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Targeting and depletion of circulating leukocytes and cancer cells by lipophilic antibody-modified erythrocytes

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Abstract

There is a great interest in targeting and selective ablation of populations of circulating cells for research or therapeutic purposes. Red blood cells (RBCs) are readily available and fully biocompatible long-circulating intravascular carriers (natural life is 120 days) that are amenable to chemical modifications, drug loading and reinjection. Here we demonstrate that using our previously described lipophilic ligand painting strategy, red blood cells (RBCs) could be in one step converted into targeted entities that selectively seek and bind various cells *in vitro* and *in vivo*. *In vitro*, RBCs modified with lipophilic anti-EpCAM or anti-CD45 antibodies efficiently bound to cancer cells and leukocytes, forming characteristic rosettes. *In vivo*, intravenously injected RBCs painted with anti-CD45 antibody immediately associated with CD45 positive cells in blood, forming RBC-leukocyte rosettes. Moreover, anti-CD45-modified RBCs, but not the same amount of anti-CD45 antibody or anti-CD45-lipid conjugate (1–2 µg/mouse), depleted over 50% of CD45+ leukocytes from circulation, with main clearance organs of leukocytes being liver and spleen with no visible deposition in kidneys and lungs. Anti-CD20 (Rituximab)-painted RBCs efficiently (over 90%) depleted CD19+/CD20+/CD45+ human lymphoma cells in mantle cell lymphoma (MCL) JeKo-1 model, while the same amount of rituximab-lipid (2 µg/mouse) was much less efficient in lymphoma cell depletion. Treatment of MCL mice with rituximab-modified RBCs carrying only 2 µg of the antibody resulted in a significant prolongation of survival as compared to the same amount of antibody-lipid control. Lipophilic ligand-painted RBCs is a novel tool that can be utilized for targeting blood borne cells for experimental immunology and drug delivery applications.

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Introduction

Selective ablation of cell populations in living subjects is one of the most useful tools in experimental immunology and clinical medicine. The existing strategy for cell depletion is based on injection of a specific antibody against cell surface markers intravenously or intraperitoneally.¹ The binding of antibody triggers the clearance of cells through enhanced macrophage recognition via Fc γ receptors, complement lysis and cytotoxic T-cell response.² One of the great examples of clinical use of antibodies for cell depletion is rituximab, a monoclonal antibody against CD20 antigen on the surface of B-lymphoma cells.² However, antibody-mediated depletion has several disadvantages, including systemic toxicity and high cost of treatment. In some cases, antibody therapy fails to reduce the counts of tumor cells in patients. Thus, some proportion of patients with multiple myeloma, mantle lymphoma and chronic lymphocytic leukemia develops resistance to Rituximab³, possibly due to the expression of complement inhibitors and/or activation of antiapoptotic pathways^{4,5}. In addition, use of systemically injected antibodies is problematic when only the circulating pool of cells needs to be targeted. For instance, antibody against epithelial cell adhesion molecule (EpCAM), albeit effective against circulating metastatic cells in mice,⁶ showed significant systemic toxicity in clinical trials.⁷

Red blood cells (RBCs) are readily available and fully biocompatible long-circulating carriers (natural life is 120 days) that are amenable to chemical modifications and drug loading.^{8,9} Previously, biomolecules and immunoconjugates were successfully coupled to RBCs,^{10–12} and efficient targeting to collagen surfaces *in vitro* and to endothelial cells *in vivo* was demonstrated.^{11–13} Our group previously demonstrated a versatile approach for painting RBC membrane with antibodies and small ligands via distearoyl anchors.¹⁴ This painting strategy allows very fast (15–30 min incubation) and efficient (up to 30,000 ligand molecules per RBC) incorporation. Depending on the amount of surface antibody, ligand painted RBCs can circulate in blood for several days.¹⁴ We wondered whether RBCs painted with targeting antibodies would bind and deplete blood borne cells, akin to previously described capture of circulating pathogens by antibody modified RBCs.^{15–17} Here we prepared and tested antibody painted RBCs targeted to blood borne cells following injection *in vivo*. We demonstrate that antibody painted RBCs efficiently and specifically bind to target cells *in vitro* and *in vivo*, deplete cells from circulation and induce therapeutic effect in the mouse model of lymphoma. The data have direct implications for drug delivery and therapy of blood-borne diseases using RBCs as drug carriers.

Results

In order to prepare lipophilic antibodies for incorporation into the RBC membrane, thiolated IgG (see Methods) was conjugated at 1:1 ratio to DSPE-PEG₃₄₀₀-maleimide as described before.¹⁴

Two different strategies were used to immobilize a targeting antibody on the RBC surface. Whenever the targeting antibody was readily available, it was directly conjugated to the lipid anchor (Fig. 1A). DSPE-PEG₃₄₀₀-IgG construct was then incubated with washed mouse RBCs for 30 min at 37°C, resulting in incorporation of ~2 μ g IgG per 5×10^8 RBCs

(approximately 16,000 IgG/RBC). In cases when the targeting antibody was not available in large quantities and therefore not easily amenable to lipid conjugation, we synthesized DSPE-PEG₃₄₀₀-anti-Fc IgG construct, incorporated it in the RBC membrane, and then immobilized the targeting antibody (Fig. 1A). Modification of RBCs with this moderate amount of lipid-antibody construct did not result in a significant hemoglobin release or aggregation (Supplemental Fig. S1). The presence of the antibody was detected with a secondary fluorescent IgG (Fig. 1B). In addition to antibodies, lipophilic cyanine dye DiI was incorporated into the RBC membrane in order to enable independent tracking of RBCs *in vivo*.¹⁴ Two-antibody capture approach often resulted in a more efficient incorporation of the targeting antibodies in the RBC membrane as determined with flow cytometry (Fig. 1C). Stability of the lipid construct in the membrane as well as clearance of RBCs in circulation was determined as described by us before.¹⁴ Following intravenous injection of DSPE-PEG₃₄₀₀-IgG/DiI-RBCs (thereafter IgG/DiI-RBCs), double-stained RBC population was detectable in blood for at least 48 h (Fig. 2A).

DSPE-PEG₃₄₀₀-IgG construct gradually disappeared from the RBC surface with a terminal half-life of 610 min (Fig. 2B), while RBCs painted with ~16,000 copies of IgG (one antibody) exhibited a elimination half-life of 670 min (Fig. 2C). Previously we found that the concentration of DSPE-PEG₃₄₀₀-IgG in the membrane affects the RBC half life (possibly due to the presence of PEG¹⁸); empirically, 16,000 IgG molecules per RBCs is a trade-off between RBC circulation half-life and the avidity for the target cells.

Targeting of ligand-painted RBCs to cells in vitro

First, we tested the ability of IgG/DiI-RBCs to bind to cells *in vitro*. Since RBCs are mainly constrained to the blood compartment, we used antibodies against intravascular targets. Thus, advanced stages of metastatic growth are characterized by large numbers of EpCAM-positive circulating tumor cells (CTC) in blood¹⁹ and disseminated tumor cells (DTC) in bone marrow.²⁰ In order to target RBCs to EpCAM+ cancer cells, we prepared anti-mouse EpCAM/DiI-RBCs using two-antibody painting approach (Fig. 1A). 4T1 mouse breast carcinoma cells (suspended in cell medium) were mixed with EpCAM-targeted RBCs (RBC/tumor cell ratio of 10:1) for 60 min. 4T1 cells formed characteristic rosettes with anti-EpCAM/DiI-RBCs, but not with control DiI-RBCs (Fig. 2A, Supplemental Fig. S2A). Interestingly, many RBCs exhibited shape distortion after attaching to tumor cell surface (Supplemental Fig. S2B). According to FACS analysis, over 95% of tumor cells had a shift in FL-2 channel fluorescence after incubation with anti-EpCAM/DiI-RBCs, but not with DiI/RBCs (Fig. 3B), suggesting that targeted RBCs specifically bind to tumor cells and there is no significant DiI transfer from non-targeted RBCs. Anti-EpCAM/DiI-RBCs also efficiently bound to adherent 4T1 cells (Supplemental Fig. S3).

In order to test the binding to 4T1 cells in blood and to confirm the specificity of the binding, we prepared a 1:1 mixture of anti-EpCAM/DiI-RBCs and IgG/DiO-RBCs and added it to mouse blood spiked with 4T1 cells (10,000 tumor cells/ml blood, RBC/tumor cell ratio 10:1). Following 60 min incubation, there was an efficient binding of targeted, DiI-labeled RBCs but not of non-targeted, DiO-labeled RBCs (Fig. 3C). In addition to 4T1 cells, we also tested the binding of anti-human EpCAM-RBCs to MDA-MB 231 human breast

carcinoma cells and PC-3 human prostate cancer cells in suspension. In this case, over 90% of tumor cells formed rosettes with targeted RBCs (Supplemental Fig. S4).

Next, we tested the binding of RBCs to leukocytes *in vitro*. Antibody against pan-leukocyte marker CD45²¹ was modified with DSPE-PEG₃₄₀₀ and directly incorporated into RBCs. In order to test the binding to leukocytes, anti-mouse CD45/DiI-RBCs or control DiI-RBCs were added to whole mouse blood at a ratio of 1:10 (targeted RBC/normal RBC) and incubated for 30 min. Following incubation, leukocytes were stained with FITC-anti-mouse CD45. We observed the presence of characteristic rosettes between anti-CD45/DiI-RBCs and CD45+ leukocytes (Fig. 3D, left), whereas no rosettes were observed in the control RBC sample (Fig. 3D, right). Flow cytometry analysis of blood mixed with targeted RBCs showed the presence of double stained population of RBC-leukocyte rosettes (Fig. 3E, left), whereas control, non-targeted RBCs did not show presence of a double stained population even when added to blood at 1:1 ratio (Fig. 3E, right). These data suggest that there is a specific binding of anti-CD45/DiI-RBCs to leukocytes in blood. Despite the fact that we stained the cells with the same antibody as the one we used for targeting, we were able to get robust cell staining in presence of bound RBCs, suggesting that not all CD45 antigens are “masked”.

Targeting of RBCs to circulating cells in vivo

In order to test whether targeted RBCs bind to circulating leukocytes *in vivo*, we injected 5×10^8 anti-CD45/DiI-RBC (total antibody approximately 2 μ g) into normal BALB/c mice (5–7% of total RBCs). Microscopic examination of FITC-anti-CD45 stained blood sample withdrawn 1 min post-injection revealed the presence of rosettes between leukocytes and targeted RBCs (Fig. 4A, **upper panel**).

Flow cytometry analysis of blood samples at 1 min post-injection showed that 65% of CD45+ cells became associated with anti-CD45/DiI RBCs (Fig. 4A, **middle panel**) as compared to non-injected mice (Fig. 4A, upper panel left). At 12 h post-injection, there was >50% decrease in the number of CD45+ cells (Fig. 4A, middle panel). Injection of 2 μ g of DSPE-PEG₃₄₀₀-anti-CD45 did not result in a significant decrease in the number of CD45+ cells at 12 h (Fig. 4A, lower panel). Injection of normal RBCs also did not result in cell depletion (Supplemental Fig. S5). Next, we measured the kinetics of depletion of CD45+ cells at 1 h, 12 h and 24 h using anti-CD45/DiI RBCs, anti-CD45 antibody or DSPE-PEG₃₄₀₀-anti-CD45. According to Fig. 4B, targeted RBCs depleted over 50% of cells at 1 h, and the depletion persisted at 24 h post-injection (albeit the levels were variable among mice). On the other hand, 2 μ g of anti-CD45 antibody (Fig. 4B, black line) and DSPE-PEG₃₄₀₀-anti-CD45 (Fig. 4B, blue line) did not produce a significant depletion of CD45+cells, and at 24 h the levels returned to the baseline.

In order to trace the fate of DSPE-PEG₃₄₀₀-anti-CD45 construct, we performed immunostaining of the liver, spleen, lungs and kidneys with secondary fluorescent antibody against rat anti-mouse CD45 (Fig. 5).

The livers of mice injected with anti-CD45/DiI-RBCs showed localization of anti-CD45 antibody on the surface of endothelial cells, Kupffer cells and also on leukocytes (Fig. 5A,

white arrow), confirming our previous finding that some of the lipophilic antibody detaches from RBCs *in vivo*.¹⁴ The liver also contained rosettes formed between anti-CD45 IgG-stained cells and DiI labeled RBCs (Fig. 5A, yellow arrow). There were a few detectable rosettes in the spleen (Fig. 5A, yellow arrow), and no detectable rosettes in the kidneys and the lungs (Fig. 5A, Supplemental Fig. S6). Remarkably, we observed significant deposition of anti-CD45 antibody on the surface of cells in the liver and spleen (Fig. 5A, white arrows). In mice injected with DSPE-PEG₃₄₀₀-anti-CD45 construct (without RBCs), the anti-CD45 antibody was deposited on endothelial cells and Kupffer cells in the liver, and on pulp cells in the spleen (Fig. 5B, white arrows). Notably, the deposition of anti-CD45 Ab in the spleen was much less widespread than in the spleen of anti-CD45/DiI-RBC injected mice. Others and us^{14,22} demonstrated that RBC-anchored ligands transfer to endothelial cells, mainly in the liver. In case of the spleen, it is possible that RBCs transfer their surface-associated DSPE-PEG₃₄₀₀-anti-CD45 following the contact with CD45+ pulp cells.

In cancers, large numbers of circulating tumor cells (CTCs) seed and disseminate the disease²³. A method to target and deplete circulating cells could bear significance on clinical oncology. Despite the fact that we demonstrated efficient binding of anti-EpCAM RBCs to epithelial tumor cell lines *in vitro*, it proved challenging for us to demonstrate the binding and depletion of epithelial CTCs by targeted RBCs *in vivo*, mainly because CTCs are extremely rare and require special methods for isolation and detection.²⁴ Therefore, we established *in vivo* model of mantle cell lymphoma JeKo-1²⁵ in SCID/NOD IL-2R gamma mouse background. In this model, intravenously injected lymphoma cells first populate the spleen and the bone marrow and within a few weeks appear in systemic circulation, in sufficient quantities to enable detection and quantification in blood with flow cytometry. Rituximab (anti-CD20) is a therapeutic antibody that is clinically approved for treatment of B-cell lymphomas.² To test the ability of RBCs to deplete JeKo-1 cells *in vivo*, we modified RBCs with DSPE-PEG₃₄₀₀-rituximab using one-antibody strategy (Fig. 1A). We injected 5×10^8 rituximab/DiI-RBCs (corresponding to approximately 2 μ g antibody) per mouse and monitored CD20+ cells in peripheral blood with flow cytometry. According to flow cytometry analysis (Fig. 6A), following the injection of rituximab/DiI-RBCs there was a visual depletion of circulating lymphoma cells at 12 h.

To confirm that RBC-mediated depletion is not due to the DSPE-PEG₃₄₀₀-rituximab that was detached from RBCs, we injected control mice with 2 μ g of DSPE-PEG₃₄₀₀-rituximab. The depletion at 12 h was much lower than with rituximab-RBCs (Fig. 6A). Kinetics of CD20+ cell depletion over time showed that both rituximab/DiI-RBCs and lipophilic rituximab decreased the numbers of CD20+ cells by 90% at 5 min post-injection (Fig. 6B). However, in the case of DSPE-PEG₃₄₀₀-rituximab the cell levels partially recovered at 24 h with 43% depletion as compared to 90% depletion by rituximab/DiI-RBCs (p-value 0.01). In order address a potential concern that the depletion rate could be overestimated due to “masking” of cell surface antigens by bound RBCs rather than due to the physical depletion, we also stained blood samples with anti-human CD45 and anti-human CD19 antibodies. According to flow cytometry analysis (Fig. 6C, D, respectively), at 12 h post-injection there were 10-fold less human CD19+ and CD45+ cells in rituximab/DiI-RBC injected mice than

in DSPE-PEG₃₄₀₀-rituximab injected mice, confirming that CD20-targeted RBCs depleted JeKo-1 lymphoma cells.

Finally, we tested whether depletion of circulating tumor cells by targeted RBCs can lead to a prolonged survival. Preliminary experiments suggested that binding of anti-mouse EpCAM-RBCs to 4T1 cells did not affect cell growth *in vitro* (Supplemental Fig. S6), and that injection of anti-mouse EpCAM-RBCs did not lead to a decrease in metastatic progression of 4T1 tumors (not shown). On the other hand, treatment of JeKo-1 lymphoma mice using rituximab-RBCs (3 times per week, 5×10^8 RBCs/mouse, 2 μ g antibody per injection) showed significant prolongation of mouse survival ($P < 0.001$) (Fig. 6E) as compared to DSPE-PEG₃₄₀₀-rituximab (2 μ g/mouse)-injected mice.

Discussion

In this work, we used our previously described lipophilic ligand painting strategy in order to convert RBCs into long circulating carriers that capture and deplete cells *in vitro* and *in vivo*. Previously, RBCs and RBC-derived particles have been used to deplete circulating antigens and toxins, respectively, *in vivo*^{15,27}, but to our knowledge this is the first demonstration of the specific approach of *in vivo* cell capture and ablation using targeted red blood cells. The impact of this work is in the following:

1. Lipophilic ligand painted RBCs can be used to target different populations of blood cells, including cancer cells, following injection into systemic circulation. With the advent of cell-based immunotherapy²⁸, stem cell therapy²⁹ and cell based imaging/drug delivery,⁸ there is a great interest in strategies for selective targeting of therapeutic cells to organs and tissues *in vivo*. For therapeutic applications, targeted RBCs can be designed to carry cytotoxic drugs or anti-inflammatory drugs; there are already examples of clinical use of dexamethasone phosphate-loaded RBCs for hormone replacement therapy,^{30,31} and RBCs were previously loaded with drugs, nucleic acids and enzymes.⁸ It is plausible to suggest that encapsulation of cytotoxic drugs inside RBCs and targeting them to tumors can improve safety and efficacy of anticancer therapies. Moreover, the strategy of lipophilic ligand painting could be applied for modification of other cell types for therapeutic applications. For instance, it could be interesting to test the surface painting strategy on NK cells and cytotoxic autologous T-cells in order to improve their ability to seek and destroy tumors^{32–34}.
2. To our knowledge, our data are the first demonstration of the therapeutic effect of antibody-painted RBCs in a mouse model of blood cancer. Despite high clinical efficacy of rituximab, significant proportion of lymphoma patients relapse and develop resistance to the antibody treatment^{4,5,35}, necessitating aggressive cytotoxic treatment (CHOP: cyclophosphamide, doxorubicin, vincristine, prednisolone³⁶). Rituximab-RBCs could be an interesting alternative to chemotherapy following lymphoma relapse. Interestingly, we did not observe any toxicity following injection of targeted RBCs and there was minimal (if any) lodgment of rosettes in the vital organs.

We observed significant differences in the binding and clearance of different cell types. While anti-EpCAM-RBCs bound to over 95% of 4T1 cells, the efficiency of binding of anti-CD45-RBCs to CD45+ cells was around 70%. In addition, the binding of anti-CD45-RBCs to CD45+ cells *in vivo* was lower than *in vitro*, and the percent of CD45+ cell depletion was lower than that of CD20+ cells. Such differences in binding and depletion efficiency could be explained by differences in antigen expression, cell size, RBC avidity, mixing conditions *in vitro* vs. *in vivo*. In addition, it is possible that under the shear force the antibody is pulled out from the RBC membrane³⁷ leading to disintegration of rosettes. Indeed, we observed numerous anti-CD45 antibody-coated, RBC-free cells in the liver and the spleen, suggesting that RBCs might become detached from the cells but leaving the antibody behind (“kiss and run”). Improving the retention of lipid-anchored antibody in the RBC membrane could potentially improve the binding and further improve the depletion efficiency.

The mechanism of depletion of cells by targeted RBCs merits further investigation. We found that RBC-leukocyte rosettes localize predominantly in the liver and spleen. While the exact mechanisms of clearance of the rosettes is not known, it is interesting that cell-attached RBCs undergo shape distortion (Fig. 2B). Liver Kupffer cells efficiently eliminate damaged RBCs through scavenger receptors.³⁸ Another interesting possibility is the enhanced recognition of RBC rosettes through immunoglobulin and complement receptors.³⁹ The mechanism of cell depletion by RBCs appears to be different from antibody-mediated depletion. Indeed, 2 µg of anti-CD45 antibody immobilized on RBCs was much more efficient in cell depletion than free anti-CD45 or DSPE-PEG₃₄₀₀-anti-CD45. Actually, lipid-antibody construct exerted a brief effect but the cell levels returned back to normal or even above the base line, suggesting the possibility that the cells are not ablated but reversibly sequestered. The depletion efficiency did not correlate with the differences in half-life between RBCs, lipid-antibody and free antibody. Thus, IgG painted RBCs have a half-life of about 10 h, while the half-life of DSPE-PEG-IgG is on the order of 5 h⁴⁰ and the half-life of free IgG is several days.^{41,42} Moreover, the main depletion occurred during the first minutes post-injection, which is less than half-life of the antibody variants tested in this work. Based on the observed differences in depletion efficiency, we suggest that the depletion is due to the specific interaction of antibody-coated RBCs with target cells rather than effect of dissociated antibody.

In conclusion, our data suggest that ligand painted RBCs present an exciting approach to immunodepletion of blood borne cells, including cancer cells, while requiring much less antibody. The lipophilic ligand painting approach could be very useful for modifications of RBCs and other cell types for selective targeting, tagging and depletion of cells.

Materials and Methods

Reagents

2-distearoyl-sn-glycero-3-phospho-ethanolamine-N-[maleimide (polyethylene glycol)-3400] (DSPE-PEG₃₄₀₀-Maleimide) was purchased from Laysan Bio, Inc. (Arab, AL, USA) and stored in chloroform as 3.4 mM solution. Traut's reagent (2-iminothiolane) was purchased from Thermo Fisher Scientific (Rockford, IL, USA). The reagent was dissolved in double-distilled water at 5 mg/ml and stored in aliquots at -20° C. AffiniPure Fc fragment specific

IgG was purchased from Jackson ImmunoResearch (West Grove, PA). Mouse anti-human and rat anti-mouse CD326 (EpCAM) antibodies were purchased from Bio Legend (San Diego, CA, USA). Rat anti-mouse anti-CD45 antibody was purchased from BioLegend (San Diego CA). 1,1'-dioctadecyl-3,3,3'-tetramethylindocarbocyanine perchlorate (DiI) was from Biotium (Hayward, CA). Anti-CD20 humanized antibody (Rituximab, Genentech) was obtained from UCSD Moores Cancer Center Pharmacy. AlexaFluor 488 goat anti-rat antibody and AlexaFluor 488 goat anti-human antibody were purchased from Life Technologies (Carlsbad, CA). FITC mouse anti-human CD19 (clone HIB19) antibody and FITC mouse anti-human CD45 (Clone H130) antibody were from BD Biosciences (La Jolla, CA). FITC labeled rat anti mouse CD45 antibody and FITC labeled rituximab antibody were synthesized in the laboratory.

Synthesis of lipophilic ligands

The synthesis was performed as described.¹⁴ Briefly, Traut's reagent was used to generate sulfhydryl groups on IgG molecules. The generation of sulfhydryl groups on the modified IgG was quantified using Ellman's Reagent (Pierce) based on the manufacturer's protocol. Generally, 40-fold of Traut's reagent (molar equivalent to IgG) resulted in 1–2 sulfhydryl groups per each IgG. DSPE-PEG-mal (1 mM in PBS, 4 μ l, molar ratio DSPE-PEG₃₄₀₀-maleimide:IgG = 1:1) were added to the desalted IgG solution and incubated at RT on a shaker. After 1h, the sample solution was filtered using a centrifugal filter device (Microcon YM-50, Millipore Co.) at 14000 g for 15 min at 4°C to remove the small molecules and suspended in 600 μ l PBS (1 mg/ml IgG). The purified DSPE-PEG-IgG was analyzed by matrix-assisted laser desorption/ionization-time-of-flight mass spectrometry (MALDI-TOF MS).

RBC painting

BALB/c mouse blood was used to prepare modified RBCs. Generally, 200 μ l of whole blood was suspended in 1000 μ l PBS and spun at 1500 g for 30 sec, repeated 4 times. Each time, buffy coat was carefully removed. Finally, RBCs were resuspended in 800 μ l PBS. The painting was performed by incubating the above RBC suspensions with DSPE-PEG₃₄₀₀-IgG construct (final concentration 20–40 μ g IgG/ml) for 15–30 min at 37°C. The mixture was kept for 5 min at room temperature, then washed 3–5 times in PBS and resuspended to a final RBC concentration of 5×10^8 cells/ml. Since anti-EpCAM antibody was not available in large quantities and therefore not easily amenable to lipid conjugation, we synthesized DSPE-PEG₃₄₀₀-anti-Fc IgG construct, incorporated it in the RBC membrane, and then immobilized anti-EpCAM antibody (Fig. 1A). In case of more available anti-CD45 IgG and anti-CD20 IgG, the antibody was directly conjugated to the lipid anchor and incorporated into RBCs (Fig. 1A). To prepare DiI-painted RBCs, the PBS-washed RBC suspension with or without IgG painting was incubated with 10 μ M DiI for 20 min at room temperature under 100 rpm. The mixture was washed with PBS for 3 times and resuspended in PBS.

In order to visualize the incorporated IgG in the RBC membrane, a corresponding Alexa Fluor® 488 secondary antibody (2 mg/ml, 2 μ l) was diluted in 200 μ l PBS and incubated with the same volume of RBCs (5×10^8 /ml in PBS) for 20 min at room temperature under

100 rpm, followed by washing and suspending in 200 μ l PBS. The stained RBCs were visualized by fluorescent microscope (Nikon Eclipse) on a glass slide. For flow cytometry analysis, blood (5.0×10^9 /ml cells in PBS, 10 μ l) was stained with secondary fluorescent antibody as described by us before,¹⁴ added to 1 ml of ice cold 0.5% BSA in PBS and the data were acquired with FACS Calibur (BD Biosciences, San Jose, CA). FlowJo software (Tree Star Inc., Ashland, OR) was used to process the flow cytometry data.

In vitro experiments

4T1 mouse breast cancer cell line was obtained from ATCC (CRL-2539) and maintained in high glucose DMEM supplemented with 10% fetal bovine serum (Mediatech), 1% L-glutamine, penicillin and streptomycin. JeKo-1 mantle cell lymphoma was obtained from the laboratory of Dr. Thomas Kipps, Moores Cancer Center UCSD and was grown in RPMI supplemented with 10% FBS, 1% pen-strep and 10 mM HEPES. For binding studies between 4T1 cells in suspension and painted RBCs, cells were detached with trypsin-free cell detaching solution (Life Technologies), washed once in full medium and resuspended in serum free DMEM. RBCs were added at 100:1 ratio and the cells were mixed at 20 rpm in eppendorf tubes. For binding to 4T1 cells grown in monolayers, cells were grown in slide chambers (NalgeNunc), washed with serum free RPMI and RBCs were added (0.5×10^8 RBC per 1.7 cm² area). Following 1h incubation, attachment of RBCs to cells was analyzed with fluorescent microscope and flow cytometry. For flow cytometry, at least 100,000 events were collected 4T1 cells and RBCs were gated based on the difference in forward and side scattering parameters. For leukocyte binding, RBCs were incubated with whole heparinated mouse blood (1:10 RBC ratio). Following incubation, blood wash washed 3 times at 3500rpm for 30 s in PBS and stained with FITC-labeled anti-CD45 antibody. Cell binding was analyzed with flow cytometry by collecting 500,000 total events.

In vivo experiments

All mouse experiments were carried out in a pathogen-free environment at the UCSD animal facility in accordance with Institutional Animal Care and Use Committee (IACUC)-approved protocol. For studies involving RBC clearance, eight- to twelve-week-old female BALB/c mice were used. Modified RBCs (equivalent to 50–100 μ l blood) were injected into the tail vein in a total volume of up to 100 μ l. Blood was collected at different time points starting at 5 min post-injection from the periorbital vein by heparinized capillaries (25 μ l volume each time). Ten microliters of blood was washed in PBS 3 times and stained with the fluorescent secondary antibody for flow cytometry analysis. The percentage of the remaining ligand and RBCs was determined using 5 min time point as 100%. Elimination curve of IgG was plotted using Prism (GraphPad) software, fitted whenever possible with bi-exponential decay equation and the ligand half-life was determined.

For *in vivo* depletion of CD45 positive cells, BALB/c mice (6–8 weeks old female) were treated with targeted RBCs, lipophilic antibody, non modified antibody, or non-modified RBCs (100 μ l of RBCs, 2 μ g IgG) by tail vein injection. Blood samples were collected at different time points and analyzed with flow cytometry as described above. For histological analysis of RBC and anti-CD45 distribution, mice were sacrificed 24 h post injection of rat anti-mouse CD45/DiI-RBCs, or DSPE-PEG₃₄₀₀-rat anti-mouse CD45. Mouse tissues (liver,

spleen, lung, and kidney) were collected, fixed in 4% paraformaldehyde overnight at 4°C, then equilibrated in 20% sucrose solution for 24 h and embedded in OCT. Tissues were cryosectioned and stained with DAPI and Alexa 488 goat anti-rat antibody.

For *in vivo* depletion of CD20+ human lymphoma cells, JeKo-1 xenograft mouse model of mantle cell lymphoma (MCL) was used. The JeKo-1 cell line was derived from a patient with blastoid variant MCL. JeKo-1 cells were cultured in RPMI 1640 medium (Invitrogen, Rockville, MD), supplemented with 100 U/ml penicillin, 2 mM L-glutamine, 100 µg/ml streptomycin, and 10% fetal bovine serum and were maintained at 37°C in a humidified atmosphere of 5% CO₂. Cell counts and viability were initially determined by trypan blue dye. Eight-to-twelve week old NSG/NOID-SCID IL-2R gamma mice were injected via the tail vein with 5×10⁶ JeKo-1 cells. *In vivo* depletion experiments were performed when CD20+ positive cell were readily detectable in peripheral blood (generally 2–3 weeks post-injection). CD20+ cell depletion experiments were performed exactly like CD45+ depletion experiments. For treatment studies, one week post-injection of JeKo-1 cells, treatment was initiated either with rituximab-RBCs or DSPE-PEG₃₄₀₀-rituximab, intravenously 2 doses per week for 3 weeks. Animals were monitored daily. Animals were euthanized if they exhibited reduction in body weight of 15% as a result of disease burden. The primary endpoint was survival defined as the time from engraftment to the development of the above defined clinical criteria leading to removal from the study.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1. Synthesis of lipophilic ligand painted erythrocytes

A, Thiolated IgG was conjugated to DSPE-PEG₃₄₀₀-maleimide as described in Methods. DSPE-PEG₃₄₀₀-IgG was incorporated in the membrane of washed mouse RBCs after incubation at 37°C. Targeting antibody was either directly conjugated to the lipid or immobilized via anti-Fc antibody; **B**, A fluorescent image of a RBC labeled with DiI and DSPE-PEG₃₄₀₀-IgG (detected via secondary antibody). Note that antibody localization is punctate, whereas DiI is homogenously distributed; **C**, Flow cytometry histogram shows that amount of IgG on RBCs (detected via secondary Alexa 488 labeled antibody) is higher for two-step painting method (green trace) than for one-step method (blue trace). Red trace are non labeled RBCs;

Fig. 2. Circulation properties of modified RBCs

A, Following i.v. injection, IgG/DiI-RBCs formed a distinct double labeled population in the upper right corner that persisted over 48 hours; **B**, Retention of lipophilic IgG on RBC surface over time monitored with flow cytometry; **C**, RBC half-life in blood as monitored with flow cytometry. Data shown as means of n=3 and SD.

Fig. 3. Binding of targeted RBCs to cells *in vitro*

A, binding of anti-mouse EpCAM/DiI-RBCs to cultured 4T1 breast carcinoma cells in suspension (RBC/tumor cell ratio of 10:1). Following 60 min incubation in cell medium, there was a formation of characteristic rosettes around tumor cells. Control, non-targeted DiI-RBCs did not show a significant binding; **B**, binding of RBCs to tumor cells was measured with flow cytometry. Red trace, 4T1+non-targeted DiI-RBCs; orange trace, 4T1+non labeled RBCs, green trace, 4T1 only; **C**, addition of 1:1 mixture of anti-mouse EpCAM/DiI-RBCs (red) and IgG/DiO-RBCs (green) to mouse blood spiked with 4T1 cells *in vitro*; **D**, anti-mouse CD45/DiI-RBCs (left) and control DiI-RBC (right) were added to whole (heparinized) mouse blood at 1:10 ratio (modified RBC: normal blood RBC). After 30 min incubation, leukocytes were stained with FITC-anti-mouse CD45 antibody. Note that FITC-anti-CD45 antibody still labeled the leukocytes despite the presence of bound RBCs; **E**, flow cytometry analysis of leukocyte binding. RBC population (upper left) is marked with black arrow, non-bound CD45+ cells are outlined with black polygon. Rosettes between targeted RBCs and CD45+ cells (left dot plot) formed double labeled population (red polygon). Even though non-targeted RBCs were added to mouse blood at 1:1 ratio (right plot), no binding to leukocytes and no significant double-stained population was observed. The experiment was repeated 3 times.

Fig. 4. Binding and depletion of CD45+ cells *in vivo* by anti-CD45 coated RBCs

Anti-CD45/Dil RBCs (5×10^8) were injected into normal BALB/c mice, blood samples were collected, stained with FITC-anti-mouse CD45 antibody, and levels of CD45+ cells in peripheral blood were determined with flow cytometry as described in Methods. **A**, flow cytometry and microscopy data, from left to right, from top to bottom: CD45+ cells before injection (inside the ROI); Blood staining 1 min post-injection of targeted RBCs shows the rosettes between targeted RBCs (red) and CD45+ cells (green); Flow cytometry of blood at 1 min post-injection (500,000 total events were collected in all experiments) shows appearance of double-stained rosettes (polygonal ROI); at 12 h post-injection of targeted RBCs the population of CD45+ cells is significantly depleted compared to before injection; in contrast, at 1 min and 12 h post-injection of DSPE-PEG₃₄₀₀-anti-CD45 (2 μ g/mouse) there was a minimal depletion of CD45+ cells. In all cases, there was a decrease in immunostaining of CD45+ cells, likely due to partial blocking of CD45 by anti-CD45; **B**, summary of flow cytometry data collected over 24 h period shows a significant depletion of CD45+ cells by anti-CD45-RBCs (red line), no depletion by 2 μ g anti-CD45 IgG (black line), and minimal depletion by 2 μ g (IgG) of DSPE-PEG₃₄₀₀-anti-CD45 (blue line). Statistical values (t-test, n=3) were calculated for anti-CD45-RBCs vs. anti-CD45. Data are shown as mean and SD.

Fig. 5. Localization of DSPE-PEG₃₄₀₀-anti-CD45 in organs

A, RBCs painted with DSPE-PEG₃₄₀₀-anti-CD45 (rat anti-mouse) and DiI were injected into Balb/C mice. 24 h later tissues were stained with AF488 goat anti-rat antibody. Kupffer cells and leukocytes (white arrow) show deposition of anti-CD45 that apparently dissociated from RBCs. Liver and spleen show presence of rosettes formed between CD45⁺ leukocytes and anti-CD45/DiI-RBCs (yellow arrow). There was a significant number of cells coated with anti-CD45 antibody in the spleen (white arrow) that presumably detached from RBCs. **B**, DSPE-PEG₃₄₀₀-anti-CD45 construct only (without RBCs) was injected into Balb/C mice. The antibody was deposited on endothelial cells in the liver and pulp cells in the spleen (white arrows). There was a much lower number of anti-CD45 positive cells in the spleen of DSPE-PEG₃₄₀₀-anti-CD45 injected mice compared to anti-CD45/DiI-RBC injected mice.

Note that yellow/orange dots in the images of lungs and kidneys are not RBCs but rather tissue autofluorescence. Representative images of 3 experiments are shown.

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Fig. 6. Depletion of JeKo-1 mantle cell lymphoma and therapeutic effect *in vivo* using anti-CD20 RBCs

Mice were injected with rituximab/DiI-RBCs or DSPE-PEG3400-rituximab (2 μ g antibody), and the numbers of lymphoma cells in the peripheral blood were monitored with flow cytometry after staining whole blood with FITC-anti-human CD20 (2,000,000 total events were collected). **A**, CD20+ cells in peripheral blood before the injection of rituximab/DiI-RBCs (left); CD20+ cells after the injection of rituximab/DiI-RBCs (center) or DSPE-PEG₃₄₀₀-rituximab (right). There was an efficient depletion of CD20+ cells by the targeted RBCs; **B**, levels of human CD20+ cells in blood over time. Rituximab/DiI-RBCs (red line) produced significant decrease in the levels of CD20+ cells as compared to DSPE-PEG₃₄₀₀-rituximab (black line) at 12 h and 24 h post-injection. (p-value 0.01, t-test, n=3). Data are presented as mean values and SD; **C**, **D**, Depletion of lymphoma cells at 12 h was verified by staining blood samples with FITC-anti-human CD45 antibody (C) or FITC-anti-human CD19 antibody (D). Left histogram, rituximab-RBCs; right histogram, DSPE-PEG₃₄₀₀-rituximab; right graph, plot of cell counts per 500,000 events by FACS (n=2). The lower number of collected events than for “A” should not affect the relative difference between treatments. Data presented as means and SD; **E**, Effect of rituximab-RBCs on survival of JeKo-1 mice. Mice were treated starting from day 10 post-implantation with rituximab-RBCs (100 μ l RBC/mouse, 2 μ g IgG) or with DSPE-PEG₃₄₀₀-rituximab (2 μ g IgG) every 3 days for 3 weeks. RBC-treated group (red line) showed significant prolongation of survival (p=0.001, n=4, log-rank test) compared to the lipophilic antibody-treated group (black

trace). Comparison of treatments rather than treatment vs. placebo is the accepted approach in clinical practice²⁶, therefore we did not include not treated control in this experiment.

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