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In Vivo Correction of Complement Regulatory Protein Deficiency with an Inhibitor Targeting the Red Blood Cell Membrane[1]

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Because of the complement system's involvement in many human diseases and potential complications associated with its systemic blockade, site-specific regulation of this effector system is an attractive concept. We report on further developments of such an approach using a single-chain Ab fragment as a vehicle to deliver complement regulatory proteins to a defined cell type. In a model system in which RBCs deficient in complement receptor 1-related gene/protein y (Crry) are rapidly cleared after injection into wild-type animals by a complement-dependent mechanism, we selectively reconstituted these cells with N- and C-terminally targeted recombinant forms of Crry. Transfusion of Crry-coated knockout RBCs into C57BL/6 mice extended their in vivo half-life from <5 min to ~2 days. Maintenance of protective levels of Crry (by a combined treatment of donor and recipient RBCs) led to nearly normal RBC survival. Uniform in vitro and in vivo coating of the RBCs and the more efficient complement inhibitory capacity of C-terminally tagged Crry were other interesting features of this experimental system. These results suggest the possibility of using the single-chain Ab fragment-mediated targeting concept of complement regulatory proteins to restrict complement inhibition to the site of its excessive activation. *The Journal of Immunology,* **2005, 175: 7763–7770.**

Expression of complement regulatory proteins on host cell membranes is essential for their protection from continuous complement activation (1, 2). Like the clotting cascade, there is a delicate balance between complement activation and its inhibition (3, 4). The reduced expression level of one or more of these membrane proteins would favor activation and amplification processes, resulting in, e.g., hemolytic uremic syndrome (5, 6) and paroxysmal nocturnal hemoglobinuria (7–11).

Two such regulators on human and mouse RBCs are the GPI-anchored proteins, decay-accelerating factor (DAF;[3] CD55) (4, 12–17) and CD59 (reviewed in Ref. 4). The former catalyzes the dissociation of the C3 and C5 convertases by its decay-accelerating activity (DAA) (18–21), whereas the latter inhibits the membrane attack complex (22–24). In primates, another important complement regulator is expressed on RBCs, complement receptor 1 (CR1; CD35) (reviewed in Ref. 25). This regulator, in addition to having DAA, possesses cofactor activity (CA) for the cleavage of C3b and C4b by the plasma serine protease factor I (26–28). Mice as well as most mammals do not express CR1 on RBCs. Protection of mouse RBCs from the complement system is in part dependent on the transmembrane-anchored protein termed CR1-related gene/protein y (Crry). It possesses, like CR1, DAA and CA (reviewed in Ref. 29). RBCs deficient in Crry (but not DAF or CD59) are rapidly cleared from the circulation upon infusion into C3-sufficient animals by the alternative pathway of complement (30, 31).

Several approaches have recently been described to facilitate attachment of membrane complement regulatory proteins to a cellular target (32–36). We attached human DAF to the mouse RBC with a targeting domain comprised of the variable regions of the mAb Ter^{119} (37) in the form of a single-chain Ab fragment (scFv). This fusion protein binds specifically to mouse glycophorin A, an RBC lineage-restricted glycoprotein (37, 38), and inhibits lysis of mouse RBCs challenged with human complement (39). A potentially important property of this targeting vehicle was that the scFv-tagged fusion proteins were evenly redistributed and did not alter the half-life of the targeted RBCs (39).

In our previous report (39), we established a heterologous human/mouse model system that did not allow for determination of the complement regulator's function in vivo. To study the in vivo applicability of this targeting concept, we used mouse Crry instead of human DAF. In this study, we demonstrate 1) reversion of a complement-sensitive Crry$^{-/-}$ RBC phenotype in vivo by reconstitution with the missing complement regulator, and 2) enhanced activity profiles for engineered Crry forms targeted to the RBC via the C terminus. Thus, by attaching in vivo a complement regulatory protein to a specific target cell type, we provide an example of ameliorating cellular damage through site-specific complement inhibition.

Materials and Methods

Animals

Mice used in this study were between 2 and 4 mo of age. C57BL/6 wild-type (WT) mice were used as recipients for RBC transfusions, to determine the levels of endogenous Crry expressed on RBCs, and as a source of WT

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[3] Abbreviations used in this paper: DAF (CD55), decay-accelerating factor; BHK, baby hamster kidney; CA, cofactor activity; CR1 (CD35), complement receptor 1; Crry, CR1-related gene/protein y; DAA, decay-accelerating activity; eYFP, enhanced yellow fluorescent protein; IRES, polyoma virus internal ribosomal entry site; KO, knockout; MFI, mean fluorescence intensity; scFv, single-chain Ab fragment; SCR, short consensus repeat; WT, wild type.

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mouse serum. Mice deficient in Crry ($Crry^{-/-}$) have been previously described and are only viable on a C3-deficient background because of the embryonic lethality of the Crry mutation (40). The deficient mice were on a mixed C57BL/6 and 129J background. Blood samples for in vitro analyses of RBC survival and Crry copy number determinations were taken from the tail vein using heparinized glass capillaries. Conditions for the in vivo protein delivery are described below. Experiments were performed in accordance with the institutional regulations of animal welfare.

Construction of expression plasmids

The expression plasmids for recombinant mouse Crry are based on pSBC-1 (41) and are arranged in a bicistronic configuration containing a polyoma virus internal ribosomal entry site (IRES)-enhanced yellow fluorescent protein (eYFP) cassette to monitor expression efficiencies (see Fig. 1*A*). The generation of mouse Crry cDNA has been described previously (42, 43). To obtain a basic construct for the insertion of the scFv-targeting domain, soluble, nontargeted Crry (short consensus repeats (SCRs) 1–5, truncation of the transmembrane and Crry's cytoplasmic domain) was generated by inserting a *Bsi*WI/*Nhe*I PCR fragment including the first 318 aa of the mature protein into the *Bsi*WI/*Xba*I linearized plasmid sT-DAF (39) (Fig. 1*B*, s-Crry5). The signal peptide of the native protein (Fig. 1*B*, Crry) was thereby replaced with that of human CD59 (44, 45). N-terminal insertion of a PCR-derived *Bsi*WI-flanked scFv Ter^{119} domain into s-Crry5 resulted in the RBC-targeted Crry form Ter-Crry5 (Fig. 1*B*). For the generation of C-terminally targeted Crry forms, three fragment ligations were performed; the *Bsi*WI/*Xba*I vector backbone of s-Crry5, a *Sac*I/*Xba*I PCR-derived scFv-targeting domain (including an 11-aa linker sequence (*L*) between SCR4/5 and the scFv), and *Bsi*WI/*Sac*I-flanked PCR-generated fragments of Crry were combined to obtain Crry5-Ter (first 318 aa of Crry) and Crry4-Ter (first 255 aa of Crry), respectively (Fig. 1*B*). PCR-derived fragments were verified by DNA sequencing to exclude the introduction of mutations during amplification reactions.

Cells, transfections, and protein production

Baby hamster kidney (BHK) cells (American Type Culture Collection; CCL-10) were used to generate the secreted Crry forms described in this study. They were maintained in DMEM (Mediatech) containing 10% FCS (Harlan Sprague-Dawley). Media were supplemented with L-glutamine (2 mM final concentration; Sigma-Aldrich), nonessential amino acids (BioWhittaker), and penicillin and streptomycin (10 U/ml and 100 μg/ml; Cellgro; Mediatech). Transfections were conducted with FuGene 6 reagent according to the manufacturer's instructions (Roche). For protein production, the medium was replaced with fresh, FCS-free DMEM 1 day after transfection, and the supernatants were harvested on the following 2 consecutive days. To obtain concentrated protein stocks, the FCS-free supernatants were applied to centrifugal filter devices with a 10-kDa molecular mass cutoff (Centricon Plus-20; Millipore).

Western blot analysis

Proteins were separated on 10% polyacrylamide gels (Novex; Invitrogen Life Technologies) under nonreducing conditions by standard procedures (46). Mouse Crry was detected with a rat anti-Crry mAb 1F2 (BD Pharmingen). After washing, the membrane was incubated with HRP-coupled anti-rat IgG (Amersham Biosciences).

In vitro and in vivo coating with RBC-targeted Crry

Depending on the experimental goal, various concentrations of mouse blood and Crry-containing culture supernatants were used. Typically, and to obtain high coating levels, 1–4 μl of mouse whole blood ($\sim$1–4 $\times$ 10^7 RBCs) was incubated with 1.5 ml of the filtered, nonconcentrated BHK culture supernatant for 2 h at room temperature, washed, and then processed for flow cytometry (see below). Supernatants from nontransfected BHK cells served as a control. One reason to switch from our initial heterologous human/mouse system to an all mouse model was to reduce the possibility of an immune response against the recombinant proteins. This was observed in our previous study for human DAF at 4–6 days after injection (39) and prevented an accurate assessment of the long-term redistribution characteristics of the complement regulator. In this study we were able to follow the copy number upon in vivo coating of mouse RBCs with soluble Ter-Crry5 for >42 days, suggesting a low immunogenicity of scFv-targeted autologous complement regulator. For the in vivo delivery of RBC-targeted mouse Crry, mice were injected i.v. via the tail vein with up to 300 μl of filtered (0.2-μm pore size) culture supernatant (either nonconcentrated or concentrated) or by i.p. injection of 450 μl of the concentrated protein preparation. Blood samples (2 μl) from a WT control mouse (to obtain the endogenous Crry level for each dataset) and from the test animals were collected by tail clipping, washed once with 1 ml of FACS buffer, and processed for flow cytometry.

In vivo RBC survival

To assess the clearance of mouse RBCs in vivo, cells (from 60–80 μl of whole blood, giving rise to between 1 and 3% labeled donor cells) from WT and $Crry^{-/-}$ mice were labeled ex vivo with the membrane dye PKH-26 (Sigma-Aldrich) according to the manufacturer's instructions. They were then introduced into WT mice via the tail vein in a 300-μl cell suspension. Blood samples (2 μl) were collected by tail clipping 5 min after RBC infusion and at various indicated time points thereafter. Collected RBCs were either directly submitted to FACS analysis without previous treatment (due to the ability of the membrane dye to spontaneously emit red fluorescence) or immunostained for Crry as described below. To exclude an adverse effect of the presence of up to 20 times the endogenous Crry level of the attached exogenous proteins, PKH-26-positive WT RBCs were treated in vitro with Ter-Crry5 or with medium alone and then transfused into WT recipients. FACS analysis of the transfused donor cells revealed that the recovery rates for both donor cell preparations were identical, establishing that the excess of exogenous Crry did not initiate an accelerated RBC clearance (data not shown).

Flow cytometry

Flow cytometry was performed to determine the expression level of endogenous Crry, to quantify the RBC-targeted recombinant Crry forms after in vitro or in vivo coating, and to quantify deposition of complement activation fragments on mouse RBCs. Typically, after the various pretreatment conditions, the cells were washed and then incubated for 30 min at 4°C with the rat mAb 1F2 described above for Western blot analysis. After washing, FITC-conjugated secondary goat anti-rat IgG pAb (BD Pharmingen) was added, and the incubation was continued for an additional 15 min at 4°C. Cells were next washed and analyzed by flow cytometry by gating on the RBCs with forward and side scatter (FACScan; BD Biosciences).

In vitro complement deposition assays

Ab-induced C3 deposition on mouse RBCs was performed as previously described (20) using a slightly modified protocol. After coating the RBCs with the targeted complement regulators (or medium alone as a control), they were sensitized with an anti-mouse CD24 mAb J11d (rat IgM; Research Diagnostics) at a dilution of 1/5,000 to 1/10,000 before exposure to mouse complement for 30 min at room temperature. To check the IgM copy number on WT and knockout (KO) RBCs after sensitization, the cells were stained with FITC-conjugated goat anti-rat secondary Ab (BD Pharmingen) and assessed by FACS. Only those cell preparations coated with equivalent IgM copy numbers were used. The RBCs were washed, resuspended in 200 μl of GVB^{2+} (Sigma-Aldrich), and then incubated for 30 min at 37°C in the presence of 5% WT mouse serum (prepared immediately before use in-house from C57BL/6 mice). The incubation was stopped by transfer of the reaction tubes to ice, and 30-μl aliquots were used to assess the complement deposition by FACS after direct immunostaining with FITC-conjugated rabbit anti-C3 polyclonal Ab ($F(ab')_2$) (Valeant Pharmaceuticals). Although heavily coated with mouse complement, RBC lysis was not observed in these experiments.

Results

Generation and characterization of mouse RBC-targeted Crry

To study regulation of complement activation in vivo, we applied the scFv-mediated RBC targeting strategy recently described for human DAF (39) to mouse Crry. We initially equipped this complement regulator at its N terminus with the scFv Ter^{119}, which specifically recognizes the abundantly expressed glycophorin A ($\sim$1 $\times$ 10^6 copies/RBC) (37). In addition, constructs were prepared in which the targeting domain was switched to the C terminus. The N-terminally tagged soluble Crry form consists of all five SCRs of the native protein, but lacks the transmembrane and cytoplasmic tail regions (Fig. 1*B*, Ter-Crry5). Two different C-terminally tagged Crry constructs were generated, containing either four or five SCRs. With respect to the proper interaction of the Ab fragment and its cellular target (glycophorin A), this configuration

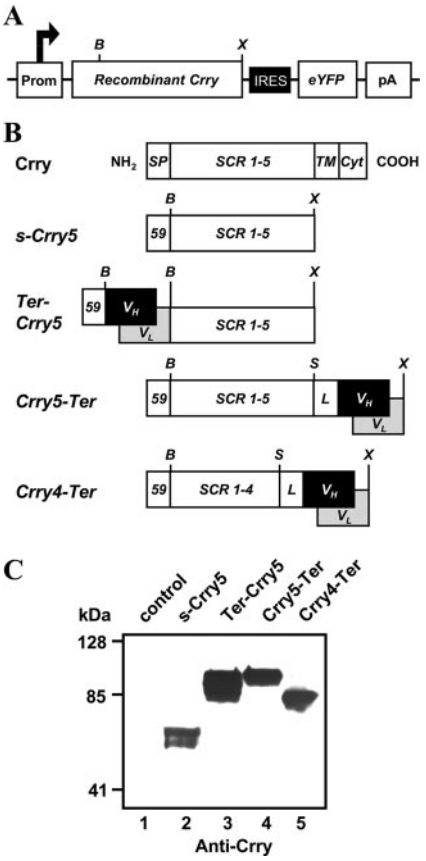

FIGURE 1. Schematic representation of the expression vector system and the Crry-containing constructs. *A*, Each vector contains a bicistronic Crry-IRES-eYFP expression cassette for monitoring transfection efficiencies controlled by the SV40 promoter and an SV40 polyadenylation signal. *B*, Diagram of the protein structures of WT and secreted Crry forms in the absence or the presence of the scFv Ter^{119} targeting epitope in which V_H and V_L refer to its variable H and L Ab fragments, respectively. An 11-aa linker sequence between Crry and the C-terminally attached scFv is denoted by L. *C*, Western blot of the secreted fusion proteins. Immunoreactive fragments were detected with a rat mAb to Crry and HRP-conjugated secondary Abs as detection reagents. Supernatant from nontransfected BHK cells served as a control. Prom, SV40 early promoter/enhancer; pA, SV40 polyadenylation signal; s, secreted; SP, native signal peptide of Crry; 59, signal peptide of CD59; Ter, scFv against mouse red cell membrane Ag glycophorin A; TM, transmembrane domain; Cyt, cytoplasmic domain. Restriction enzymes: B, *Bsi*WI; S, *Sac*I; X, *Xba*I.

with the complement regulator fused to the N terminus of the variable H chain of the scFv was anticipated to cause a loss or reduction in binding affinity. We therefore included an 11-aa linker sequence between the last SCR and the scFv. These two constructs were designated Crry5-Ter and Crry4-Ter, respectively. The recombinant fusion constructs were transiently transfected in mammalian BHK cells, and the proteins were secreted into the culture supernatant. Western blot analysis confirmed the expected molecular mass of ~100 kDa for Ter-Crry and Crry5-Ter and ~85 kDa for Crry4-Ter, respectively (Fig. 1*C*, *lanes 3–5*).

In vitro reconstitution of deficient target cells with recombinant Crry forms

In our previous study (39), the scFv Ter^{119} membrane-targeting moiety attached uniformly to mouse RBCs. Using the same targeting domain, we anticipated this result for the three forms of mouse Crry. Initially, we determined the normal expression level of Crry on C57BL/6 WT RBCs. Staining of these cells for native Crry demonstrated a uniform signal peak with a mean fluorescence intensity (MFI) of 58, reflecting its endogenous expression profile (Fig. 2*A*). Immunostaining of RBCs from Crry KO mice, which are only viable on a *C3*-deficient background as stated previously (40), confirmed the absence of Crry from these cells (Fig. 2*A*, dotted lines). Upon in vitro treatment with Ter-Crry5, reconstitution of

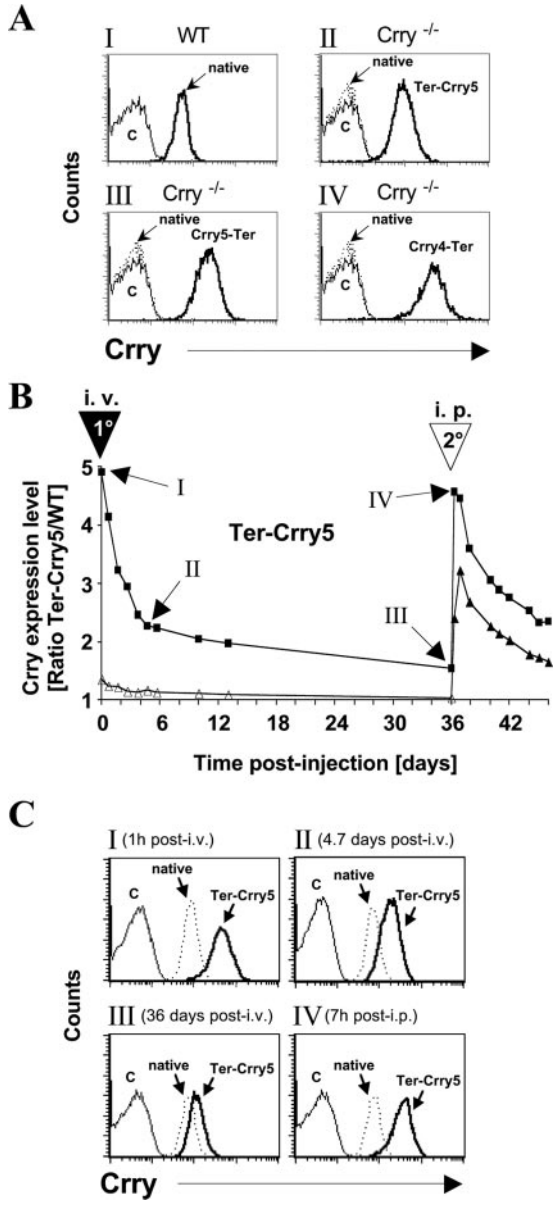

FIGURE 2. Coating of mouse RBCs in vitro and in vivo with targeted Crry forms. *A*, RBCs from C57BL/6 mice were used to determine the expression level of native Crry (I). Crry$^{-/-}$ RBCs incubated in vitro with supernatants from BHK cells containing Ter-Crry5 (II), Crry5-Ter (III), or Crry4-Ter (IV). Bound proteins were detected by indirect immunostaining using an anti-Crry primary mAb. As controls (c), WT RBCs were treated with chromophore-conjugated secondary Ab, whereas Crry$^{-/-}$ RBCs were incubated with primary and secondary Ab. *B*, Coating of WT RBCs in vivo by i.v. (primary injection, day 0) and i.p. (secondary injection, day 36) administration of Ter-Crry5. The signal intensity was monitored by FACS as described in *A*. Two different concentrations were used for the primary infusions (■, high; △, low). Note that both mice received the same high-dose treatment at the second i. p. injection (■ and ▲). Data are expressed as the ratio of the signal intensity of exogenous to endogenous Crry (obtained from freshly isolated WT control RBCs for each dataset). *C*, Selected FACS histograms (I–IV) used to generate the graph in *B*.

Crry was achieved (Fig. 2*A*). Similar results were observed for the C-terminally tagged Crry forms, Crry5-Ter and Crry4-Ter (Fig. 2*A*). These results establish that the switch of the scFv-targeting domain to the C terminus of the complement regulator does not influence its interaction with glycophorin A.

In vivo RBC targeting with Ter-Crry5

The chimeric fusion protein Ter-Crry5 was administered i.v. into WT recipient mice, followed by a second i.p. injection 36 days later (Fig. 2, *B* and *C*). A homogeneous staining pattern was observed, as was the case in the in vitro coating experiments (compare Fig. 2, *A* and *C*). In the examples shown, the increase in copy number of Ter-Crry5 on the target cells was between 1.4-fold of the endogenous expression level (nonconcentrated supernatant; open symbols) and 5-fold (concentrated supernatant; closed symbols; Fig. 2*B*). Selected histograms demonstrated a homogeneous staining pattern throughout the follow-up period (Fig. 2*C*). These profiles are in agreement with a cell-to-cell transfer of the targeted proteins noted previously (39). Interestingly, recombinant Ter-Crry5 (high-dose treatment) was detectable on circulating RBCs 36 days after injection (~1.5-fold of the native regulator; Fig. 2, *B* and *C*). In addition, a homogeneous staining pattern was maintained, without the appearance of a cell population exhibiting only the endogenous Crry expression level (Fig. 2*C*). As an alternate administration procedure, we injected via the i.p. route a concentrated protein preparation into the mice initially receiving a differential i.v. treatment (high and low dose; Fig. 2*B*). The Crry expression level peaked at 4.5- and 3.2-fold over the level in its WT counterpart, comparable to levels seen with i.v. administration.

The clearance curve of Ter-Crry5 was biphasic. There was a more rapid initial phase of signal decay with a half-life of ~3 days (Fig. 2*B*; days 0–6; exponential regression analysis, $r^2 = 0.968$). This was followed by a second phase with a slower half-life (Fig. 2*B*; days 6–36). The signal decay of Ter-Crry5 in which both mice received a second high-dose injection between days 36 and 46 closely resembled that of the first 6 days after the initial injection of the concentrated supernatant (Fig. 2*B*). The reason for this biphasic clearance pattern of Ter-Crry5 is unknown. However, equilibration with Ter^{119}-positive RBC precursors in the bone marrow (because these cells express glycophorin A beginning at the proerythroblast stage) (37) and/or with the extravascular space could be envisioned. Ter^{119}-positive cells from the bone marrow compartment are indeed targets for the infused reagents, because ~50% of these cells were coated as quickly as the peripheral RBCs (our unpublished observations). Additional biodistribution experiments are in progress to study the fate of the injected proteins.

Using indirect immunostaining, the FACS signals used to generate the data presented in Fig. 2*B* were specific and were not the result of a cross-reactivity of the secondary Ab with an induced anti-mouse immune response against the recombinant proteins. This possibility was addressed by staining the RBC samples with an FITC-conjugated goat anti-mouse polyclonal Ab. These were negative (data not shown), indicating a lack of an immune response to the infused scFv-containing autologous complement regulator. Immunogenicity of our constructs cannot be ruled out after multiple infusions, especially because the targeting scFv is of rat origin.

Activity profiles of RBC-targeted Crry forms

To assess the relative complement regulatory activity of RBC-targeted vs native Crry, we reconstituted $Crry^{-/-}$ RBCs with increasing amounts of Ter-Crry5, Crry5-Ter, and Crry4-Ter. After classical pathway complement activation, we performed a FACS-based C3-deposition assay. As a reference, we included sensitized WT RBCs and found considerable C3-deposition, much higher than on nonsensitized control cells (MFI, 247 vs 6; Fig. 3, dotted line). As expected, complement activation was even higher in the complete absence of Crry (MFI, 3400 vs 247; Fig. 3). After incubation with increasing amounts of Ter-Crry5, the C3 deposition progressively decreased in a dose-dependent manner. Between a 5- and 10-fold excess of Ter-Crry5 was required to limit the C3 deposition to that of the WT reference cells (Fig. 3, ♦). This means that 5–10 times more recombinant Ter-Crry5 was necessary to achieve the identical complement regulatory effect as that observed for the native protein. However, in the case of the C-terminally tagged Crry forms between 1.5 and 3 times more of the regulators were required to limit the C3 deposition to that of the WT RBCs (Fig. 3, open symbols). Of interest, coating the KO RBCs with high copy numbers of exogenous regulators protects even better against complement activation compared with WT cells (Fig. 3, below dotted line). Similarly, coating of WT RBCs with any of the Crry constructs abolished complement activation (data not shown). In addition, these proteins completely blocked complement activation via the alternative pathway (data not shown).

RBC-targeted Crry as a complement therapeutic

The preceding results indicate that Crry, attached to the RBC via a scFv-targeting moiety fused either to its N or C terminus, retains functional complement regulatory activity in vitro. A major goal though of our current study was to demonstrate the functionality of this concept in an in vivo animal model. Because $Crry^{-/-}$ RBCs are rapidly cleared in a complement-dependent process from the circulation upon transfusion into WT recipient mice (30, 31), we studied the survival of these complement-sensitive cells in vivo after being coated in vitro with Ter-Crry5.

The infused donor RBCs were labeled before injection with the membrane dye PKH-26 to allow discrimination from the host cell pool. To obtain the clearance pattern of normal cells, PKH-labeled WT RBCs were injected into WT recipients. These cells demonstrated a $t_{1/2}$ of ~10.5 days (Fig. 4*A*, extrapolated from linear

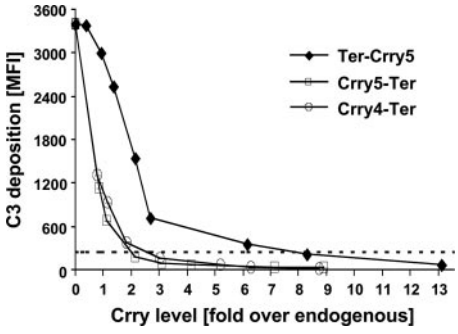

FIGURE 3. Dose-dependent inhibition of classical pathway-mediated complement activation by RBC-targeted Crry forms. $Crry^{-/-}$ RBCs were coated in vitro with increasing amounts of Ter-Crry5, Crry5-Ter, and Crry4-Ter and then sensitized with complement-fixing anti-mouse mAb J11d (rat IgM anti-mouse CD24). As controls, WT (background complement activation) and $Crry^{-/-}$ RBCs were incubated with medium alone and sensitized accordingly. The RBCs were then washed, resuspended in GVB^{2+}, and incubated for 30 min at 37°C in the presence of 5% WT mouse serum, followed by assessment of the complement deposition by direct immunostaining with FITC-conjugated rabbit anti-C3 polyclonal Ab ($F(ab')_2$). Complement deposition is expressed as the MFI. The dotted line represents the C3 deposition on sensitized WT RBCs. The copy number of exogenous Crry on $Crry^{-/-}$ RBCs is expressed in multiples of the native protein as quantified by FACS of WT control RBCs. A representative experiment is shown ($n = 4$).

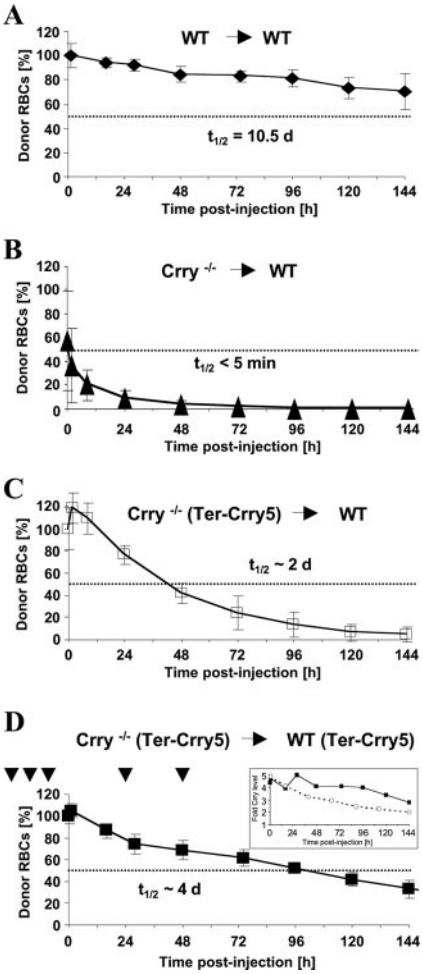

FIGURE 4. Ter-Crry5 provides long-term in vivo protection against complement attack. RBCs (WT and Crry$^{-/-}$) were labeled with the membrane marker PKH-26 for in vivo tracking purposes. All donor RBCs were infused via the tail vein into nontreated WT recipient mice (*A–C*), except in *D*, in which the mice were additionally treated with Ter-Crry5 by i.p. injection before and after transfusion of the RBCs (arrowheads at −24, −16, −8, 24, and 48 h). The following RBC types and conditions were used: nontreated WT (*A*), nontreated Crry$^{-/-}$ (*B*), and Ter-Crry5-coated Crry$^{-/-}$ (*C* and *D*). The *inset* in *D* shows the corresponding copy number profile of Ter-Crry5 on the host RBCs during the course of the experiment (solid line) in comparison with the profile obtained from a single injection as per Fig. 2*B* (dotted line). All mice received equivalent numbers of RBCs, and the data are normalized to the recovery rate of WT RBCs at 5 min after injection ($n = 3$ for each condition). The time needed to reduce the starting concentration of the donor RBCs by 50% was used to determine their half-life (dotted line). Error bars indicate the SD.

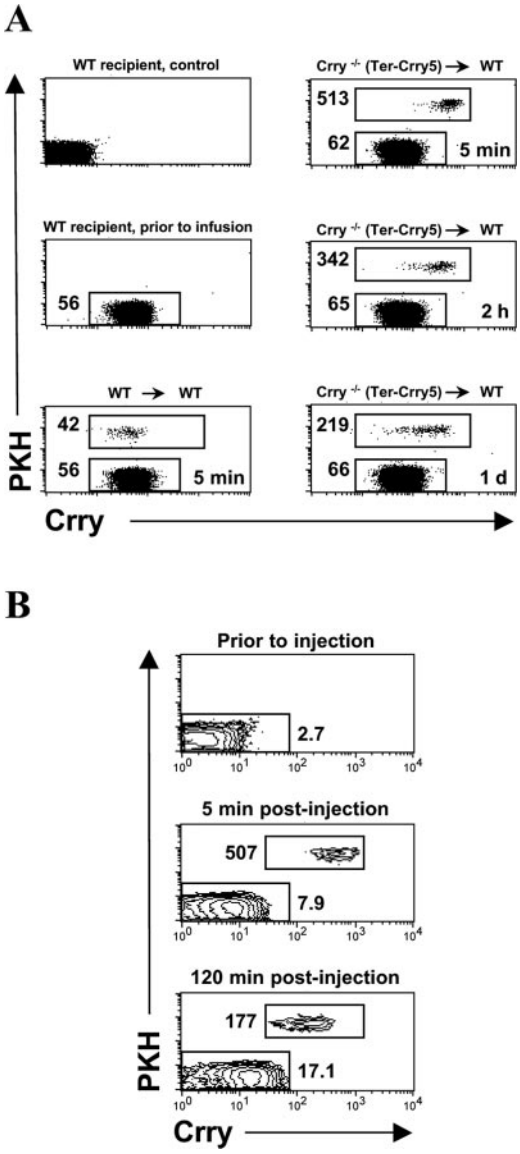

FIGURE 5. ScFv-Crry transfers from precoated donor RBCs to the host's RBC acceptor pool during in vivo circulation. PKH-labeled Crry$^{-/-}$ RBCs were coated in vitro with Ter-Crry5 and then analyzed for their Crry level after i.v. injection into WT (*A*) and Crry$^{-/-}$/C3$^{-/-}$ double-KO mice (*B*). At the indicated time points, samples were taken, and indirect immunostaining was performed as described in Fig. 2 with an anti-Crry mAb. The numbers next to the gates represent the Crry signal intensities (MFIs) of the PKH-positive infused RBCs (*upper gates*) and PKH-negative host RBCs (*lower gates*). *A*, To obtain the background fluorescence signal, WT RBCs were incubated only with secondary anti-mouse FITC-conjugated Ab. *B*, As in *A*, but additionally treated with primary anti-Crry mAb. Dot plots (*A*) and contour plots (*B*) from a representative experiment are shown ($n = 3$).

regression analysis; $r^2 = 0.9606$) in accord with the reported $t_{1/2}$ of 8–15 days (47, 48) of C57BL/6 RBCs. When nontreated Crry$^{-/-}$ RBCs were injected into WT mice, ~50% were eliminated shortly after injection (by 5 min), and most of the remaining cells were cleared by 24 h (Fig. 4*B*), consistent with previous reports (30, 31). To study the effect of membrane-targeted Crry, we coated the RBCs with Ter-Crry5 at ~11 times the endogenous Crry level in vitro before infusion into the WT mice. This pretreatment led to a half-life of nearly 2 days (Fig. 4*C*). However, the clearance rate of Ter-Crry5-coated Crry$^{-/-}$ RBCs was still approximately five times faster than that of WT cells (Fig. 4, *A* and *C*; $t_{1/2}$, 10.5 vs ~2 days). This result was not unexpected if one considers the progressive transfer of regulator proteins from the infused cells to the RBC pool of the host (39) (see below).

The above experiments were performed by injecting precoated RBCs into nontreated recipient animals. We next treated not only the KO donor RBCs with Ter-Crry5, but also the WT recipients. We therefore sequentially increased the exogenous Crry copy number on the WT RBC pool by injecting concentrated protein preparations before and after the mice were transfused with the in vitro coated RBCs (Fig. 4*D*, denoted by arrowheads at −24, −16, −8, 24, and 48 h; see solid line of the *inset* for the Crry expression profile on the host RBCs). As a result, the in vivo half-life of circulating Crry$^{-/-}$ RBCs increased from ~2 days (Fig. 4*C*, nonpretreated recipients) to ~4 days (Fig. 4*D*). These results are in

agreement with the dose-dependent activity profiles of recombinant Crry forms (Fig. 3); that is, the higher copy number of the complement regulator on the target cell provides greater protection against complement activation and thus extends the in vivo half-life of KO RBCs injected into precoated recipient mice (Fig. 4*D*).

In vivo transfer of Ter-Crry to the host RBC pool

We next sought to explain the more accelerated clearance from the circulation of in vitro-coated Crry$^{-/-}$ RBCs compared with that of WT cells. To do this, we compared the Ter-Crry5 copy number on donor (PKH-positive) and host RBCs (PKH-negative). During the first 5 min after injection of Ter-Crry5-coated Crry$^{-/-}$ RBCs into a WT host (Fig. 5*A*), the copy number on the donor cells decreased from an MFI of 900 (determined before injection of the coated donor cells; data not shown) to an MFI of 513 (Fig. 5*A*, 5 min point). At the same time, the endogenous Crry expression level of the recipient's WT RBCs increased slightly from an MFI of 56 to an MFI of 62.

To confirm this rather minimal increase in Crry copy number on RBCs already expressing the native regulator (relatively high baseline MFI), we performed a similar experiment using Crry$^{-/-}$/C3$^{-/-}$ double-KO recipient mice (Fig. 5*B*). As expected from the results shown above, the regulator loss from the precoated donor RBCs (representing ~2% of the host population) coincided with a definitive increase in the signal intensity of the PKH-negative host cell pool. These data provide conclusive evidence for a transfer of Ter-Crry5 from RBC to RBC.

Discussion

In this study, we adopted our recently described scFv-mediated targeting concept of chimeric complement regulators to an in vivo model of complement regulatory protein deficiency using autologous Crry. In this system Crry$^{-/-}$ RBCs are cleared by a complement-dependent mechanism after infusion into WT recipient mice. However, after in vitro or in vivo reconstitution with mouse RBC-specific, scFv-containing Crry, these cells were rescued from rapid clearance.

In our current study we verified our previous results with human DAF that after injection of an RBC-targeted homologous complement regulator into WT recipient mice, a uniform coating of the entire RBC pool with Ter-Crry5 was observed and maintained. Although protected initially against autologous complement attack after i.v. infusion, the Ter-Crry5-coated KO donor RBCs did not establish a WT survival profile. This finding was explained by a progressive loss of the regulatory protein from the donor cell population to the uncoated host RBCs (Fig. 5). To take advantage of this equilibration process, we also coated the recipient's (WT) RBC pool with Ter-Crry5 before the animals received the injection of in vitro precoated Crry$^{-/-}$ RBCs. This pretreatment further extended the circulatory half-life of the infused cells due to a reduction in the copy number gradient between donor and host cells, i.e., the equilibrated state has been shifted to a higher level (Fig. 2*C*, *panel I*). The activity profile of Ter-Crry5 (only 10–20% as potent as the native protein; Fig. 3) and a copy number excess <5-fold throughout the RBC survival experiment (Fig. 4*D*, *inset*, solid line) explains the accelerated clearance pattern of Ter-Crry5-coated Crry$^{-/-}$ RBCs compared with those from WT mice (Fig. 4*A*). The ability to jump from cell to cell in vivo ensures redistribution of Ter-Crry5 exclusively among the target cell population, a favorable pharmacological property that is most likely related to the binding affinity of the scFv targeting domain.

The experiments described in this article involve the conversion of a transmembrane-anchored complement regulator to a soluble form. This regulator was then attached to the cell surface via a scFv that binds glycophorin A. Because these alterations may influence its functional properties, we performed a comparison of native and RBC-targeted Crry. Attachment of Crry to mouse glycophorin A via an N-terminal scFv led to a reduction (~5- to 10-fold) in complement regulatory activity. However, this loss of activity could be compensated by coating the target RBCs with higher amounts of exogenous Ter-Crry5. One explanation for the reduced activity profile of RBC-targeted vs WT Crry is its attachment to glycophorin A. Because glycophorin A forms homodimers on the RBC membrane (49, 50) and associates with the cytoskeletal network (38, 51), it may be relatively immobile compared with native Crry (Fig. 6*A*). Attached to glycophorin A, the targeted recombinant Crry forms will take on the mobility features of this membrane protein (Fig. 6*B*).

Another explanation for the decreased activity of scFv-targeted Crry forms is their orientation on the plasma membrane. For example, the SCRs of N-terminally tagged Ter-Crry5 probably result in an inverse configuration of Crry (Fig. 6*B*, *left panel*). In an attempt to improve the activity profiles of scFv-targeted Crry, we switched the targeting domain from the N to the C terminus. This approach led to an ~4-fold enhanced activity for Crry5-Ter and Crry4-Ter in C3 deposition assays compared with the N-terminally tagged form, Ter-Crry5. Of particular interest was the increased activity of C-terminally targeted Crry (~50–80% as active as native Crry), suggesting a more WT-like configuration (Fig. 6*B*, *right panel*). This finding might therefore be applicable to other family members of the regulators of complement activation. In fact, if the same targeting domain was fused to the C terminus of human DAF, we observed an ~2-fold increase in activity compared with

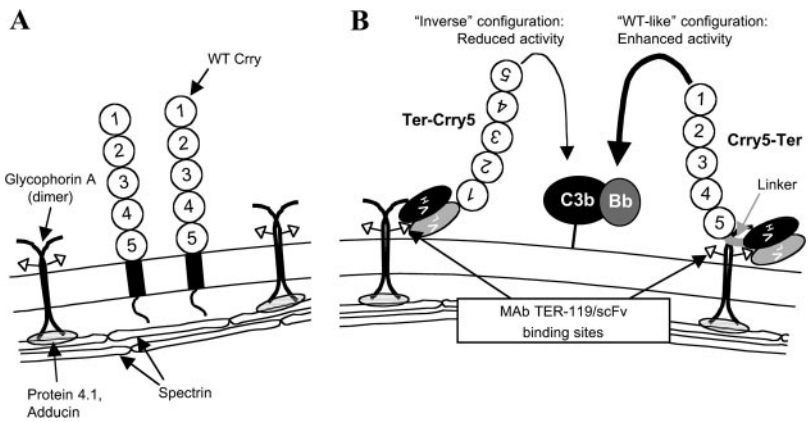

FIGURE 6. Characteristics of WT and glycophorin A-targeted Crry forms: a proposed model for their differential activity profiles. *A*, Glycophorin A is associated with the cytoskeletal network (spectrin) via several adapter proteins, including adducin and protein 4.1, which presumably reduces its lateral mobility on the surface of the RBC. In contrast, WT Crry is not known to be involved in such interactions and probably has a higher surface mobility. *B*, Proposed configuration relative to the membrane of glycophorin A-targeted Crry in an inverse (N-terminally tagged Ter-Crry5) and a WT-like orientation of its SCRs (C-terminally tagged Crry5-Ter). The C-terminally tagged Crry more closely resembles that of the native protein, which may explain its increased activity/affinity for its interaction with membrane-bound C3b or C4b (the alternative pathway C3 convertase is shown as an example).

the N-terminally targeted DAF form (D. Spitzer and J. P. Atkinson, unpublished observation). Along this line, when the same targeting strategy was applied to N-terminally tagged mouse DAF (the human homologue with only DAA), no protection of Crry-deficient RBCs was observed in vivo. Of interest, the same coating level of DAF efficiently inhibited complement activation in vitro (D. Spitzer and J. P. Atkinson, unpublished observation). Whether the remaining 20–50% difference in regulatory activity between C-terminally targeted and native Crry can be attributed to the nature of the membrane anchor (plasma membrane environment and/or mobility characteristics of glycophorin A) or the chimeric regulator itself is currently under investigation.

To translate our concept into the generation of a reagent for the treatment of a human disease, a suitable target Ag has to be first identified, ideally having a unique and an abundant expression profile on the target cell or tissue. An scFv targeting vehicle would then be generated from a characterized mAb or via phage display technology (reviewed in Ref. 52). In the case of a nonhuman hybridoma, humanization would have to follow. More recently, the generation of transgenic mice, genetically engineered to produce human Abs, would be an alternative to further minimize the immunogenicity of the targeting domain (53, 54). The targeted constructs, after i.v. injection (intended for more acute clinical applications) or produced by autologous cells after in vivo gene transfer (to maintain a long-term protein supply), would now deliver the complement inhibitor to the site of undesirable complement activation. Based on the results presented in this study, we believe that the concept of targeted complement regulators has the potential for developing therapeutic reagents, and that cofactor activity might be favored over those regulators with DAA.

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Disclosures

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