chimeras and DAF^{low} Crry$^{-/-}$/C3$^{-/-}$ → wild-type BM chimeras had different explanations. Whether reduced plasma C3 level in the DAF^{low} Crry$^{-/-}$/C3$^{-/-}$ → wild-type BM chimeras was caused by transcriptional regulation of the C3 gene or increased spontaneous C3 activation and consumption is not clear, nor is it known if other complement proteins were also present at lower levels in the plasma of these mice. The phenomenon of reduced systemic complement activity in these chimera mice is reminiscent of the phenotype of DAF$^{-/-}$/Crry$^{-/-}$C3$^{+/-}$ mice (44). By crossing female DAF$^{-/-}$/Crry$^{-/-}$C3$^{-/-}$ mice with male DAF$^{-/-}$/Crry$^{+/-}$C3$^{+/-}$ mice, we previously generated viable DAF$^{-/-}$/Crry$^{-/-}$C3$^{+/-}$ mice and found them to have diminished plasma complement activity, caused at least in part by spontaneous C3 activation and consumption (44). Our finding here with the DAF^{low} Crry$^{-/-}$/C3$^{-/-}$ → wild-type BM chimeras suggested that Crry deficiency on the hemopoietic system alone was sufficient to reduce systemic complement activity.

Apart from illuminating the relative activity of DAF and Crry on mouse erythrocytes, our study also highlights the feasibility of using retroviral-mediated gene transduction of hemopoietic stem cells to rectify membrane complement regulator defects on blood cells in vivo. An alternative strategy involving single-chain Ab fragment (scFv)-mediated cell surface tagging of complement regulators has been explored by other investigators (45). In this strategy, a Crry-scFv fusion protein, with the scFV displaying specificity toward glycophorin A, an erythrocyte cell surface Ag, was successfully targeted onto RBC in vitro and in vivo (45). Experiments with Crry-deficient mouse erythrocytes showed that scFV-directed tagging of recombinant Crry prolonged their survival in complement-sufficient host (45). However, scFV-Crry fusion protein targeted onto erythrocytes was subjected to intercellular redistribution in vivo, which could potentially limit its effectiveness short of repeated administration

(45). In contrast, retrovirus-mediated gene transduction of hematopoeitic stem cells led to stable and persistent expression of DAF on erythrocytes. The expression efficiency can be further improved by selective sorting of the transduced stem cells. In principle, a similar approach, involving retrovirus-mediated transduction of hemopoietic stem cells with genes encoding engineered transmembrane forms of human DAF and CD59, may be employed to correct DAF and CD59 deficiencies on the blood cells of paroxysmal nocturnal hemoglobinuria patients (46).

Acknowledgments

We thank Dr Hector Molina of Washington University at St. Louis, MO for providing the Crry$^{-/-}$C3$^{-/-}$ founder mice.

Disclosures

The authors have no financial conflict of interest.

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