

Human CD59 Inhibitor Sensitizes Rituximab-Resistant Lymphoma Cells to Complement-Mediated Cytolysis

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Abstract

Rituximab efficacy in cancer therapy depends in part on induction of complement-dependent cytotoxicity (CDC). Human CD59 (hCD59) is a key complement regulatory protein that restricts the formation of the membrane attack complex, thereby inhibiting induction of CDC. hCD59 is highly expressed in B-cell non-Hodgkin's lymphoma (NHL), and upregulation of hCD59 is an important determinant of the sensitivity of NHL cells to rituximab treatment. Here, we report that the potent hCD59 inhibitor rILYd4 enhances CDC *in vitro* and *in vivo*, thereby sensitizing rituximab-resistant lymphoma cells and primary chronic lymphocytic leukemia cells (CLL) to rituximab treatment. By defining pharmacokinetic/pharmacodynamic profiles of rILYd4 in mice, we showed that by itself rILYd4 does not adversely mediate *in vivo* hemolysis of hCD59-expressing erythrocytes. Increasing expression levels of the complement regulators CD59 and CD55 in rituximab-resistant cells occur due to selection of preexisting clones rather than *de novo* induction of these proteins. Moreover, lymphoma cells overexpressing CD59 were directly responsible for the resistance to rituximab-mediated CDC therapy. Our results rationalize the use of rILYd4 as a therapeutic adjuvant for rituximab treatment of rituximab-resistant lymphoma and CLL. Furthermore, they suggest that preemptive elimination of CD59-overexpressing subpopulations along with rituximab treatment may be a useful approach to ablate or conquer rituximab resistance. *Cancer Res*; 71(6); 2298–307. ©2011 AACR.

Introduction

In the last 10 years, the chimeric antibody (Ab) of rituximab, which specifically targets CD20 on the B lymphocyte membrane, has led to significant progress in the treatment of B-cell non-Hodgkin's lymphoma (NHL; ref. 1). However, a subset of NHL patients do not respond to rituximab, despite expressing CD20 (2), and many patients who initially respond develop resistance to further treatment over time (3). The mechanisms suspected to mediate therapeutic effects of rituximab include (i) complement-dependent cytotoxicity (CDC; refs. 4–7); (ii) Ab-dependent cellular cytotoxicity (ADCC; refs. 2, 8–10) involving phago-

cytosis (11) and/or Fc:FcR-dependent mechanisms (12); and (iii) apoptosis (1, 9, 12).

The role of the CDC on rituximab-mediated lymphoma therapy has been extensively investigated *in vivo* and *in vitro* (9). Complement depletion by cobra venom factor or C1q-deficiency significantly reduces the antitumor activity of rituximab in mouse models (6, 13–15). Consistently, complement consumption has been observed *in vitro* and *in vivo* after rituximab administration (5, 16), and addition of fresh-frozen plasma as a source of complement can increase the therapeutic response to rituximab in CLL-refractory patients (17, 18). The importance of CDC in B-cell lymphoma response to rituximab was further confirmed by the finding that Abs that abrogate the function of membrane complement regulatory proteins (mCRP) such as CD46, CD55, and CD59 enhance the therapeutic effect of rituximab in animal models of the disease (2, 4, 19–24).

The complement system is the principal part of the innate immune system and plays an important role in host defense. To prevent the potentially harmful effect of complement activation on normal cells, some mCRPs including CD46, CD55, and CD59 have evolved to restrict complement activation at different stages of the complement cascades (9, 25). CD59, a glycosylphosphatidylinositol (GPI)-anchored mCRP, restricts formation of the membrane attack complex by preventing C9 polymerization through binding to C8 and C9 (26). CD55, another GPI-anchored mCRP, inactivates the C3 and C5 convertases by accelerating the decay of these

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proteases (27–29), whereas CD46, a non-GPI-anchored membrane protein, acts as a cofactor for inactivation of cell-bound C4b and C3b by serum factor I (30). Not only do these mCRPs protect normal cells from bystander complement attack but also they confer protection to cancer cells by limiting complement activation by a therapeutic Ab such as rituximab. Numerous findings indicate that CD59 is the most effective mCRP protecting B-cell lymphomas from rituximab-mediated CDC (2, 4, 21, 31). Dalle and colleagues have recently found that CD59, but neither CD46 nor CD55, is overexpressed in an *in vivo* model of rituximab-resistant (RR) follicular lymphoma (FL)-derived tumor cells isolated from a patient (32). Moreover, in a clinical study of chronic lymphocytic leukemia (CLL), Bannerji and colleagues found a significant increase in hCD59 expression in patients who failed to clear CLL cells from peripheral blood after initiation of rituximab treatment (33). Taken together, these results suggest that the overexpression of mCRPs, and especially CD59, contributes to the resistance of lymphoma and CLL cells to rituximab therapy (34, 35). For these reasons, the development of a molecule capable of abrogating CD59 function in cancer cells is likely to fulfill an unmet clinical need.

Recently, we have generated a specific high-affinity inhibitor of hCD59 denoted as rILYd4 (36), which is the recombinant 114 amino acid peptide representing domain 4 (D4) of intermedilysin (ILY), a cytolytic toxin secreted by *Streptococcus intermedius*. We have shown that rILYd4 binds to the hCD59 functional site and thereby abrogates hCD59 function (36). Here, we further show that rILYd4 sensitizes RR B-cell NHL and primary CLL cells to rituximab treatment *in vitro*, *ex vivo*, and *in vivo*. The results indicate that rILYd4 may provide a novel therapeutic approach as an adjuvant to rituximab for the treatment of lymphoma.

Materials and Methods

Additional information is available in Supplementary Materials and Methods.

Mice

Animal studies were approved by the Harvard Medical School Institutional Animal Care and Use Committee.

Balb/C nude mice used for the determination of rILYd4 *in vivo* efficacy were purchased from Charles River Laboratory. Mice specifically expressing hCD59 as a transgene in the erythrocytes of *mCd59a* and *mCd59b* double-knockout mice (*hCD59^{RBC+/-}/mCd59ab^{-/-}*) were used for pharmacokinetic/pharmacodynamic (PK/PD) and toxicity studies. *hCD59^{RBC+/-}/mCd59ab^{-/-}* were generated by crossing the *mCd59a* and *mCd59b* knockout mouse (*mCd59ab^{-/-}*; ref. 37) with a hCD59 transgenic mouse (*ThCD59^{RBC}*), in which hCD59 was specifically expressed under the control of the hemoglobin promoter at a level comparable with that seen in human erythrocytes (38).

B-lymphoma and primary CLL cells and reagents

Human Burkitt's B-cell lymphoma Ramos, Daudi, and Raji cells were purchased from American Type Culture Collec-

tion and cultured in the RPMI-1640 medium supplemented with 10% heat-inactivated FBS, 100 U/mL penicillin, and 100 µg/mL streptomycin. These cell lines were authenticated by the supplier, obtained within 6 months of their use, and passaged less than 50 times. We did not reauthenticate the cell lines.

The CLL patients had been previously enrolled on Dana-Farber Harvard Cancer Center (DFHCC) protocol 99-224. All participants signed informed consent prior to sample collection. The blood from CLL patients was then separated on a Ficoll gradient and peripheral blood mononuclear cells (PBMC) were frozen. The frozen PBMCs from 6 patients participating in this study (Supplementary Table 1) were cultured in IMDM medium (Invitrogen) supplemented with 10% human AB serum (GemCell; Gemini Co.), 50 µg/mL transferrin (Roche Applied Science), 5 µg/mL human insulin (Roche Applied Science), 100 U/mL penicillin, and 100 µg/mL streptomycin.

Intact ILY and rILYd4 tagged with HisX6 at its N-terminals were purified as described elsewhere (36, 38).

Generation of RR and hCD59-negative Ramos cell lines

We used a previously reported procedure (31) to develop Ramos cells resistant to CDC at different concentrations of rituximab. The cells generated were denoted as RR0.2, RR0.8, RR3.2, RR12.8, and RR51.2 because they could survive complement attack induced by rituximab (Biogen Idec) at concentrations of 0.2, 0.8, 3.2, 12.8, and 51.2 µg/mL, respectively, all in the presence of 10% normal human serum (NHS; Valley Biomedical). We followed the same procedures to generate rituximab-resistant NHL Daudi and Raji cells.

The hCD59-expressing subpopulations in all of the aforementioned Ramos cell lines were removed with repeated intact ILY treatment (5 µg/mL, on 3–5 occasions), and the surviving Ramos cell subpopulations were regarded as hCD59-negative cells, which was further confirmed by fluorescence-activated cell sorting (FACS) analysis.

Isolation of CD59-expressing population by cell sorting

Parental or original Ramos (OR) cells were stained with anti-hCD59 Ab and fluorescein isothiocyanate-conjugated secondary Ab. Cells staining positive for CD59 (0.38% of the total population) were collected in the first round by cell sorting (CS) and then grown in culture medium. When the cells reached 5×10^6 cells/mL density, a second round of CS was carried out to collect cells positive for CD59 (5% of the total population) for further experiments. Cells sorted through 2 rounds of selection of the CD59-positive population are termed CS cells.

Rituximab-mediated CDC on Ramos cells and primary CLL cells

Cell viability was determined by either Trypan blue or Alamar blue assay (4). A total of 5×10^4 Ramos cells per well were seeded on 96-well plates. Rituximab (10 µg/mL) and 10% NHS were added to the wells (total volume: 200 µL/well) in the absence and presence of different concentrations of rILYd4, and plates were incubated at 37°C. For the Trypan

blue assay, complement activation was stopped after a 1-hour incubation on ice, followed by staining with 0.04% Trypan blue. The dead cells identified by the blue staining were counted in a blinded fashion, and cytolysis was expressed as the fraction of dead cells out of the total number of cells. For the Alamar blue assay, cells were treated for 4 hours and then 30 μ L Alamar blue and 70 μ L culture medium were added to each well and incubated overnight. Cytolysis was assessed by reading the plates in an F-2000 fluorescence spectrophotometer (Hitachi; excitation: 560 nm; emission: 590 nm). The medium alone (without cells), NHS alone, and heat-inactivated human serum (IHS) alone were used as control for background in the relevant calculation. The positive control, considered 100% cytolytic, was Triton X-100 (0.1% in Dulbecco's PBS)-treated wells and negative control were cells without any treatment. Percent lysis in each well was calculated as follows: [(fluorescence in negative control well – fluorescence in test well)/fluorescence in negative control well] \times 100.

The primary CLL cells spontaneously die after culturing for several days. Thus, we had to carry out the experiments individually. Primary CLL cells were seeded on 96-well plates at 2×10^6 cells/mL. Different concentrations of rILYd4, 20 μ g/mL rituximab, and 20% NHS (total 200 μ L/well) were added to the corresponding wells and incubated at 37°C. After 3 hours of incubation, plates were placed on ice to stop complement activation, followed by staining with 0.04% Trypan blue. Blue stained dead cells were counted by 2 independent investigators, and cytolysis was estimated from the fraction of dead cells as described earlier.

Anticancer efficacy of rILYd4 *in vivo*

To ensure the resistance of the RR51.2 cell line, we treated RR51.2 cells with 51.2 μ g/mL rituximab and 10% NHS for 1 hour at 37°C before xenografting the cells into the nude mice. A total of 1×10^7 cells suspended in 100 μ L 50% Matrigel (BD Biosciences) diluted in serum-free culture medium were implanted subcutaneously on the left flank of each Balb/C nude mouse. An early development model (the mice had no measurable tumor) or an established tumor model (the mice had an average 0.15 g tumor) was used to evaluate the *in vivo* efficacy of rILYd4. After grafting, treatment was carried out at day 6 in the development model and, at day 18, in the established tumor model. Rituximab (2 mg/kg) without or with rILYd4 (2 mg/kg) was injected intraperitoneally (i.p.) on 3 occasions 4 days apart (Q4D). Tumors size was measured with calipers and tumor mass was calculated by the following formula: (width)² \times length/2 (39, 40). Tumor-free rate in the established tumor model was expressed as the fraction of the number of the mice without the tumor at 35 days after the treatment out of the total number of mice with the tumor before the treatment.

Data analysis

The difference between means of paired samples on primary CLL cells was evaluated by Wilcoxon's signed-rank test.

In the other experiments, the comparison between 2 or 3 groups was examined with a nonparametric Mann–Whitney test. A value of $P < 0.05$ was considered significant.

Results

rILYd4 sensitizes RR cells to a rituximab-mediated CDC effect *in vitro*

We generated 5 RR Ramos cell lines and showed that the increase in CD59 expression levels correlated with the degree of resistance to rituximab-mediated CDC (Fig. 1A and B). In contrast, the level of CD20 expression was not altered by the selection of rituximab resistance (Supplementary Fig. 1). These results confirmed functionally the generation of RR cells.

Intact ILY binds exclusively to hCD59 and rapidly lyses hCD59-expressing cells (38, 41). Repetitive exposure of OR and RR Ramos cells to ILY was used to enrich for the subpopulation of CD59-negative cells. Elimination of CD59-expressing cells was verified by FACS analysis (Fig. 1C). All these hCD59-negative cells were very sensitive to rituximab-mediated CDC, as shown by the complete lysis obtained even with the lowest dose of rituximab (0.2 μ g/mL; Fig. 1D). Taken together, these *in vitro* results indicate that the CD59-expressing subpopulation in Ramos cells may be responsible for the resistance to rituximab-mediated CDC. This interpretation provides a strong rationale for the utilization of rILYd4 as an adjuvant in rituximab therapy: rILY4 would sensitize RR cells by abrogating hCD59 function and thereby enhancing rituximab-mediated CDC.

The effectiveness of this approach was tested using RR51.2 cells, which express the highest level of CD59 and are resistant to CDC effect mediated by rituximab at the highest concentration (51.2 μ g/mL). The results showed that rILYd4 increased rituximab-mediated CDC in a dose-dependent manner (ED_{50} of rILYd4 = 0.614 μ g/mL or 33 nmol/L; Fig. 2A and Supplementary Fig. 2A). Treatment of the same cells with escalating concentrations of rILYd4 in the absence of a complement source (either no addition of NHS or the addition of inactivated HS) did not induce cell lysis (Supplementary Fig. 2B). This result rules out a direct toxic effect of rILYd4 and confirms the complement-dependent nature of the rituximab-mediated cytolysis (Supplementary Fig. 2B).

rILYd4 sensitizes primary CLL cells to rituximab-mediated CDC *ex vivo*

The clinical relevance of the aforementioned findings was shown by the capacity of rILYd4 to sensitize primary CLL cells to rituximab-mediated CDC effects *ex vivo*. The selection of primary CLL cells instead of B-cell NHL was dictated by their greater accessibility. Treatment of CLL primary cells obtained from 6 different patients (Supplementary Table 1) with escalating doses of rILYd4 resulted in a dose-dependent increase in the rituximab-mediated CDC effect in all the samples (Fig. 2B and C). Determination of the levels of CD20 and CD59 on the surface of the CLL cells from each patient by FACS analysis showed that the cells from subject CLL-1 that express CD20 at the highest level are also the most sensitive to the induction of rituximab-mediated CDC by rILYd4 (Fig. 2D and

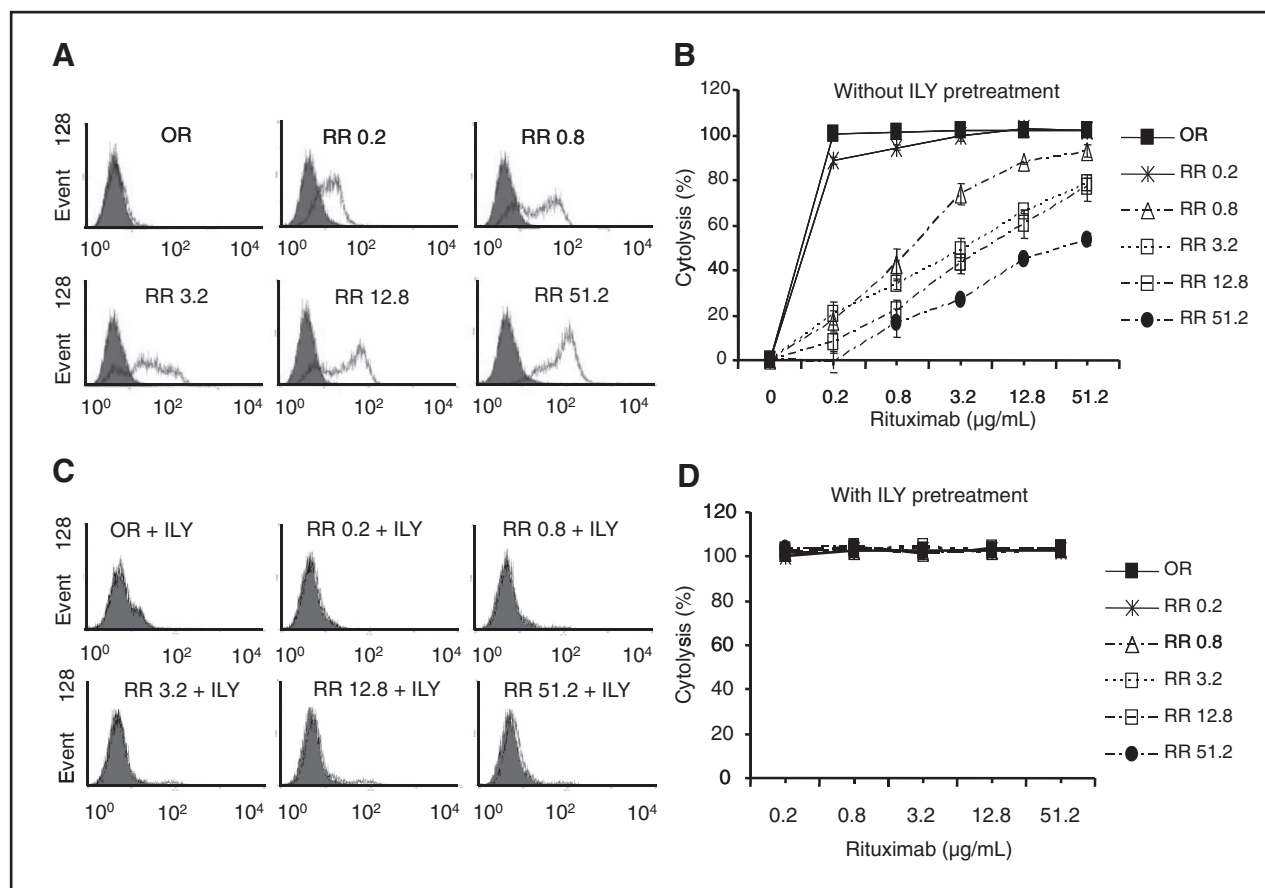


Figure 1. Expression of CD59 on OR and RR cells with or without ILY pretreatment and their response to rituximab-mediated CDC *in vitro*. A, physical detection of CD59 expression on OR and RR cells by FACS. Cells were stained with anti-hCD59 (black lines) or isotype-matched Ab (solid gray lines). B, functional confirmation of RR cell lines in CDC treatment ($n = 4$). C, physical absence of hCD59 expression on ILY-pretreated OR and RR cells. D, the hCD59-negative cells enriched by ILY pretreatment (5 μg/mL for 5 times) are very sensitive to rituximab-mediated CDC. Results are presented as mean \pm SEM of 4 independent Alamar blue assays.

Supplementary Fig. 3). This observation suggests that CD20 level may be associated with the sensitivity to rILYd4 treatment. Taken together, these results indicate that rILYd4 sensitizes RR NHL and CLL cells to rituximab-mediated CDC *in vitro* and *ex vivo*, respectively.

Potential mechanism of upregulation of CD59 in RR Ramos cells

Here, we document that the levels of GPI-anchored proteins such as mCRP CD59 (Fig. 1) and CD55, as well as non-mCRP CD48, but not non-GPI-anchored proteins such as CD20 and mCRP CD46 gradually increased on the surface of these RR Ramos cells (Supplementary Fig. 1). These results are consistent with the findings as reported previously by Takei and colleagues (31). More interestingly, after removing the CD59-expressing subpopulation with ILY pretreatment, the expression of the other GPI-anchored proteins CD55 and CD48 disappeared simultaneously in the residual CD59-negative subpopulation whereas the expression of non-GPI-anchored proteins such as CD20 and CD46 remained unchanged (Fig. 1C and Supplementary

Fig. 4). To investigate the potential mechanism of the upregulation of CD59 in RR Ramos cells, we used a CS method to enrich and characterize the CD59-expressing population from OR cells. The enriched CD59-positive cell population expressed CD20, CD46, CD59, CD55, and CD48 (Fig. 3A) at a similar level and had the same sensitivity to rituximab-mediated CDC as the RR51.2 cells (Figs. 1A, 2B, and 3C and Supplementary Fig. 1). In addition, rILYd4 also sensitized CD59-enriched CS cells to rituximab-mediated CDC in a dose-dependent manner (Fig. 3D). Furthermore, the CD59-negative subpopulation obtained from ILY-treated CS cells (Fig. 3B) was sensitive to rituximab-mediated CDC (Fig. 3E). Taken together, these results indicate that increased detection of GPI-anchored proteins in CD59-positive Ramos cells by multiple challenges with rituximab and complement may result from serial enrichment of the CD59-positive cells but not from induction of CD59 expression. Therefore, the elimination of the CD59-expressing subpopulation by combination treatment with rituximab and CD59 inhibitor such as rILYd4 may be a powerful approach to conquer rituximab resistance.

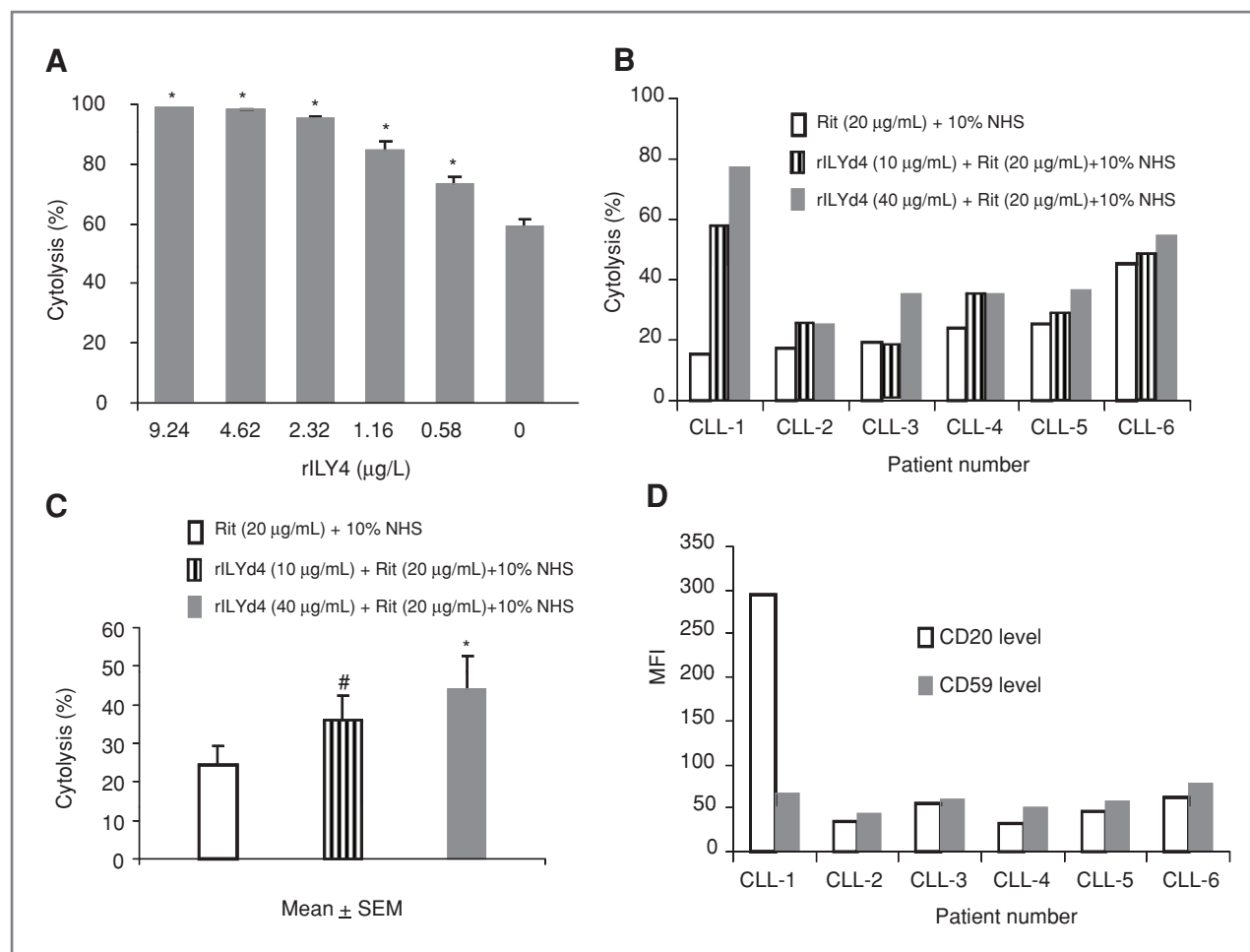


Figure 2. rILYd4 is an effective adjuvant for rituximab against RR cells or CLL cells. A, rILYd4 sensitizes RR51.2 cells to rituximab (Rit; 10 μg/mL)-mediated CDC effects *in vitro* as measured by Alamar blue assay. *, $P < 0.05$ versus no rILYd4 treatment. Data represent mean \pm SEM from 4 experiments. B, rILYd4 sensitizes CLL cells to rituximab-mediated CDC effects *ex vivo* and (C) in a dose-dependent manner. D, expression levels of CD20 and CD59 were detected by FACS analysis. #, $P < 0.05$ versus no rILYd4 treatment. *, $P < 0.05$ versus no rILYd4 treatment cells. Results are presented as mean \pm SEM from 6 patients (CLL-1–CLL-6). MFI, mean fluorescent intensity.

rILYd4 sensitizes RR51.2 cells to rituximab-mediated CDC effect *in vivo*

Next, we used both developing and established orthotopic xenograft models to investigate whether rILYd4 sensitizes RR Ramos cells to rituximab treatment *in vivo*. We implanted RR51.2 cells into nude mice following published protocols (39, 40). In the developing xenograft model, we observed that combination treatment with rituximab and the adjuvant rILYd4 dramatically slowed down tumor growth as compared with rituximab alone (Fig. 4A). Importantly, treatment with only rILYd4 did not affect tumor growth significantly (Fig. 4A). A similar observation was made in the established xenograft model, in which combination treatment with rituximab and the adjuvant rILYd4 resulted in significant reduction in tumor size as compared with treatment with rituximab alone (Fig. 4B). Furthermore, the tumor-free rates in the established xenograft model after treatment with vehicle, rILYd4 alone, rituximab alone, and combination of rituximab with rILYd4

were 0%, 0%, 8.3%, and 50%, respectively, suggesting that treatment with rILYd4 as an adjuvant can lead to elimination of tumor in 50% of the animals (Fig. 4C).

PK/PD profiles of rILYd4

The circulating half-life ($t_{1/2}$) and volume of distribution of rILYd4 were determined after intravenous (i.v.) tail vein injection of rILYd4 into $mCd59ab^{-/-}/ThCD59^{RBC+/-}$ mice. The serum level of rILYd4 showed a biphasic decay curve, with a fast initial distribution followed by a slower elimination phase. PK parameters are shown in Figure 5A. The half-life of rILYd4 *in vivo* was 2.71 hours, with a distribution volume of 383 mL/kg. The distribution of rILYd4 indicated by the V_{ss} (distribution volume) is likely due to the binding of rILYd4 to hCD59 on the erythrocytes of compound mice $mCd59ab^{-/-}/ThCD59^{RBC+/-}$.

PD study was carried out to evaluate the functionality of rILYd4. Theoretically, the rILYd4 bound to hCD59 should

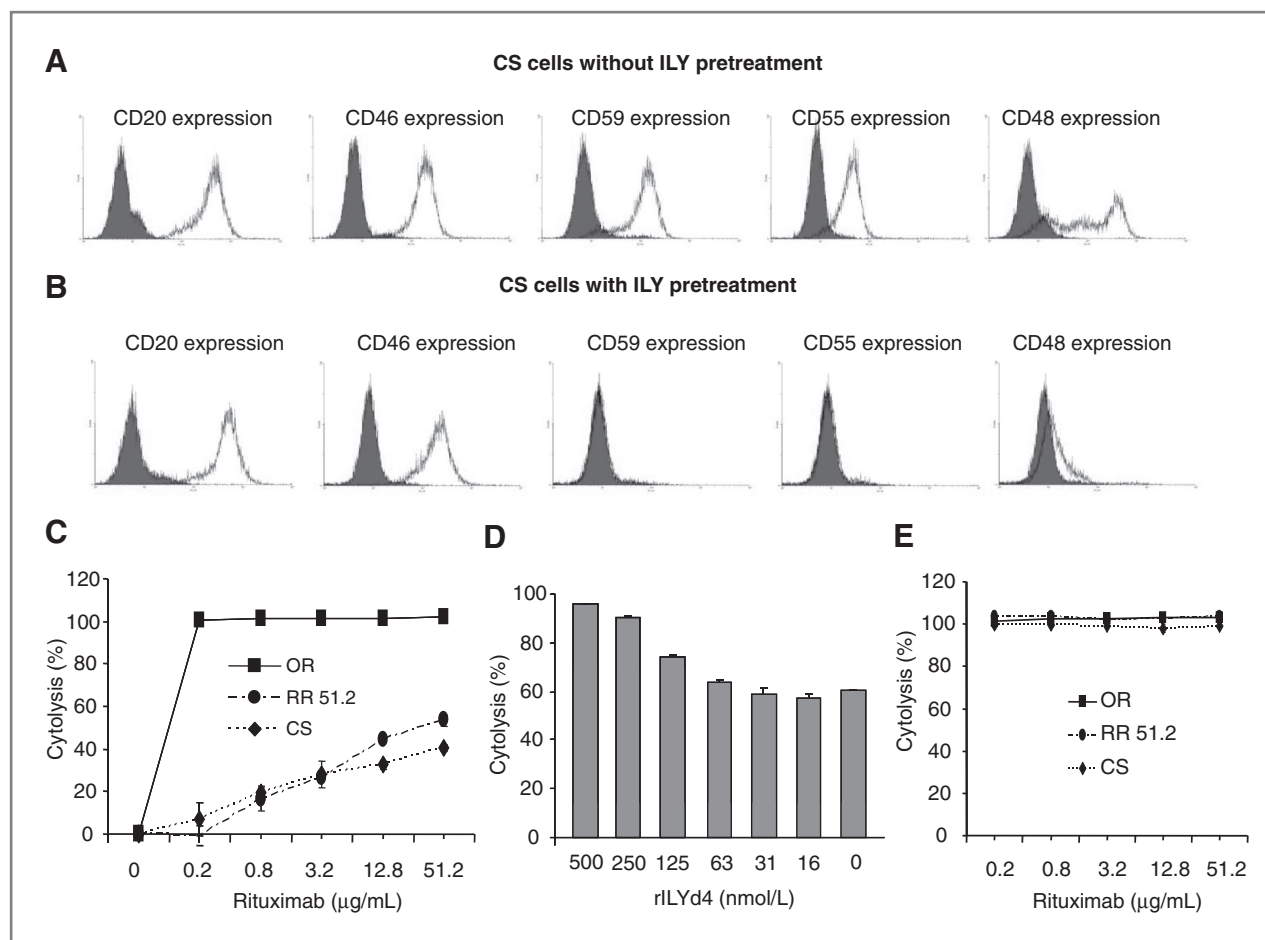


Figure 3. Expression of CD20, CD46, CD59, CD55, and CD48 on CD59-enriched CS cells with and without ILY pretreatment and their response to CDC effect induced by rituximab *in vitro*. FACS analysis on CS cells without ILY pretreatment (A) and with ILY pretreatment (B). Cells were stained with anti-CD20, CD46, CD59, CD55, and CD48 Ab (black lines) or isotype-matched Ab (solid gray lines). CS cells are resistant to CDC effect as measured by Alamar blue assay, which is similar to RR51.2 cells (C). rILY4 sensitizes CD59-expressing cells to the CDC effect of rituximab as measured by Alamar blue assay (D). The hCD59-negative cells enriched from CS cells by the ILY pretreatment are very sensitive to rituximab-mediated CDC (E). Results are mean \pm SEM of 4 different experiments.

inhibit the hemolysis induced by intact ILY (36). Erythrocytes collected at different time points following treatment with 10 mg/kg rILYd4 showed a clearly time-dependent decline in protection against ILY-induced hemolysis as measured by the concentration of ILY required for 50% hemolysis (Fig. 5B). Concomitant decay of rILYd4 function, as shown by the PD study, largely overlapped the decline in rILYd4 exposure in serum as determined in the PK study. Twenty-four hours after one 10 mg/kg i.v. dose of rILYd4, we could not detect any additional protection by rILYd4 against the hemolytic activity of ILY (Fig. 5B).

rILYd4 alone did not mediate hemolytic anemia in *mCd59ab^{-/-}/ThCD59^{RBC+/-}* mice

We have previously reported (36) and also confirmed in this study that rILYd4 alone has no direct lytic effect on hCD59-expressing cells *in vitro* (Supplementary Fig. 2B). To further assess its toxicity profile *in vivo*, we utilized *mCd59ab^{-/-}/ThCD59^{RBC+/-}* mice. In an acute toxicity study, we found

that there were no significant differences in the levels of hemoglobin content or free plasma hemoglobin (data not shown) between *mCd59ab^{-/-}/ThCD59^{RBC+/-}* mice treated with rILYd4 or vehicle (Fig. 6A). In the subchronic toxicity study with administration of rILYd4 for 1 month, rILYd4 did not result in any significant difference in hemoglobin levels, reticulocyte counts, and body weight or pathologic changes in tissues (data not shown) as compared with vehicle alone (Fig. 6B–D). These results suggest that transient inhibition of hCD59 function by rILYd4 does not induce hemolysis or any negative side effects and that rILYd4 is neither acutely nor subchronically toxic.

Discussion

Here, we show that the administration of rILYd4 abrogates hCD59 function in RR cells and restores the sensitivity of these resistant Ramos and primary CLL cells to rituximab-mediated CDC effects *in vitro*, *ex vivo*, and *in vivo*. We also document that

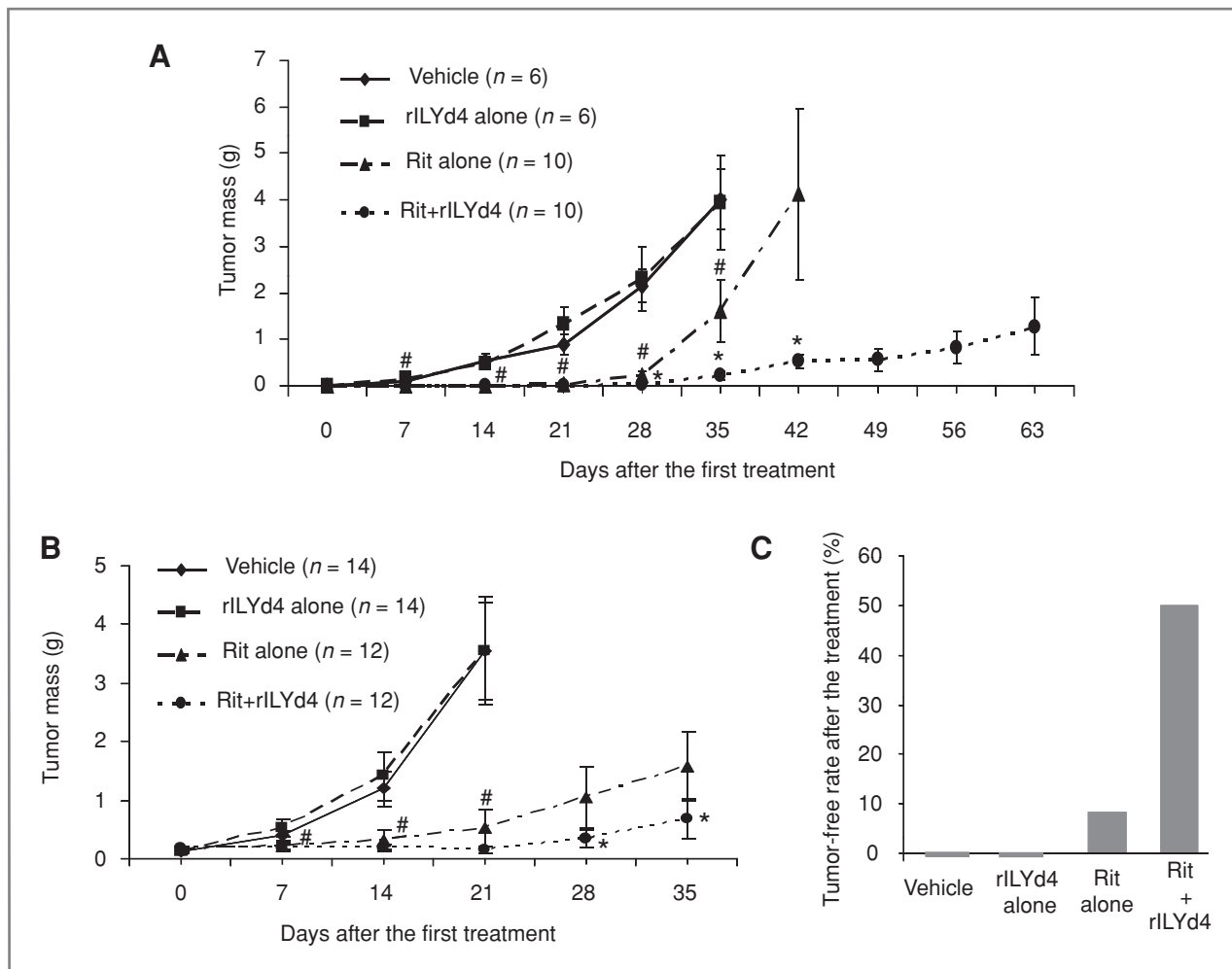


Figure 4. rILYd4 is an effective adjuvant against RR cells *in vivo*. A, a developing xenograft mouse model. B, an established xenograft mouse model. C, tumor-free rate in an established xenograft mouse model after treatment. #, $P < 0.05$ versus vehicle treatment. *, $P < 0.05$ versus rituximab (Rit)-alone treatment. Data represent mean \pm SEM.

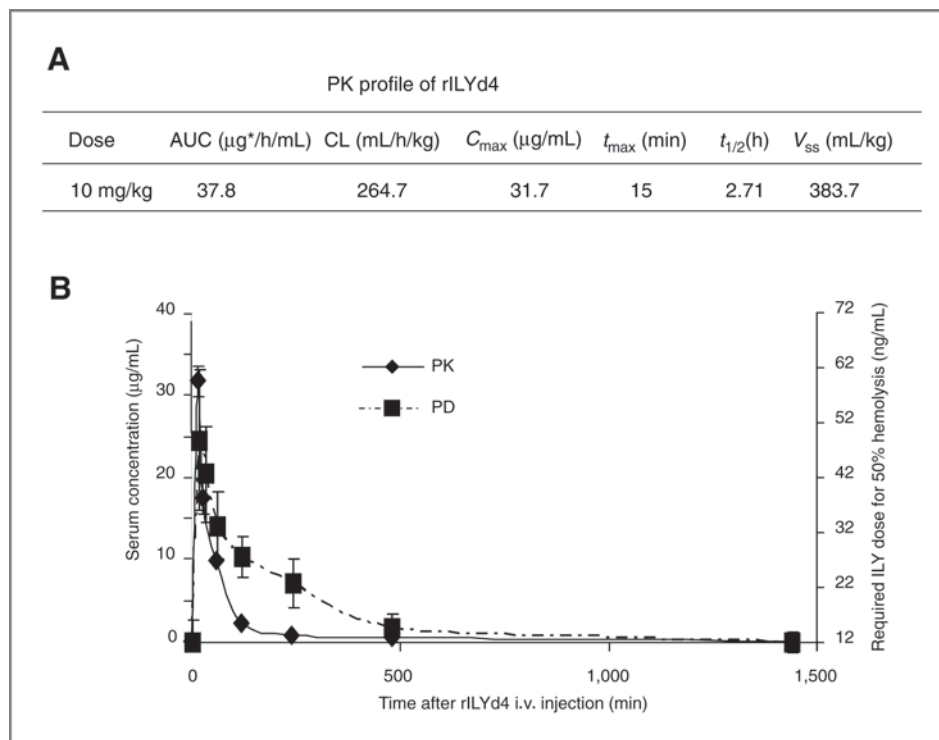
the hCD59-overexpressing population of Ramos cells is responsible for the resistance to rituximab-mediated CDC *in vitro*. These results highlight the critical role of CD59 in the development of rituximab resistance and indicate that rILYd4 may provide a new approach to enhance the therapeutic efficacy of rituximab by abrogating hCD59 activity. These interpretations are also supported by our recent findings that rILYd4 could sensitize the lymphoma cell line RL-7 or the multiple myeloma cell line ARH-7 to rituximab-mediated CDC (42).

The role of CDC in rituximab therapy was challenged previously by the findings that the expression of mCRPs does not predict clinical outcome after rituximab treatment in follicular NHL (43). Recently, Weiner's group reported that the C3b component of complement could inhibit NK cell activation and ADCC effects during rituximab treatment (10) and thus C3 depletion improves rituximab antitumor activity (20). Also, several researches have shown that both CDC and ADCC play major roles in the antitumor activity of rituximab

(2, 8–10). Furthermore, CDC-resistant cells are sensitive to ADCC, and vice versa (9, 14, 44). Differences in the relative importance of CDC and ADCC following rituximab treatment may result from the different types of tumors used, expression levels of CD20 and mCRP, tumor-inoculating methods, and tumor growth period (9, 11). Although the investigation of the relative roles of CDC and ADCC is beyond the scope of our studies, it will be very helpful for drug design in future and therefore warrants further investigation.

Since CD59 is universally expressed in human cells with a relative high level in erythrocytes, potential side effects such as hemolysis are the potential hurdles for the development of clinically useful hCD59 inhibitors. Monoclonal antibodies (mAb) directed against hCD59 are useful tools for the study of CD59 function *in vitro* (4, 19, 31). Despite that it is impossible to apply them to cancer therapy, as they bind not only cancer cells but also many other cells that express hCD59, potentially inducing ADCC, or CDC, or apoptosis in normal cells. To address this problem, Ziller and colleagues identified

Figure 5. PK and PD profiles of rILYd4. A, PK profile of rILYd4. AUC, area under the curve; CL, clearance; C_{max} , maximum concentration of a compound in blood; t_{max} , time at which C_{max} is reached; $t_{1/2}$, time required for the concentration of a compound in blood or plasma to decrease by 50%; and V_{ss} : distribution volume. B, correlation between the PK and PD profiles of rILYd4. Data represent mean \pm SEM.



mini Abs that specifically blocked hCD59 and hCD55 function (23). They reported that these mini Abs facilitated rituximab-mediated lymphoma treatment *in vivo*; however, although alone they failed to activate complement, they killed about 25% of cancer cells through ADCC (24). Therefore, to limit this unwanted side effect on normal cells and improve specific targeting of cancer cells, they administered biotin-labeled rituximab in combination with avidin-labeled MB55-MB59 to target the mini anti-CD59 Abs to the cancer cells (24). In contrast to this approach, rILYd4 restores sensitivity of the RR lymphoma cells to rituximab treatment *in vitro* and *in vivo*. Moreover, rILYd4 itself does not trigger lysis (36) or ADCC effect in cells that are nontargeted by the Ab (data not shown) *ex vivo* and *in vivo*. Therefore, rILYd4 may be a safe and effective adjuvant for rituximab therapy.

The higher expression of CD59 in cancer cells such as lymphoma and rituximab-pretreated lymphoma cells has been long recognized, but its mechanism remains unclear (32, 33). Our results indicate that RR Ramos cells overexpressing GPI-anchored proteins including CD59, CD55, and CD48 may result from the positive selection of preexisting cells that highly express GPI-anchored proteins rather than from the induction of GPI-anchored proteins. We therefore postulate that tumor heterogeneity may be responsible for the increase in the population of highly expressing CD59 lymphoma cells, a contributor to rituximab resistance. In support of this hypothesis, we show that two other NHL cell lines (Raji and Daudi) resistant to rituximab-mediated CDC also express a higher level of CD59 than their parental cell lines (Supplementary Fig. 5). Together, these results indicate that the ablation of

those preexisting resistant tumor cells by early treatment with combination of rILYd4 and rituximab may effectively limit the expansion of RR lymphoma cells through the abrogation of this subpopulation of cells expressing high levels of hCD59. Furthermore, rILYd4 may also sensitize tumor cells that have acquired resistance to rituximab after multiple therapies administered for the anticancer activity of rituximab.

We also designed and executed a PK/PD study to evaluate drug exposure and other kinetic parameters such as $t_{1/2}$, C_{max} , and volume of distribution in $mCd59ab^{-/-}/ThCD59^{RBC+/-}$ mice. The avid binding of rILY4 to hCD59 most likely explains its larger volume of distribution ($V_{ss} = 383$ mL/kg). For the PD study, we took advantage of the fact that the preformed rILYd4-hCD59 interaction on erythrocytes could compete with binding of the full-length ILY to hCD59 and exert protection against ILY-mediated hemolysis (36). The comparable PK and PD profiles suggest that rILYd4 bound to erythrocytes remains functional throughout the *in vivo* exposure. These data provide insight and guidance for the further engineering of rILYd4 to better suit biological therapy.

The toxicity profile is critical for identifying a dosing window to get good efficacy with tolerable side effects. Here, we show that i.p. injection of 3-fold the effective dose (6 mg/kg, Q4D \times 8 times) of rILYd4 did not induce erythrocyte lysis in hCD59 transgenic mice in a mCd59-deficient background. No other notable pathologic changes have been observed either. Overall, at the doses tested, rILYd4 did not show any sign of unwanted side effects. It remains to be seen whether other toxic effects emerge upon reaching the maximum tolerated dose in mice. Immunogenicity is another critical aspect for the

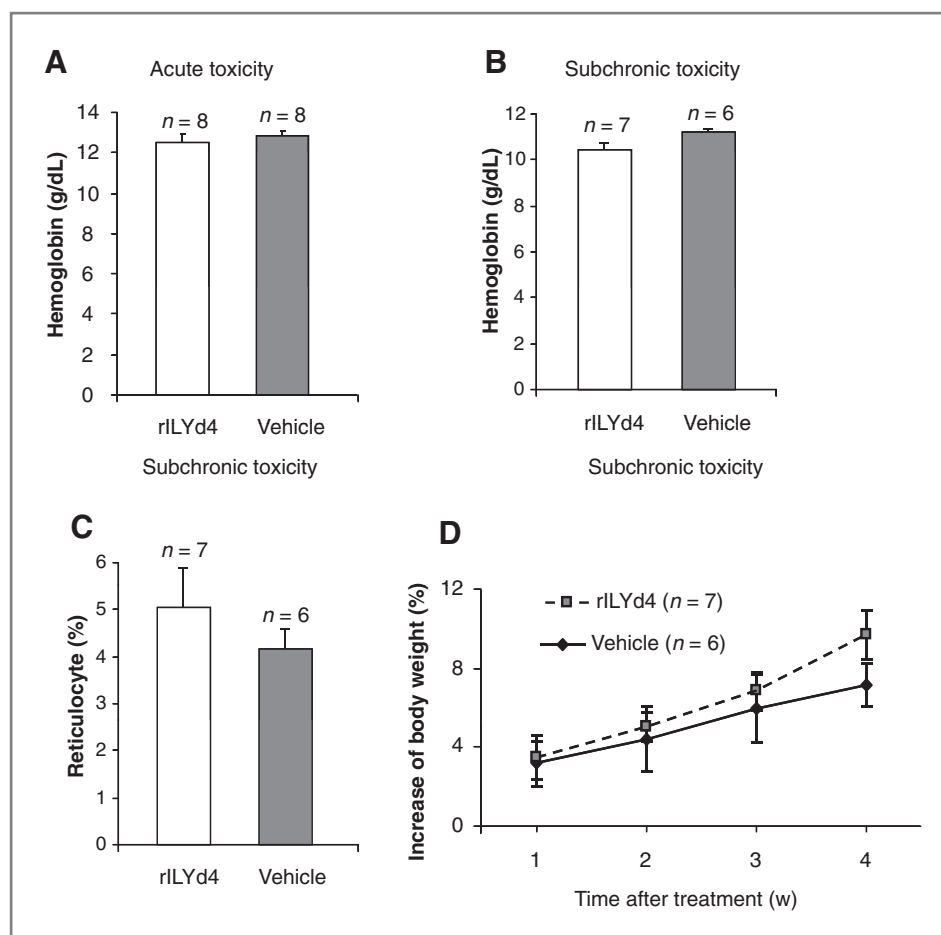


Figure 6. rILYd4 does not mediate acute or subchronic toxicity in *mCd59ab^{-/-}/ThCD59^{RBC+/-}* mice. A, hemoglobin content in acute toxicity study. B–D, hemoglobin content (B), reticulocyte count (C), and body weight (D) in subchronic toxicity study. Results are presented as mean \pm SEM.

effective development of a protein drug. Previous findings indicate that rILYd4 has low immunogenicity (45, 46). However, a low- or even nonimmunogenic form of rILYd4 or ILYd4-derived peptides will be essential for clinical application and requires further investigation and development.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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