Engineered red blood cells as carriers for systemic delivery of a wide array of functional probes

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We developed modified RBCs to serve as carriers for systemic delivery of a wide array of payloads. These RBCs contain modified proteins on their plasma membrane, which can be labeled in a sortase-catalyzed reaction under native conditions without inflicting damage to the target membrane or cell. Sortase accommodates a wide range of natural and synthetic payloads that allow modification of RBCs with substituents that cannot be encoded genetically. As proof of principle, we demonstrate site-specific conjugation of biotin to in vitro-differentiated mouse erythroblasts as well as to mature mouse RBCs. Thus modified, RBCs remain in the bloodstream for up to 28 d. A single domain antibody attached enzymatically to RBCs enables them to bind specifically to target cells that express the antibody target. We extend these experiments to human RBCs and demonstrate efficient sortase-mediated labeling of in vitro-differentiated human reticulocytes.

mammalian cell engineering | sortaggable GPA and Kell | survival time in circulation

Red blood cells are the most numerous cell type in blood and account for a quarter of the total number of cells in the human body. RBCs possess many unique characteristics that make them attractive candidates for in vivo delivery of natural and synthetic payloads (1): (*i*) extensive circulatory range; (*ii*) removal of old or damaged RBCs by cells of the reticuloendothelial system; (*iii*) biocompatibility; (*iv*) a long circulatory half life (~120 d in humans and ~50 d in mice); (*v*) a large surface area of ~140 μm^2 with a favorable surface-to-volume ratio; and (*vi*) the absence of a nucleus, mitochondria, and any DNA. Thus, any modification made to the DNA of RBC precursors is eliminated upon their enucleation and cannot lead to abnormal growth or tumorigenicity after their transfusion into a recipient.

Engineered RBCs have been generated using encapsulation (2–4), by noncovalent attachment of foreign peptides, or through installation of proteins by fusion to a monoclonal antibody specific for an RBC surface protein (5, 6).However, modified RBCs have limitations if intended for application in vivo. Encapsulation allows the entrapment of sizable quantities of material but does so at the expense of disrupting plasma membrane integrity, with a concomitant reduction in circulatory half life of the modified RBCs. Osmosis-driven entrapment limits the chemical nature of materials that can be encapsulated successfully, the site of release is difficult to control, and encapsulated enzymes are functional only at the final destination, compromising reusability at other sites (5, 6). Targeting of cargo to RBCs by fusion to an RBC-specific antibody, (e.g., anti-glycophorin antibody), has limitations because this mode of attachment to the RBC is noncovalent and dissociates readily, thus reducing circulatory half life and mass of cargo available for delivery (5, 6). Other developments that exploit RBCs for targeted delivery include nanoparticles enveloped by an RBC-mimicking membrane and RBC-shaped polymers (1). The short in vivo survival rate of these RBC-inspired carriers (~7 d maximum) may limit their therapeutic utility.

There is a need to develop new methodology for engineering RBCs so that they can carry a wide variety of useful cargoes to specific locations in the body. We describe an approach that involves minimal modification of the RBCs, with preservation of plasma membrane integrity. The method involves sortase-mediated site-specific covalent attachment of payloads to specific RBC surface proteins.

Bacterial sortases are transpeptidases capable of modifying suitably modified proteins in a covalent and site-specific manner (7, 8). Sortase A from *Staphylococcus aureus* recognizes an LPXTG motif positioned close to the substrate's C terminus and cleaves between T and G to form a covalent acyl-enzyme intermediate. This intermediate is resolved by a nucleophilic N-terminal glycine residue on an appropriately designed probe (9) with concomitant formation of a peptide bond between substrate and probe. Conversely, a protein may be labeled at its N terminus by extending it with suitably exposed glycine residues and using a probe with the LPXTG motif for the sortase reaction. These probes can be designed to contain proteins, peptides, small molecules (organic or inorganic), carbohydrates, lipids, and other substances (10).

We engineered RBCs that contain sortase-modifiable proteins on their plasma membrane. These engineered RBCs can be labeled in a sortase-catalyzed reaction under native conditions without inflicting damage to the membrane or cell. The site-specificity of the sortase reaction and its ability to accommodate a wide range of substituents offer distinct advantages, including modification of RBCs with substituents that cannot be encoded genetically. We demonstrate the conjugation of biotin to in vitro-

Significance

Engineered human RBCs are attractive carriers for the introduction of novel therapeutics, immunomodulatory agents, and diagnostic imaging probes into the human body. Normal murine and human RBCs can be produced in tissue culture from progenitors; we have introduced genes into these cells encoding surface proteins that can be covalently and site-specifically modified on the cell surface. The engineering and labeling processes do not damage the cells or affect their survival in vivo. Most importantly, the engineered RBCs can be labeled with a wide array of functional probes, including small molecules, peptides, and proteins, and thus have the potential to be carriers of a variety of therapeutic substances into the bloodstream.

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differentiated mouse erythroblasts as well as to mature in vivo-differentiated mouse RBCs; these modified RBCs remain detectable in the bloodstream for at least 28 d. Also, we attach a single-domain antibody to RBCs and demonstrate the ability of the engineered cells to bind specifically to target cells that express the antibody target protein. Finally, we extend these mouse proof-of-concept experiments to human RBCs and demonstrate efficient sortase-labeling of in vitro differentiated human reticulocytes. This platform creates new possibilities for engineered RBC-based applications in humans.

Results

Design of Sortase-Modifiable Proteins on the RBC Surface. RBCs lack a nucleus and at their mature stage cannot be genetically modified. Therefore we engineered erythroid precursors to express sortase-modifiable proteins that are retained on the plasma membrane of mature RBCs. These modified substrates must be chosen so that they do not inhibit erythroid differentiation and are not targeted for degradation during the extensive membrane remodeling that occurs during enucleation and at the later reticulocyte stage (11). For example, RBC precursors express high levels of the transferrin receptor (Tfr), a type II membrane protein, but because it is no longer present on mature RBCs, Tfr is not a suitable target for modification. Therefore we chose to express two sortase-modifiable membrane proteins, Kell and glycophorin A (GPA), in erythroid progenitors. The blood group antigen Kell is also a type II membrane protein with an extracellularly exposed C terminus and was selected as a target for C-terminal labeling. GPA, a type I membrane protein with its N terminus extracellularly disposed, is the most abundant protein on the RBC surface and was chosen for N-terminal modification.

The Type-II Membrane Protein Kell Is Expressed and Can Be Sortase-Labeled on the RBC Surface. Extension of the Kell C terminus with the sortase-recognition motif LPXTG is the minimal modification required to render it sortase-modifiable. We constructed a retroviral construct encoding human Kell (hKell), C-terminally modified by extension with LPETG, followed by an HA epitope tag. A sortase reaction performed on thus modified Kell using a glycine-based probe leads to conjugation of the probe onto the C terminus of Kell, with concomitant loss of the HA tag (Fig. 1A). To test the expression and modification of Kell by sortase, we used an in vitro erythroid differentiation system (12). Culture of murine fetal liver-derived progenitors in vitro for ~48 h allows approximately four terminal cell divisions and the formation of hemoglobin-containing erythroblasts, about 30–50% of which undergo enucleation to yield reticulocytes. Expression of hKell-LPETG-HA did not inhibit the in vitro differentiation process. Almost half of both nucleated erythroblasts and enucleated reticulocytes displayed modified Kell at the cell surface, and a large fraction of these could be sortagged with a biotin-containing probe as shown by flow cytometry, immunoblotting, and immunofluorescence (Fig. S1).

Reticulocytes obtained by in vitro differentiation not only must undergo expulsion of remaining organelles but also must execute the membrane reorganizations that lead to the biconcave disk shape of mature RBCs. To ensure that this maturation step does not lead to loss of modified Kell, murine fetal liver lineage-negative cells were infected with retroviral vectors expressing engineered hKell-LPETG-HA and were transplanted into lethally irradiated mice (Fig. S2). Mature RBCs were harvested from transplanted mice 4 wk after transplantation and were analyzed for the presence of sortase-modifiable Kell on their surface. We routinely found that ~20–50% of mature RBCs in these chimeric mice contained sortaggable hKell on their surface (Fig. 1B) and that these cells could be covalently modified with biotin using sortase (Fig. 1C) with efficiency of 81 ± 28% as determined by flow cytometry. We observed a high level of
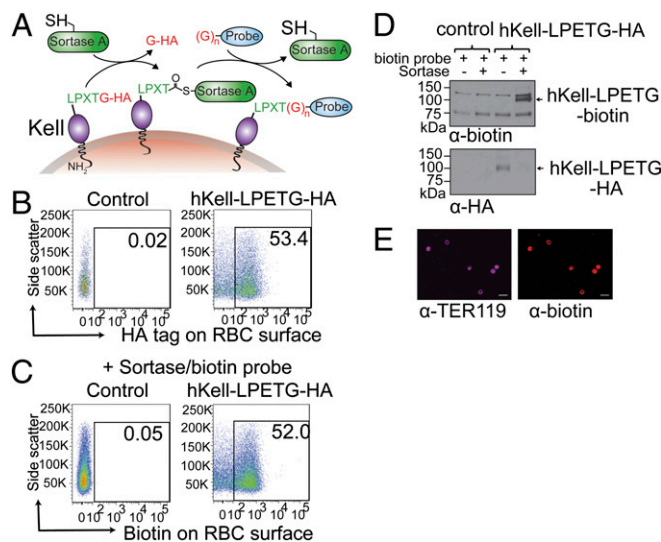
Fig. 1. Expression and sortase-mediated C-terminal labeling of Kell on the surface of mature mouse RBCs. (A) Schematic for Kell C-terminal sortase labeling. Kell was extended at the C terminus to include the sortase-recognition motif LPETG followed by an HA epitope tag. Incubation of sortase with cells containing Kell-LPETG-HA on the surface leads to peptide cleavage between T and G in the recognition motif and the formation of an acyl-enzyme intermediate between cysteine on sortase and the sortase motif on Kell. Addition of a nucleophilic probe with N-terminal glycine residues [GGG-K(biotin)] for biotin conjugation resolves the intermediate, thus ligating the probe to the C terminus of Kell. The probe can be a peptide, protein, lipid, carbohydrate, small molecule, or other entity. (B) Evaluation of mature RBCs for the presence of Kell-LPETG-HA on the cell surface by staining blood from control mice and from mice that have undergone bone marrow transplantation to express Kell-LPETG-HA with α-HA–tagged antibody and analyzing via flow cytometry (gated on mature RBCs). (C) Evaluation of mature RBCs for sortase labeling by incubating control blood or Kell-LPETG-HA blood with sortase and the biotin-containing probe, staining with α-biotin antibody, and analyzing via flow cytometry (gated on mature RBCs). (D) Evaluation of RBCs for sortase labeling by immunoblotting. Control or Kell-LPETG-HA blood was incubated with biotin probe with or without sortase. Total cell protein was resolved by SDS/PAGE and immunoblotted for an α-HA tag and α-biotin. Loss of the HA tag upon sortase labeling indicates complete modification. (E) Immunofluorescence images show biotin (labeled with red) conjugated to the C terminus of hKell on mature RBCs (labeled with Ter119 antibody, purple). (Scale bars, 10 μm.)

conjugation of the biotin probe onto the Kell C terminus upon sortagging, as evidenced by complete loss of the HA tag on the immunoblot (Fig. 1D). Immunofluorescence microscopy (Fig. 1E) confirms the presence of biotin conjugated to the surface of RBCs. We conclude that Kell when extended at its C terminus with the sortase recognition motif does not inhibit erythroid differentiation, is retained on the plasma membrane of mature RBCs, and can be labeled in a sortagging reaction.

Genetically Modified Type-I Membrane Protein Human GPA Is Expressed and Can Be Sortase-Labeled on the Murine RBC Surface. Extension of the N terminus of GPA with glycine residues is the minimal modification needed to render GPA a suitable sortase substrate. In the modified version, the N-terminal signal sequence was retained, followed by glycine residues and a myc epitope tag. Cleavage of the signal peptide would yield (Gly)$_n$-myc tag-GPA. Incubation of cells carrying the modified GPA with sortase A from *S. aureus* and an LPETG-based probe leads to conjugation of this probe to the N terminus of GPA (Fig. 2A). We constructed four retroviral constructs for GPA, encoding products extended at their N terminus with either three (3G) or five (5G) N-terminal glycine residues, using either mouse GPA (mGPA)
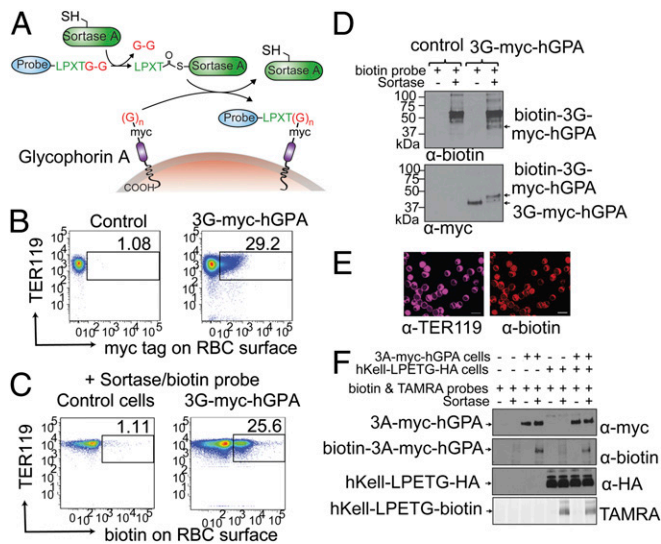
Fig. 2. Expression and sortase-mediated N-terminal labeling of GPA on the surface of mature mouse RBCs. (*A*) Schematic for GPA N-terminal sortase labeling. GPA was extended at the N terminus to include three glycine residues and a myc tag. Preincubation of sortase with a probe containing a C-terminal sortase-recognition motif, LPETG, leads to cleavage between T and G and formation of an acyl-enzyme intermediate between cysteine on sortase and threonine on the probe. Nucleophilic attack of the N-terminal glycine on GPA resolves the intermediate, thus ligating the probe to GPA. The probe can be a peptide, protein, lipid, carbohydrate, a small molecule, or another entity. For conjugation of biotin to GPA, the probe is K(biotin) LPRTGG. (*B*) Evaluation of mature RBCs for the presence of 3G-myc-hGPA on the cell surface by staining blood from control mice and from mice that have undergone bone marrow transplantation to express 3G-myc-hGPA with α-TER119– and α-myc–tagged antibodies and analyzing via flow cytometry. (*C*) Evaluation of mature RBCs for sortase-labeling by incubating control blood or 3G-myc-hGPA blood with sortase and the biotin-containing probe, staining with α-TER119 and α-biotin antibodies, and analyzing via flow cytometry. (*D*) Evaluation of RBCs for sortase-labeling by immunoblotting. Control or 3G-myc-hGPA blood was incubated with the biotin probe with or without sortase, and total cell protein was resolved by SDS/PAGE and immunoblotted for the α-myc tag and α-biotin. The shift in molecular mass of GPA upon biotin conjugation in the α-myc tag immunoblot indicates almost complete modification. Biotin-conjugated GPA is indicated in the α-biotin immunoblot with an arrow. (*E*) RBCs conjugated with biotin were sorted by flow cytometry. Immunofluorescence images show biotin (labeled with red) at the N terminus of hGPA on mature RBCs (labeled with Ter119 antibody, purple). (*F*) HEK293T cells were transfected with 3A-myc-hGPA, hKell-LPETG-HA, or both. Cells were incubated with *S. pyogenes* sortase and a biotin probe followed by incubation with *S. aureus* sortase and a TAMRA-containing probe. Specific conjugation of biotin to GPA and of TAMRA to Kell is demonstrated by immunoblotting and fluorescence imaging.

or human GPA (hGPA). We used the murine in vitro erythroid differentiation system (12), as described above for Kell, to test GPA expression and modification by sortase. Upon retroviral transduction of progenitors, none of the four GPA constructs affected the differentiation process, because in each case we obtained normal numbers of enucleated, Ter119^{+} erythroblasts that expressed the modified GPA on their surface (Figs. S3 and S4 *A* and *B*). We tested whether these GPA constructs are sortase-modifiable by expressing them in HEK293T cells, followed by sortagging with a biotin-containing probe (Fig. S4C). Although we observed high levels of expression of the mouse constructs, we failed to obtain biotinylation of the encoded products. In contrast, 3G-myc-hGPA was readily sortagged and was used for subsequent experiments. Expression of 3G-myc-hGPA in erythroid progenitors yielded ∼36% nucleated erythroblasts and ∼67% enucleated reticulocytes that bear the modified GPA on the surface upon in vitro differentiation. Almost all

the modified GPA on both nucleated and enucleated cells can be sortagged with a biotin-containing probe, as monitored by flow cytometry, immunoblotting, and immunofluorescence (Fig. S5).

As with Kell, we infected murine fetal liver lineage-negative cells with retroviral vector expressing 3G-myc-hGPA and transplanted them into lethally irradiated mice (Fig. S2). After 4 wk, 20–50% of the RBCs in these transplanted mice were Ter119^{+}, discoid-shaped mature RBCs expressing the modified GPA (Fig. 2*B*; $n = 10$) and could sortagged with a biotin-containing probe with an efficiency of $85 \pm 5\%$ as determined by flow cytometry (Fig. 2*C*). The observed reduction in gel mobility of 3G-myc-hGPA upon sortagging with biotin can be used as a readout for reaction efficiency and indicates that most of the GPA is modified by sortase (Fig. 2*D*). Immunofluorescence microscopy (Fig. 2*E*) confirms that all these modified RBCs indeed have biotin conjugated to their surface. We conclude that sortaggable GPA is retained on the surface of mature RBCs and can be labeled efficiently in a sortase-catalyzed reaction.

By using sortases with distinct substrate specificity, it is possible to combine N-terminal and C-terminal labeling strategies (13) to generate multilabeled RBCs. Unlike sortase A from *S. aureus*, sortase A derived from *Streptococcus pyogenes* recognizes LPXTA motifs and accepts oligo-alanine probes as nucleophiles. Therefore, the sortase reactions of both enzymes can be performed as orthogonal reactions. For the initial proof-of-concept experiments, we infected HEK293T cells with two constructs: AAA-myc-hGPA and hKell-LPETG-HA. We then incubated the cells sequentially with two different types of sortase A in the presence of either biotin-LPETA (for GPA sortagging via *S. pyogenes* sortase) or GGG-tetramethylrhodamine (TAMRA) (for Kell sortagging via *S. aureus* sortase), giving rise to HEK293T cells containing both the biotinylated GPA and TAMRA-modified Kell (Fig. 2*F*). Moreover, we generated mature RBCs with cell-surface expression of both 3A-myc-hGPA and hKell-LPETG-HA. Although the expression levels of hKell and hGPA on individual RBCs were somewhat variable, we successfully sortagged hGPA with biotin and hKell with Alexa-647 on the RBC surface (Fig. S6). Dual labeling thus can be used to attach two different functional moieties onto the surface of RBCs.

Survival of Engineered RBCs in Circulation. To assess whether the process of sortagging RBCs, including incubation with sortase, centrifugation, and washing, affects their in vivo survival, we labeled wild-type RBCs and wild-type RBCs that had undergone a mock C-terminal sortagging reaction with or without sortase (no conjugation of a probe) with carboxyfluorescein succinimidyl ester (CFSE), a fluorescent dye that stably stains cytosol of live cells. We then transfused the RBCs into normal recipient mice and periodically assessed cell survival in circulation using CFSE fluorescence. We did not observe any difference in the in vivo survival of RBCs between the experimental groups (Fig. 3*A*), indicating that the sortagging procedure does not cause significant damage to the cells that would lead to their premature removal.

To expedite RBC survival experiments, we created mice using CRISPR/Cas-9 genome-editing technology (14, 15), with LPETG inserted at the C terminus of the murine endogenous Kell gene (mKell-LPETG) (Tables S1–S3). These mice appear normal, have normal RBC parameters, and reproduce normally (Fig. S7). We CFSE-stained control RBCs, mKell-LPETG RBCs, and mKell-LPETG RBCs sortagged with biotin, transfused them into wild-type recipient mice, and monitored RBC survival in circulation. The modification of mKell with LPETG at its C terminus did not affect the survival of the modified RBCs in vivo (Fig. 3*B*). We did observe a slight but significant difference in the survival of mKell-LPETG RBCs sortagged with a biotin probe.
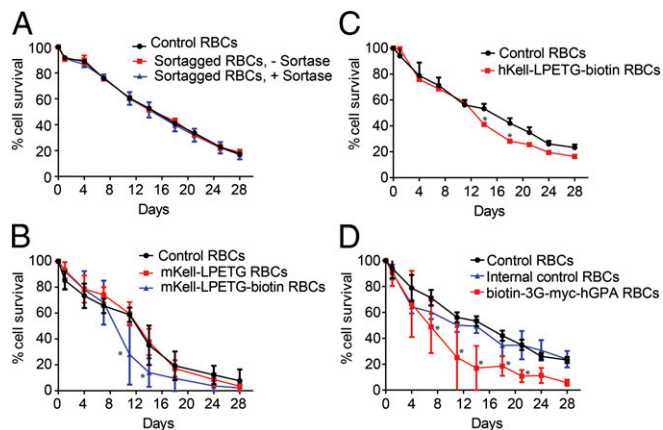
Fig. 3. Survival of engineered RBCs in vivo. (*A*) Wild-type RBCs subjected to mock C-terminal sortagging reacting with or without sortase (*n* = 3, each group) or left untreated (*n* = 3) were reacted with CFSE and transfused into recipient mice via i.v. injection. RBC in vivo survival was tracked via CFSE fluorescence and flow cytometry. (*B*) mKell-LPETG RBCs were obtained from mice genetically engineered to have endogenous Kell extended at the C terminus with an LPETG sequence. Wild-type RBCs (*n* = 6), mKell-LPETG RBCs (*n* = 6), and mKell-LPETG RBCs sortagged with biotin (*n* = 6) were stained with CFSE and were transfused into recipients, and their survival was monitored via CFSE fluorescence or anti-biotin staining using flow cytometry. (*C*) CFSE-stained wild-type RBCs (*n* = 6) and hKell-LPETG RBCs isolated from transplanted mice (*Materials and Methods*) and sortase labeled with biotin (*n* = 2) were transfused into recipient mice, and RBC survival in circulation was tracked via CFSE fluorescence (wild-type RBCs) or anti-biotin staining (hKell-LPETG-biotin RBCs) using flow-cytometry. (*D*) Wild-type RBCs (*n* = 6) and sortase-labeled biotin-3G-myc-hGPA RBCs (*n* = 6) were CFSE stained, transfused into mice, and tracked via CFSE fluorescence or anti-biotin staining using flow cytometry. Internal control RBCs are wild-type cells originating from 3G-myc-hGPA–transplanted mice and subjected to the sortagging reaction and CFSE staining. Error bars represent SD; *$P < 0.01$.

We also harvested hKell-LPETG RBCs from transplanted mice, sortagged them with biotin, and transfused them into recipients. Similarly, engineered biotin-labeled Kell-LPETG RBCs last more than 28 d in circulation, although their survival is slightly lower than that of wild-type RBCs (Fig. 3*C*).

We conducted similar experiments comparing the in vivo survival of normal RBCs with sortase-labeled biotin-3G-myc-hGPA RBCs. The results (Fig. 3*D*) echoed the trend seen with Kell-LPETG RBCs: Sortagged RBCs had a slightly but significantly lower survival rate (Fig. 3*D*). The reason for the somewhat enhanced clearance of sortase-biotinylated RBCs is uncertain, and we do not yet know whether the reduced survival is payload specific. Nevertheless, the lifespan of the engineered RBCs in normal mice, including the RBCs conjugated with biotin, is at least 28 d and thus is drastically longer than that of other RBC-based carriers designed thus far.
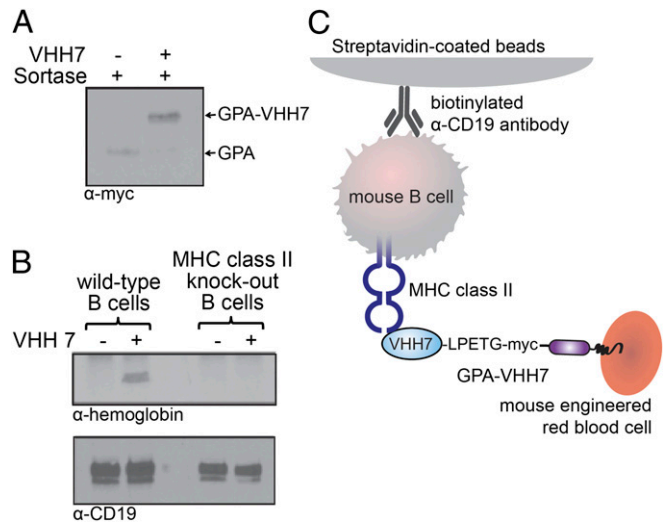
Conjugation of a Single-Domain Antibody to Engineered RBCs for Cell Type-Specific Targeting. One potential application of modified RBCs is to provide them with a targeting moiety that would enable their delivery (and the delivery of an attached or incorporated payload) to particular tissues or cell types. Such targeting might be accomplished by the installation of proteins or other entities, such as ligands for specific receptors, that can participate in the specific recognition of the intended target. To test the conjugation of a functional protein onto the surface of RBCs, we used VHH7-LPETG, a sortaggable alpaca-derived single-domain antibody specific for murine class II MHC molecules, and covalently attached it to RBCs with 3G-myc-hGPA on their surface. As shown by the shift in gel mobility of the 3G-myc-hGPA, the sortagging reaction is stoichiometric (Fig. 4*A*).

Incubation of these modified RBCs with class II MHC-positive B cells immobilized on beads results in binding of the RBCs to B cells. Specific binding was inferred from the presence of hemoglobin, which copurifies with B cells after washing. Neither incubation of wild-type B cells with unmodified RBCs nor incubation of modified RBCs with B cells derived from class II MHC knock-out animals led to binding of the two cell types (Fig. 4 *B* and *C*).

Sortagging and Cytosolic Modification of Terminally Differentiated Human RBCs. To extend the utility of the method described here, we investigated whether erythroid progenitor modification and sortase-mediated cell-surface labeling are feasible for human cells. For these experiments we used an in vitro human erythroid differentiation system (16). Granulocyte-colony stimulating factor (G-CSF)–mobilized CD34$^+$ peripheral blood stem cells were differentiated in vitro in 18 d into hemoglobin-containing reticulocytes, with an enucleation efficiency of ~50%. We constructed a lentiviral vector that encodes a version of 3G-myc-hGPA fused to eGFP at its C terminus (3G-myc-GPA-eGFP). This construct provides an example of a cytoplasmically expressed protein domain designed to be retained in mature RBC via its genetic fusion to GPA. Both the empty (control) vector and the 3G-myc-hGPA-GFP vector encoded a GFP moiety 3′ of the internal ribosome entry site sequence. Cells transduced successfully with 3G-myc-hGPA-GFP, which contains an additional eGFP attached to the cytoplasmic domain of GPA, express a significantly (~2×) higher level of GFP signal, as indicated by flow cytometry (Fig. 5*A*, *Left*). Because GFP and hGPA have a molecular mass of 32.7 kDa and 37 kDa, respectively, the mobility of the 3G-myc-hGPA-GFP on SDS/PAGE also indicates that the modified GPA proteins are expressed in monomeric and dimeric

Fig. 4. Conjugation of a single-domain antibody to engineered RBCs and cell type-specific targeting. (*A*) Mouse mature RBCs engineered to contain 3G-myc-GPA on the cell surface were incubated with sortase with or without VHH7-LPETG, a single-domain antibody with binding specificity for mouse MHC class II molecules and a sortase motif complementary to 3G. An immunoblot against the myc epitope tag on GPA reveals fusion of VHH7 to GPA as an increase in GPA molecular mass. (*B*) Wild-type mouse B cells (expressing MHC class II) or B cells derived from MHC class II knock-out mice were immobilized on magnetic streptavidin beads via biotinylated α-CD19 antibody, incubated with 3G-myc-GPA RBCs or RBCs that contained sortase-labeled VHH7-LPETG-myc GPA on their surface, and washed. Immobilization of B cells in all experimental set-ups was tested by the presence of CD19 in an α-CD19 immunoblot, and binding between B cells and RBCs was evaluated by the presence of hemoglobin in the α-hemoglobin immunoblot. (*C*) Schematic for experiment shown in *B*.
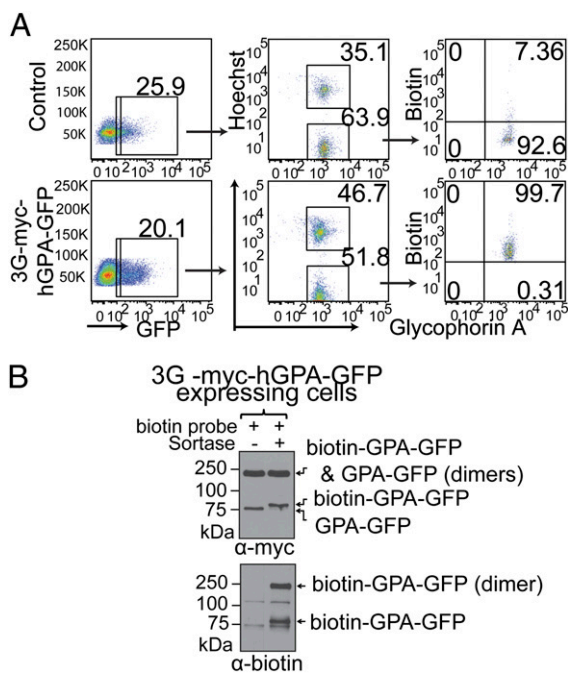
Fig. 5. Expression and sortase-mediated labeling of GPA on the surface of human in vitro-differentiated reticulocytes. (*A*) Human G-CSF–mobilized peripheral blood CD34⁺ stem cells were virus transduced with either empty vector or a vector containing 3G-myc-GPA-eGFP and were differentiated for 18 d. Flow cytometry analysis of reticulocytes reveals the percentage of transduced cells (GFP⁺) and the percentage of enucleated cells (*Lower Center*). Reticulocytes, either control or 3G-myc-GPA-eGFP transduced, were incubated with sortase and a biotin-containing probe, stained for biotin with α-biotin–phycoerythrin antibody, analyzed via flow cytometry, and gated on enucleated cells. (*B*) 3G-myc-GPA-eGFP transduced and in vitro-differentiated reticulocytes were incubated with a biotin probe with or without sortase, and total cell protein was immunoblotted for the myc tag and biotin. Both monomers and dimers (higher molecular mass) of 3G-myc-GPA-eGFP are visible in both immunoblots. A shift in GPA-GFP molecular mass upon biotin conjugation in the α-myc tag immunoblot indicates complete modification.

forms (Fig. 5*B*). Viral transduction of human CD34⁺ cells with 3G-myc-hGPA-GFP did not affect their differentiation, because the percentages of enucleated reticulocytes (50–60%) were similar to those in cultures infected with an empty (control) vector (Fig. 5*A, Center*). All enucleated reticulocytes that contain the modified GPA at the surface could be sortase-labeled with a biotin-containing probe, as monitored by flow cytometry (Fig. 5*A*). Furthermore, all the 3G-myc-hGPA-GFP present on the reticulocyte surface was modified by biotin sortagging, as evidenced by the shift in gel mobility of the 3G-myc-hGPA-GFP construct (Fig. 5*B*). This experiment therefore establishes that mature human RBCs can be equipped with a cytoplasmically disposed protein of interest while retaining the ability to target the RBCs selectively to an intended target by sortagging of the modified GPA with a targeting moiety of interest.

Discussion

Engineered RBCs have several advantages as i.v. carriers for therapeutic delivery. Mature RBCs lack a nucleus so that, upon terminal differentiation, modified erythroid progenitors leave no trace of introduced genetic alterations. Thus the possibility of tumorigenicity, a key risk of stem cell-based therapies (17) is eliminated. Further attractive characteristics of RBCs include their long survival (a lifespan of ~120 d) and their presence throughout the macro- and microcirculation. Modification of RBCs with bioavailable therapeutics thus might lead to prolonged

efficacy and coverage of all areas perfused by the circulation in vivo.

Current methods used to generate engineered RBCs mainly involve osmosis-driven encapsulation and attachment of payloads to RBC-targeting antibodies or peptides. Osmosis-driven encapsulation often leads to membrane fragility, has not been combined with surface coupling, and is limited in the types of payloads suitable for delivery. Covalent or noncovalent attachment of a payload to RBC-targeting antibodies results in noncovalent surface coupling that is prone to dissociation in the circulation, affecting both success of delivery and circulatory half life. RBC ghosts have been used to encapsulate functional nanoparticles but survive only 7 d, at most, in the circulation.

Here we combined our understanding of RBC biology with sortase-mediated protein modification and in vitro erythroid differentiation to engineer RBCs that can be modified with virtually any substituent of choice under native conditions and with minimal risk of damaging the RBC in the process, offering an advantage over the osmosis-driven encapsulation method. In the course of terminal differentiation, some plasma membrane proteins present on erythroid precursors are degraded selectively, whereas other proteins integral to the function of mature RBCs are retained. Both Kell and GPA are expressed on erythroid precursors and are retained on mature RBCs. We generated modified versions of GPA and Kell to render them suitable for modification by sortase, and it is likely that other RBC membrane proteins will lend themselves to similar treatment. Although sortase-mediated cell-surface labeling has been exploited previously [e.g., to explore trafficking of influenza glycoproteins (18, 19)], here we apply this method to primary cells as a platform for diagnostic or therapeutic purposes. We used a sortase A variant from *S. aureus* active at 0 °C (20) to reduce the risk of inflicting cellular damage in the course of labeling. Experiments performed on mouse erythroid progenitors served as a proof of concept for further experiments that applied in vitro-differentiation protocols to human erythroid progenitors. We thus demonstrated the applicability of this method to human erythrocytes as well.

The ability to guide in vitro differentiation of human hematopoietic stem cells and progenitors to reticulocytes has shown great progress, although genetic manipulation of the progenitors and functionalization of the reticulocytes derived from them remain to be explored more fully. Our experiments establish that it is possible to modify both murine and human erythroid precursors genetically to enable sortase-mediated engineering of RBCs. The modifications we made to the RBCs are minimal. The covalently attached payload remains detectable in circulation for at least 28 d, much longer than RBCs engineered by other methods.

The approach presented here has many other possible applications; the wide variety of possible payloads, ranging from proteins and peptides to synthetic compounds and fluorescent probes, may serve as a guide. We have conjugated a single-domain antibody to the RBC surface with full retention of binding specificity, thus enabling the modified RBCs to be targeted to a specific cell type. We envision that sortase-engineered cells could be combined with established protocols of small-molecule encapsulation (3). In this scenario, engineered RBCs loaded with a therapeutic agent in the cytosol and modified on the surface with a cell type-specific recognition module could be used to deliver payloads to a precise tissue or location in the body. We also have demonstrated the attachment of two different functional probes to the surface of RBCs, exploiting the subtly different recognition specificities of two distinct sortases. Therefore it should be possible to attach both a therapeutic moiety and a targeting module to the RBC surface and thus direct the engineered RBCs to tumors or other diseased cells. Conjugation of an imaging probe (i.e.,

a radioisotope), together with such a targeting moiety also could be used for diagnostic purposes.

Further advances in the in vitro generation of human RBCs and genetic engineering of their precursors will provide a robust platform for the use of this surface-engineering method, possibly in conjunction with cytosolic modification, in clinical applications (11, 21–26). Moreover, the established safety of blood transfusions inspires confidence that these engineered RBCs indeed will find use in humans.

Materials and Methods

All animal experiments followed the protocols approved by the Division of Comparative Medicine, Massachusetts Institute of Technology. Detailed materials and methods are found in *SI Materials and Methods*.

Creation of Sortaggable hGPA and hKell. A cDNA encoding hGPA was modified by insertion of nucleotides encoding five or three glycine residues immediately after the signal peptide, followed by a myc epitope tag for detection. To create sortaggable hKell, a cDNA encoding hKell was modified by insertion of nucleotides encoding the peptide LPETGG at the C terminus, followed by an HA epitope tag and the stop codon.

Creation of Human and Murine Reticulocytes with Cell-Surface Expression of Engineered hGPA and hKell. Using retrovirus vectors, we transduced cDNAs encoding sortaggable or control hGPA or hKell into murine erythroid progenitors purified from embryonic day 14.5 fetal livers. Transduced erythroid progenitors were cultured in erythroid maintenance medium overnight to allow gene expression, followed by a 48-h culture in RBC differentiation medium, at which time most of the cells had differentiated into enucleated reticulocytes.

Using a lentivirus vector, we transduced cDNAs encoding sortaggable or control hGPA into human CD34$^+$ cells. After 18 d differentiation in vitro,

most transduced hCD34$^+$ cells had differentiated into enucleated reticulocytes with cell-surface expression of sortaggable hGPA.

Creation of Murine Mature RBCs with Cell-Surface Expression of Sortaggable GPA and Kell. Sortaggable hKell and hGPA were transduced into mouse hematopoietic stem and progenitor cells using infection with a retroviral vector. The transduced cells were transplanted into irradiated mice, resulting after 1 mo in the generation of mature RBCs with cell-surface expression of engineered hKell or hGPA.

To create RBCs with endogenous sortaggable Kell, a DNA sequence encoding LPETGG was genetically attached to the C terminus of the mKell gene by the Crisper-Cas technique. RBCs from the transgenic mice were all sortaggable.

Sortase Labeling Reaction. To label the GPA N terminus with biotin, sortase and the K(biotin)LPRTGG peptide were preincubated on ice for 15 min and then were added to a suspension of sortaggable reticulocytes or mature RBCs. To label the GPA N terminus with VHH7 (a nanobody that recognizes MHC class II), sortase, and VHH-LPETG-His6 were preincubated as above and then were added to sortaggable reticulocytes or mature RBCs. To label the Kell C terminus with biotin, sortase and a GGGK(biotin) peptide were incubated with sortaggable reticulocytes or mature RBCs directly.

Transfusion and Survival of Engineered RBCs. After mature RBCs were sortagged, they were labeled with CFSE, washed, and transfused into CD45.1$^+$ mice. The first blood sample was collected 1 h after transfusion and was labeled "day 0." Subsequent blood samples were collected every 3 d from day 1 until day 28 and were analyzed by FACS.

1. Yoo JW, Irvine DJ, Discher DE, Mitragotri S (2011) Bio-inspired, bioengineered and biomimetic drug delivery carriers. *Nat Rev Drug Discov* 10(7):521–535.
2. Biagiotti S, Paoletti MF, Fraternale A, Rossi L, Magnani M (2011) Drug delivery by red blood cells. *IUBMB Life* 63(8):621–631.
3. Godfrin Y, et al. (2012) International seminar on the red blood cells as vehicles for drugs. *Expert Opin Biol Ther* 12(1):127–133.
4. Muzykantov VR (2010) Drug delivery by red blood cells: Vascular carriers designed by mother nature. *Expert Opin Drug Deliv* 7(4):403–427.
5. Murciano JC, et al. (2003) Prophylactic fibrinolysis through selective dissolution of nascent clots by tPA-carrying erythrocytes. *Nat Biotechnol* 21(8):891–896.
6. Zaitsev S, et al. (2010) Sustained thromboprophylaxis mediated by an RBC-targeted pro-urokinase zymogen activated at the site of clot formation. *Blood* 115(25): 5241–5248.
7. Popp MW, Antos JM, Grotenbreg GM, Spooner E, Ploegh HL (2007) Sortagging: A versatile method for protein labeling. *Nat Chem Biol* 3(11):707–708.
8. Popp MW, Antos JM, Ploegh HL (2009) Site-specific protein labeling via sortase-mediated transpeptidation. *Current Protocols in Protein Science* 56(15.3):15.3.1–15.3.9.
9. Popp MW, Ploegh HL (2011) Making and breaking peptide bonds: Protein engineering using sortase. (Translated from eng). *Angew Chem* 50(22):5024–5032.
10. Antos JM, Miller GM, Grotenbreg GM, Ploegh HL (2008) Lipid modification of proteins through sortase-catalyzed transpeptidation. *J Am Chem Soc* 130(48):16338–16343.
11. Liu J, Guo X, Mohandas N, Chasis JA, An X (2010) Membrane remodeling during reticulocyte maturation. *Blood* 115(10):2021–2027.
12. Zhang J, Socolovsky M, Gross AW, Lodish HF (2003) Role of Ras signaling in erythroid differentiation of mouse fetal liver cells: Functional analysis by a flow cytometry-based novel culture system. *Blood* 102(12):3938–3946.
13. Antos JM, et al. (2009) Site-specific N- and C-terminal labeling of a single polypeptide using sortases of different specificity. *J Am Chem Soc* 131(31):10800–10801.
14. Jinek M, et al. (2012) A programmable dual-RNA-guided DNA endonuclease in adaptive bacterial immunity. *Science* 337(6096):816–821.
15. Wang H, et al. (2013) One-step generation of mice carrying mutations in multiple genes by CRISPR/Cas-mediated genome engineering. *Cell* 153(4):910–918.
16. Hu J, et al. (2013) Isolation and functional characterization of human erythroblasts at distinct stages: Implications for understanding of normal and disordered erythropoiesis in vivo. *Blood* 121(16):3246–3253.
17. Gruen L, Grabel L (2006) Concise review: Scientific and ethical roadblocks to human embryonic stem cell therapy. *Stem Cells* 24(10):2162–2169.
18. Popp MW, Karssemeijer RA, Ploegh HL (2012) Chemoenzymatic site-specific labeling of influenza glycoproteins as a tool to observe virus budding in real time. *PLoS Pathog* 8(3):e1002604.
19. Sanyal S, et al. (2013) Type I interferon imposes a TSG101/ISG15 checkpoint at the Golgi for glycoprotein trafficking during influenza virus infection. *Cell Host Microbe* 14(5):510–521.
20. Chen I, Dorr BM, Liu DR (2011) A general strategy for the evolution of bond-forming enzymes using yeast display. *Proc Natl Acad Sci USA* 108(28):11399–11404.
21. Cong L, et al. (2013) Multiplex genome engineering using CRISPR/Cas systems. *Science* 339(6121):819–823.
22. Mali P, et al. (2013) RNA-guided human genome engineering via Cas9. *Science* 339(6121):823–826.
23. Giarratana MC, et al. (2011) Proof of principle for transfusion of in vitro-generated red blood cells. *Blood* 118(19):5071–5079.
24. Douay L, Giarratana MC (2009) Ex vivo generation of human red blood cells: A new advance in stem cell engineering. *Methods Mol Biol* 482:127–140.
25. Leberbauer C, et al. (2005) Different steroids co-regulate long-term expansion versus terminal differentiation in primary human erythroid progenitors. *Blood* 105(1):85–94.
26. Griffiths RE, et al. (2012) Maturing reticulocytes internalize plasma membrane in glycophorin A-containing vesicles that fuse with autophagosomes before exocytosis. *Blood* 119(26):6296–6306.