

Correction

IMMUNOLOGY AND INFLAMMATION

Correction for “Engineered erythrocytes covalently linked to antigenic peptides can protect against autoimmune disease,” by Novalia Pishesha, Angelina M. Bilate, Marsha C. Wibowo, Nai-Jia Huang, Zeyang Li, Rhogerry Dhesycka, Djenet Bousbaine, Hojun Li, Heide C. Patterson, Stephanie K. Dougan, Takeshi Maruyama, Harvey F. Lodish, and Hidde L. Ploegh, which appeared in issue 12, March 21, 2017, of *Proc Natl Acad Sci USA* (114: 3157–3162; first published March 7, 2017; 10.1073/pnas.1701746114).

The authors note that the author name Rhogerry Dhesycka should instead appear as Rhogerry Deshycka. The corrected author line appears below. The online version has been corrected.

Novalia Pishesha, Angelina M. Bilate, Marsha C. Wibowo, Nai-Jia Huang, Zeyang Li, Rhogerry Deshycka, Djenet Bousbaine, Hojun Li, Heide C. Patterson, Stephanie K. Dougan, Takeshi Maruyama, Harvey F. Lodish, and Hidde L. Ploegh

www.pnas.org/cgi/doi/10.1073/pnas.1705149114

Engineered erythrocytes covalently linked to antigenic peptides can protect against autoimmune disease

Novalia Pishesha^{a,b,c}, Angelina M. Bilate^a, Marsha C. Wibowo^{a,d}, Nai-Jia Huang^a, Zeyang Li^{a,c}, Rhogerry Dhesycka^{a,b}, Djenet Bousbaine^{a,c,d}, Hojun Li^a, Heide C. Patterson^a, Stephanie K. Dougan^a, Takeshi Maruyama^a, Harvey F. Lodish^{a,b,d,1}, and Hidde L. Ploegh^{a,c,d,1,2}

^aWhitehead Institute for Biomedical Research, Cambridge, MA 02142; ^bDepartment of Biological Engineering, Massachusetts Institute of Technology, Cambridge, MA 02142; ^cProgram in Cellular and Molecular Medicine, Boston Children's Hospital, Boston, MA 02115; and ^dDepartment of Biology, Massachusetts Institute of Technology, Cambridge, MA 02142

Contributed by Hidde L. Ploegh, February 6, 2017 (sent for review October 7, 2016; reviewed by Douglas A. Melton, Maki Nakayama, and Jing Zhang)

Current therapies for autoimmune diseases rely on traditional immunosuppressive medications that expose patients to an increased risk of opportunistic infections and other complications. Immunoregulatory interventions that act prophylactically or therapeutically to induce antigen-specific tolerance might overcome these obstacles. Here we use the transpeptidase sortase to covalently attach disease-associated autoantigens to genetically engineered and to unmodified red blood cells as a means of inducing antigen-specific tolerance. This approach blunts the contribution to immunity of major subsets of immune effector cells (B cells, CD4⁺ and CD8⁺ T cells) in an antigen-specific manner. Transfusion of red blood cells expressing self-antigen epitopes can alleviate and even prevent signs of disease in experimental autoimmune encephalomyelitis, as well as maintain normoglycemia in a mouse model of type 1 diabetes.

sortase | engineered red blood cells | antigen-specific tolerance | autoimmune diseases

The incidence of diseases with an immune component continues to increase. Examples include not only ~80 autoimmune diseases, but also life-threatening conditions caused by immune responses to protein replacement therapies, or by attack of the host's immune system on transplanted tissues or transferred cells. Treatment of these conditions often depends on prolonged use of immunosuppressants, which lack antigen specificity. Because sustained immunosuppression increases the risk of infection, an important goal remains the development of antigen-specific immune intervention to achieve tolerance, while sparing desirable effector immune responses, such as those directed against pathogens (1). Administration of soluble, disaggregated proteins or peptides, apoptotic cells, or micro/nanoparticles chemically conjugated with antigenic peptide, as well as antibody fusion constructs, have been used with varying degrees of success (2–8). A challenge in the development of antigen-specific immune intervention is the delivery of the antigenic payload to the correct destination for processing, to establish long-lasting peripheral tolerance. Adding to this challenge, the tolerogenic doses of different antigens vary greatly, when tolerance can be achieved at all. Furthermore, the introduction of nonnative materials, such as micro/nanoparticles, might lead to unpredictable adverse effects.

Apoptotic cells are tolerogenic, presumably by displaying self-antigens in a noninflammatory context to antigen-presenting cells, leading to anergy or deletion of immune effector cells (9). Expansion of the regulatory T-cell compartment may also contribute to curtailing autoimmunity (10). Each second, millions of red blood cells (RBCs) are cleared from the circulation by phagocytic cells in the spleen and by the reticuloendothelial system, without obvious signs of inducing an immune response. We exploit this natural route of RBC removal for induction of tolerance. We have found that transfusion of RBCs, covalently modified with an antigenic payload, can induce antigen-specific tolerance in naïve recipients. We use genetically engineered RBCs—as well as their unmodified counterparts—as substrates for sortase, a transpeptidase, to covalently

attach peptides from disease-relevant autoantigens. This approach has prophylactic and therapeutic efficacy in experimental autoimmune encephalomyelitis (EAE), a mouse model of multiple sclerosis. Similarly, transfusion of RBCs modified with an insulin-derived peptide in the nonobese diabetic (NOD) mouse model of type 1 diabetes (T1D) allows a majority of animals to remain normoglycemic. These results suggest application of this strategy to other autoimmune diseases.

Results

Sortagging Is a Robust, Efficient, and Simple Method to Covalently Modify RBCs Without Compromising Their Biological Properties. An important aspect of our strategy is to preserve the biological properties of labeled RBCs, so that they remain as close to their native state as possible. We used a sortase A-mediated reaction (“sortagging”) to minimize damage to the RBC membrane (11, 12). Sortase A recognizes an LPXTG motif and cleaves the peptide bond between the threonine and glycine residues in this motif to yield a thioester acyl-enzyme intermediate. A nucleophile that contains a suitably exposed N-terminal glycine, (G)_n, can resolve this intermediate, covalently linking the two motifs via a peptide bond (Fig. 1A). We have used retroviral and lentiviral vectors that encode membrane proteins appended with sortase motifs to

Significance

Immune-mediated diseases are prevalent, debilitating, and costly. Unfortunately, current treatments rely on nonspecific immunosuppression, which also shuts down a protective immune response. To circumvent this, we exploited the noninflammatory natural means of clearance of red blood cells (RBCs), in combination with sortase-mediated RBC surface modification to display disease-associated autoantigens as RBCs' own antigens. We found that this strategy holds promise for prophylaxis and therapy, as shown in a mouse model of multiple sclerosis and of type 1 diabetes.

Author contributions: N.P., A.M.B., H.F.L., and H.L.P. designed research; N.P., A.M.B., M.C.W., N.-J.H., Z.L., R.D., D.B., H.L., H.C.P., S.K.D., and T.M. performed research; N.P., A.M.B., H.F.L., and H.L.P. analyzed data; and N.P., H.F.L., and H.L.P. wrote the paper.

Reviewers: D.A.M., Harvard University; M.N., University of Colorado, Denver; and J.Z., University of Wisconsin–Madison.

Conflict of interest statement: H.L.P. serves as a paid consultant and owns equity in Rubies, a company that seeks to apply modified red blood cells for treatment of disease. H.F.L. and H.L.P. serve as advisors and have equity in Rubius, a biotechnology company that seeks to exploit but does not provide financial support for the technology described in this paper.

Freely available online through the PNAS open access option.

¹To whom correspondence may be addressed. Email: lodish@wi.mit.edu or hidde.ploegh@childrens.harvard.edu.

²Present address: Program in Cellular and Molecular Medicine, Boston Children's Hospital, Boston, MA 02115.

This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1701746114/-DCSupplemental.

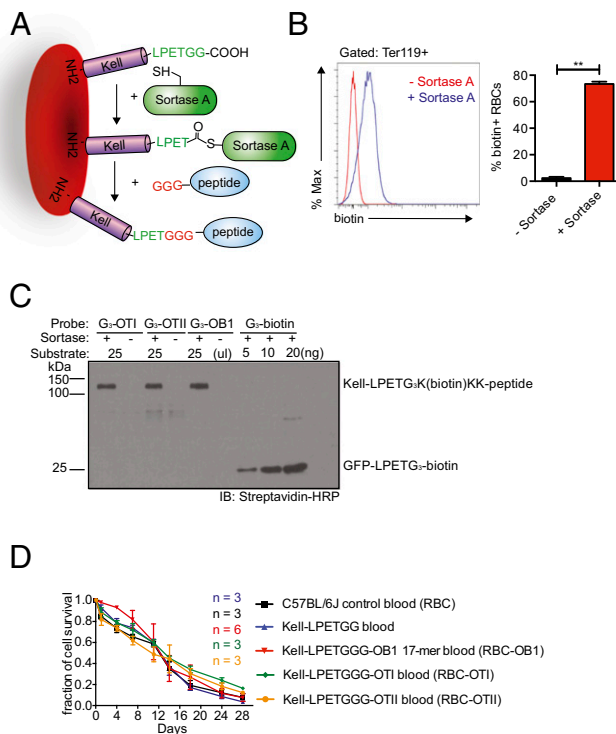


Fig. 1. Designs and characterization of engineered RBCs. (A) Schematic for Kell C-terminal sortase labeling with GGG-carrying antigens peptides. (B) Evaluation and quantification of mature Kell-LPETGG RBCs for sortase labeling by incubation of RBCs with biotin-containing probes in the presence or absence Sortase A. Cytofluorimetry was performed with anti-TER119, a RBC surface marker, and anti-biotin antibodies. ($n = 3$; $**P < 0.01$, unpaired t test with Holm-Sidak adjustment). (C) Quantification of sortase labeling of Kell-LPETGG RBCs with different biotin-containing peptides by immunoblotting (IB) using streptavidin-HRP. GFP-biotin carrying a single biotin/mole of GFP was used a reference. (D) CFSE-labeled RBCs from C57BL/6J and Kell-LPETGG mice were transfused into recipient mice. Kell-LPETGG blood samples were also subjected to sortagging with the three different OVA-derived peptides before transfusion. RBC survival in the circulation was tracked via CFSE fluorescence by flow cytometry.

generate red cells that have variable numbers of sortagable proteins on their surface (12, 13). Because tolerogenic doses vary among different antigens, it is important to have a source of RBCs that can be modified consistently and reproducibly with a known quantity of antigen. To this end we used CRISPR/Cas9 to generate mice whose RBCs carry the Kell protein extended at its C terminus to include an LPETGG motif, referred to here as Kell-LPETGG mice. Kell-LPETGG mice bred to homozygosity for this modification served as blood donors for the transfusion experiments described below (Fig. S1A). Insertion of the sortase motif does not cause hematological abnormalities, as inferred from complete blood count data (Fig. S1B). Kell-LPETGG is expressed under the control of its endogenous promoter, restricting its expression to the RBC compartment. Neither WT RBCs, nor white blood cells isolated from Kell-LPETGG mice could be labeled with $(G)_3$ -K(biotin) in a sortagging reaction, as distinct from Kell-LPETGG RBCs, which were modified with an efficiency of $\sim 80\%$, likely an underestimate (Fig. 1B and Fig. S1C). The conditions for the sortagging reaction are mild and no major damage to RBCs was apparent, as assessed by the absence of PtdSer externalization (Annexin V staining) (Fig. S1D). The morphology of sortagged RBCs, regardless of attached payload, was normal (Fig. S1E).

Using three biotinylated peptides of different sequence, we enumerated the number of sortase-modifiable Kell molecules per

cell. We performed sortagging reactions on 25 μ L of fresh Kell-LPETGG RBCs with GGGK(biotin)KK-OT-I, GGGK(biotin)KK-OT-II, and GGGK(biotin)KK-OB1 peptides as nucleophiles. These peptides represent three different immunodominant peptides of ovalbumin (OVA). They are diverse in length and biophysical properties (see Table S1 for the list of antigenic adducts synthesized and attached to Kell-LPETGG RBCs). Sortagging yields a consistent number of the various biotinylated payloads attached (Fig. 1C). Using monobiotinylated GFP as a reference, we quantified the number of peptides covalently attached to the surface of Kell-LPETGG RBCs; there were $\sim 9,000$ Kell proteins consistently modified per RBC (Fig. 1C).

We next characterized the *in vivo* persistence of sortagged RBCs by assessing their circulatory half-life. We stained the modified RBCs (i.e., RBC-OT-I, RBC-OT-II, and RBC-OB1) with carboxyfluorescein succinimidyl ester (CFSE) before transfusion. Their survival was equal to that of unmodified RBCs, regardless of the identity of the payload attached (Fig. 1D). Because the OB1 peptide linked to Kell is biotinylated, we were able to track its disappearance. Indeed, the loss of the Kell-LPETGGG-K(biotin)KK-OB1 signal obtained by immunoblotting corresponded with the disappearance of CFSE signal (Fig. S24). Modification by sortase therefore does not accelerate removal of engineered RBCs, which retain the attached peptide while in circulation. We hypothesized that the circulatory persistence (> 28 d) of antigen-decorated RBCs creates a window of opportunity for the induction of more complete peripheral tolerance by editing out antigen-specific effector cells.

Engineered RBCs Blunt Specific B, CD4, and CD8 T-Cell Responses.

Autoimmunity can result from abnormal behavior of three major immune effectors: B cells, CD4 T cells, CD8 T cells, or a combination thereof. To eliminate potential variables related to diverse T-cell receptor (TCR) or B-cell receptor repertoires, and the potential for self-reactivity, we used the model protein antigen OVA. There are three clonal derivatives of OVA-specific immune effector

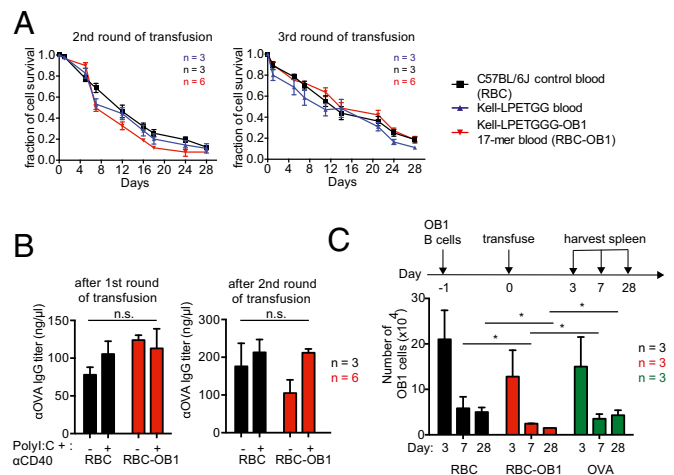


Fig. 2. OB1 peptide-decorated RBCs blunt responses of OB1-specific B cells. (A) CFSE-labeled RBCs from C57BL/6J and Kell-LPETGG mice were transfused into recipient mice. One set of Kell-LPETGG blood samples were also subjected to sortagging with OB1 peptides before transfusion. Following the first transfusion (as in Fig. 1D), the same cohort is subjected to two more transfusions with a 1-wk gap between each transfusion. RBC survival in the circulation was tracked via CFSE fluorescence by flow cytometry. Repeated transfusions of sortagged RBCs into C57BL/6J mice do not induce faster clearance. (B) A cohort of BALB/c mice was transfused with either C57BL/6J or RBC-OB1. OVA-specific IgG titers at the end of each transfusion were measured by ELISA. (C) Flow cytometry of the total number of adoptively transferred OB1 B cells in spleen, harvested 3, 7, and 28 d after RBC, RBC-OB1, or OVA transfusion. $*P < 0.05$; ns, not significant.

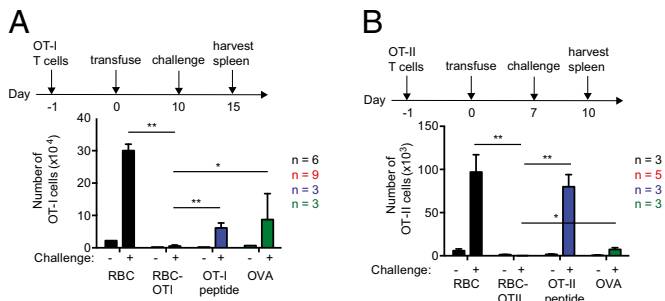


Fig. 3. Engineered RBCs blunt responses of OVA-specific CD4 and CD8 T-cell responses. (A) Total number of OT-I T cells in spleen following a challenge with saline (–) or OT-I peptide/CFA (+) at day 10. (B) Absolute number of OT-II T cells in spleen, 3 d after challenge with saline (–) or OVA/CFA (+) at day 7. Data are shown as mean \pm SD and represent at least two independent experiments. Statistically significant differences are indicated by asterisks: * $P < 0.05$; ** $P < 0.01$ (unpaired t test with Holm–Sidak adjustment).

cells: the CD8 TCR transgenic mouse (OT-I) recognizes the H-2K^b-SIINFEKL complex; the CD4 TCR transgenic mouse (OT-II) recognizes the I-A^b-ISQAVHAAHAEINEAGR complex; and the OVA-specific B-cell transnuclear mouse (OB1) recognizes the FGD-centered epitope contained in the 17-mer FDKLPGFGDSIEAQQGK (14–16).

To determine whether a B-cell-specific OVA-derived epitope can be viewed as self when attached to the surface of a RBC, we attached it to Kell-LPETG RBCs using sortase. Repeated transfusions of CFSE-stained RBC-OB1 into a cohort of C57BL/6J recipients did not accelerate the rate of clearance of OB1-modified RBCs (Figs. 1D and 2A). The multiple transfusions did not elicit an antibody response against intact OVA protein (Fig. S2B). To further test the immunogenicity of RBC-OB1, we carried out repeated transfusions of sortaged RBCs into BALB/c mice, which show a Th2-skewed response, favoring IgG1 production in the presence of the adjuvants polyI:C and anti-CD40, administered intraperitoneally (Fig. S2C). Once again, these multiple transfusions of RBC-OB1 did not elicit an antibody response against intact OVA protein (Fig. 2B and Fig. S2C). Finally, we transferred OB1-specific B cells that recognize and respond to the 17-amino acid OB1 peptide. Transferred OB1 B cells disappeared at a faster rate in mice treated with RBC-OB1 than in animals exposed to OVA or to unmodified RBCs, indicating induction of B-cell tolerance (Fig. 2C).

Next, to determine whether a CD8 T-cell-specific epitope can also be viewed as self when attached to the surface of a RBC, we adoptively transferred CFSE-labeled OT-I T cells, followed by transfusion of RBC-OT-I, RBC or free OT-I peptide 1 d later (Fig. S3A). In mice that received RBC-OT-I, transferred OT-I CD8⁺ T cells showed only modest expansion at first, compared with mice receiving an equivalent number of unmodified RBCs or an equimolar amount of OT-I peptide, as judged from the absolute number of cells recovered from spleen and by CFSE dilution (Fig. S3B and C). OT-I T cells disappeared after several divisions in both RBC-OT-I and OT-I peptide-treated mice, but at day 3 posttransfer, surviving T cells in RBC-OT-I recipients displayed characteristics of nonresponsive (tolerant) cells: they failed to down-regulate CD62L while remaining CD44⁺ (Fig. S3D). Upon *in vitro* restimulation with OT-I peptide, the surviving OT-I T cells produced fewer proinflammatory cytokines, TNF- α , and IFN- γ , than OT-I T cells from mice that received OT-I peptide alone (Fig. S3E). Surviving OT-I T cells showed higher levels of apoptotic and exhaustion markers, such as Fas, PD-1, and LAG-3 (Fig. S3F). Transfusion of RBC-OT-I thus imposes peripheral tolerance in a manner that resembles T-cell exhaustion (17), but may include physical removal as well. By days 6 and 9, far fewer OT-I T cells

were detected in RBC-OT-I-transfused mice than in animals that received control RBCs (Fig. S3B). After a subsequent challenge of mice with OT-I peptide in complete Freund's adjuvant (CFA), a strong adjuvant, the OT-I T cells in mice transfused with RBC-OT-I failed to respond, whereas OT-I T cells in mice injected with an equimolar amount of OT-I peptide, OVA, or an equal number of control RBCs proliferated as expected (Fig. 3A). In the RBC-OT-I-transfused mice we saw no prominent change in the regulatory T-cell compartment (Fig. S3G).

We next assessed whether a similar tolerogenic effect applied to the CD4 T-cell compartment. We adoptively transferred CFSE-labeled OT-II CD4⁺ T cells, followed the next day by transfusion of RBC, RBC-OT-II, or administration of OT-II peptide. Only transfusion of RBC-OT-II led to rapid division of the transferred OT-II T cells, followed by disappearance of the transferred OT-II T cells by day 6 (Fig. S4A–C). The surviving OT-II T cells did not respond to a challenge with OVA in CFA (Fig. 3B). There was little change in the regulatory T-cell compartment for mice transfused RBC-OT-I (Fig. S4G). OT-II T cells in mice that received RBC-OT-II also expressed apoptotic markers and resembled anergic T cells (Fig. S4D–F). Abortive activation or deletion of T and B cells can therefore occur as early as day 3, at a time when >90% of transfused RBCs remain. These results are reminiscent of those obtained through systemic administration of an antigenic payload attached to a peptide adduct designed to bind glycoprotein A noncovalently (7, 18). Based on these three OVA models, we conclude that induction of antigen-specific tolerance by modified RBCs can apply to B, CD4 T cells, and CD8 T cells.

RBCs Carrying MOG_{35–55} Not Only Confer Protection Against EAE but Can Even Reverse Early Clinical Signs of EAE. We next tested the ability of RBCs modified with the immunodominant peptide of myelin oligodendrocyte glycoprotein (MOG; residues 35–55), a major central nervous system protein, to affect the course of EAE. Immunization of C57BL/6 mice with MOG_{35–55} in the presence of CFA and pertussis toxin elicits clinical signs of this multiple sclerosis-like condition within 10–14 d (19). Administration of modified Kell-LPETGG RBCs sortaged with MOG_{35–55} peptide (RBC-MOG_{35–55}) 7 d before induction of disease delayed onset, if not completely suppressed EAE (Fig. 4A). In contrast, all mice that received Kell-LPETGG RBCs sortaged with an irrelevant peptide

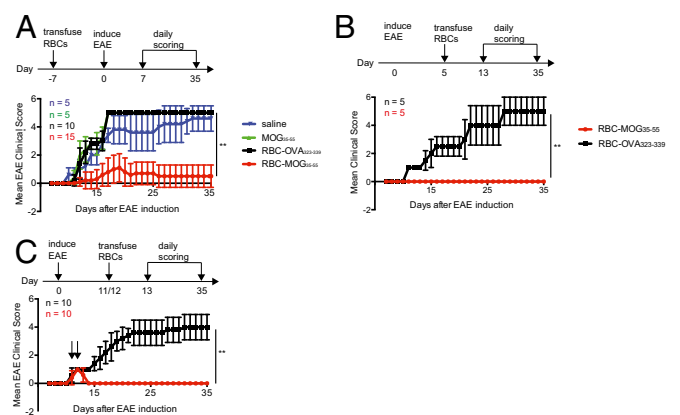


Fig. 4. Engineered RBCs in EAE mouse models. (A) Mean EAE clinical scores of mice subjected to transfusion with RBC-MOG_{35–55}, RBC-OVA_{323–339}, unconjugated MOG_{35–55} peptide, or saline at 7 d before induction of EAE. Mean clinical scores of mice subjected to therapy by transfusion of RBC-MOG_{35–55} (or RBC-OVA_{323–339} as control) into (B) mice at preclinical stage and (C) mice with a clinical EAE score of 1; the black arrows indicate time of RBC administration. ** $P < 0.01$, two-way ANOVA with repeated measures. All pair-wise comparisons were performed; RBC-MOG_{35–55} was shown to be significantly different from other treatment groups.

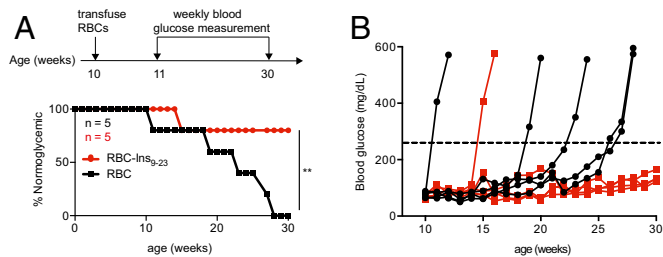


Fig. 5. Engineered RBCs in T1D mouse models. (A) Schematic for prophylactic T1D treatment. Blood glucose levels were measured to monitor T1D progression in NOD mice, considered diabetic when glucose levels were >250 mg/dL, ** $P < 0.01$ (log-rank test). (B) Individual blood glucose level measurement in mice treated with RBC or RBC-Ins₉₋₂₃.

(RBC-OVA₃₂₃₋₃₃₉), unconjugated MOG₃₅₋₅₅ peptide, or saline progressed to severe disease (Fig. 4A). Histology of spinal cord sections confirmed the presence of inflammatory nodules and demyelination in mice treated with RBC-OVA₃₂₃₋₃₃₉ (Fig. S5A and B). Cells that infiltrate the spinal cord of mice transfused with RBC-OVA₃₂₃₋₃₃₉ comprise inflammatory Th1 and Th17 CD4 T cells. Although we noted the presence of Foxp3⁺ regulatory T cells, these failed to suppress disease progression. Spinal cords from RBC-MOG₃₅₋₅₅-treated mice lacked both infiltrating inflammatory and regulatory T cells (Fig. S5C and D).

We examined whether RBC-MOG₃₅₋₅₅ could interfere in the course of EAE by transfusing mice with RBC-MOG₃₅₋₅₅ during the induction period (i.e., at day 5 after disease induction) (Fig. 4B). Although transfusion of RBC-MOG₃₅₋₅₅ prevented EAE, its RBC-OVA₃₂₃₋₃₃₉ counterpart did not (Fig. 4B). To determine whether RBC-MOG₃₅₋₅₅ could reverse incipient EAE, we transfused RBC-MOG₃₅₋₅₅ into mice that had already developed a limp tail (EAE clinical score of 1). Transfusion of RBC-MOG₃₅₋₅₅ into these mice halted progression and alleviated clinical symptoms of EAE (Fig. 4C). This effect—that is, amelioration of EAE symptoms—was rapid. We saw no changes in cellular composition of the inguinal lymph nodes and spleen, but noted a decrease in Th17 cells in spinal cord infiltrates (Fig. S6).

RBCs Carrying Ins₉₋₂₃ Confer Protection Against T1D. We investigated whether this strategy could also be applied to T1D in the NOD/ShiJ mouse model. These mice develop T1D as early as 12 wk of age, as manifested by insulinitis and low pancreatic insulin content (20). Mice are considered diabetic when their plasma glucose levels rise to >250 mg/dL. Insulin B-chain peptide 9–23 (Ins₉₋₂₃) is an immunodominant self-antigen, the recognition of which can mediate autoimmune destruction of pancreatic β -cells and impair insulin production and release (21). A single prophylactic transfusion of Kell-LPETGG RBCs sortagged with the Ins₉₋₂₃ at 10 wk of age protected NOD/ShiJ from T1D in 80% of animals until week 30, whereas injection of unmodified RBCs did not (Fig. 5). Incomplete protection may be because of epitope spreading (22). The pancreas of mice treated with RBC-Ins₉₋₂₃ retained insulin-expressing pancreatic islets at 30 wk of age, even with evident infiltration of CD4 and CD8 T cells, whereas in RBC-treated (control) mice, there were no or only very few pancreatic islets that remained (Fig. S7). We attribute this protection to insulin-specific tolerance. The CD8 and CD4 T cells observed in the pancreas most likely are of other specificities. Identification of an expanded set of autoantigens would thus be required to further improve disease outcome.

Application of the Sortase Modification Strategy to Human RBCs. Translation of this approach to human RBCs could rely on the production of RBCs genetically modified to enable a site-specific sortase-catalyzed modification, as we have done for mouse Kell.

Unfortunately, production of human RBCs from hematopoietic precursors in vitro, genetically modified to display an LPXTG motif at their surface, remains expensive and time-consuming (12, 23, 24). Because completion of the sortase reaction involves a GGG-containing nucleophile, endogenous proteins on the RBC surface that bear an exposed glycine at their N terminus could serve as a nucleophile, and be used in conjunction with a peptide modified with a C-terminal LPETGG sequence (Fig. 6A) (25). To test this theory, we sortagged mouse C57BL/6 RBCs with various LPETGG-containing biotinylated peptides. Using monobiotinylated GFP as reference, we found that $\sim 4,500$ LPET-containing peptides can be attached to normal C57BL/6J mouse RBCs (Fig. 6B). BALB/c RBCs behave similarly in this reaction (Fig. S8A). Moreover, transfusion with RBC-MOG₃₅₋₅₅, C57BL/6 RBCs sortagged with MOG₃₅₋₅₅-LPETGG reversed clinical signs of EAE to levels commensurate with RBCs genetically engineered to contain a sortase motif (Fig. 6C).

Human RBCs likewise possess endogenous proteins that can serve as nucleophiles and resolve the LPET-sortase covalent intermediate. RBCs from unrelated donors sortagged with LPETGG-biotin yielded very similar labeling profiles; $\sim 3,000$ peptides were attached to each human RBC (Fig. 6D and Fig. S8B). As assessed in an immunoblot, sortagging of endogenous RBC proteins yields a similar banding profile for two unrelated donors. In principle, this strategy allows the transfusion of enzymatically modified autologous RBCs within 1 h of obtaining the RBC population.

Discussion

We use sortase to modify the surface of human and mouse RBCs by covalently attaching peptides and other payloads. In one line of

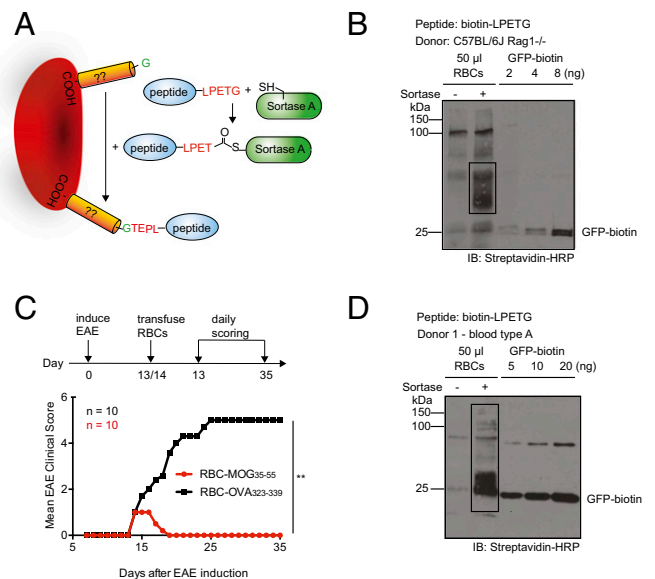


Fig. 6. Translatability of engineered RBCs in human system. (A) Schematic for sortagging of endogenous RBC membrane proteins containing a suitably exposed N-terminal glycine with LPETGG-equipped peptide. (B) Installation of biotin-LPETGG peptide onto endogenous sortase substrates on mouse RBCs, analyzed by SDS PAGE followed by immunoblotting using streptavidin-HRP. Endogenous biotinylated substrates are boxed. (C) Mean EAE clinical scores of mice subjected to therapy by transfusion of C57BL/6J RBCs from Rag2^{-/-} mice, sortagged with MOG₃₅₋₅₅-LPETGG (or C57BL/6J RBCs Rag2^{-/-} as control) into mice with clinical EAE score of 1; time of RBC administration is indicated by the black arrow. ** $P < 0.01$, two-way ANOVA with repeated measures. (D) Installation of biotin-LPETGG peptide onto endogenous sortase substrates on human RBCs type A, analyzed by SDS PAGE followed by immunoblotting using streptavidin-HRP. Endogenous biotinylated substrates are boxed.

experiment, we used CRISPR/Cas9 to introduce into the murine germ line the LPETGG sortase motif at the C terminus of the Kell protein. Fresh red cells from these mice can be incubated with sortase and any payload bearing an N-terminal (G)_n sequence, allowing attachment of ~9,000 payloads per cell. The entire process from bleeding to transfusion takes no more than 60 min.

Our second application uses unmodified mouse or human red cells, and presents a more viable option for a clinical setting. Instead of relying on an added nucleophile equipped with N-terminal glycines, we performed the sortagging reaction by relying on endogenous RBC surface proteins with one or more exposed N-terminal glycines. We provided the antigenic payload as a peptide equipped with an LPXTG extension at its C terminus. Both Kell-LPETGG and unmodified RBCs yield RBCs decorated with several thousand copies of the desired antigen per cell. These red cells confer tolerance not only against OVA, but also against MOG and the immunodominant peptide of insulin. For both EAE and T1D, we achieved prophylaxis, as well as amelioration of clinical signs of disease. Both can confer protection with a single administration.

Several alternative approaches have been used to achieve antigen-specific tolerance in an autoimmune disease setting, with varying degrees of success. DNA vaccination usually requires multiple dosing, at times requiring coadministration of immunosuppressants (26–29). Although efficacious prophylactically, therapeutic efforts present more of a challenge (30). Inadvertent activation of innate immunity caused by the delivery vector, as well as antivector immunity, are additional confounding factors (31).

To achieve tolerance, intravenous peptide delivery necessitates the administration of multiple doses, depending on the disease model examined (32–34). Peptides, proteins, or conjugated peptides (e.g., peptides conjugated to anti-DEC205) delivered systemically do not benefit from specific targeting, as in the case of our sortase-modified RBCs. Oral tolerance likewise requires administration of large amounts of antigen and multiple doses (35–38), but orally administered peptide treatments have so far failed in human clinical trials (39).

Dying cells—including aged RBCs—are phagocytosed by macrophages or dendritic cells, often at specific anatomical locations. The identity and context of phagocytes that ingest the antigen-loaded RBCs could lead to different outcomes, in terms of both antigen presentation and stimulation of an immune response (40–42). Phagocytosis triggers elaborate signals that might either induce tolerance or an immune response (9, 10). Splenocytes or peripheral blood mononuclear cells chemically modified with peptides have been explored as tolerogens, but these require the use of isogenic cells (6, 43, 44). Chemical modification using carbodiimide- or maleimide-based coupling strategies shows considerable variation

in conjugation efficiency, and modify surface proteins without necessarily leading to the formation of the desired adducts (43, 44). Using modifiers that target RBCs noncovalently, such as a module that recognizes glycophorin A, can lead to uneven distribution of the payload by dissociation (7). Cell types other than the intended phagocytes may acquire the antigen, leading to uncertain outcomes (9, 45, 46). When using nanoparticles/microparticles as a vehicle for the delivery of autoantigens (46–49), one must consider delivery to many different sites depending on size and other biophysical properties of these preparations.

In comparing our method to other means of tolerance induction, ours specifically addresses the issue of autoimmunity in a polyclonal setting using enzymatically modified RBCs. An advantage of using Kell-LPETGG RBCs lies in the amount of antigen that can be attached covalently to a well-defined target on the RBC surface, and in a reproducible and controlled manner. Rh-negative, blood group O RBCs could be stockpiled as a source of universal donor RBCs. Given the broad acceptance and safety profile of RBC transfusions, this antigen-specific tolerance strategy promises a lack of adverse effects. Furthermore, our approach offers the use of a wide breadth of antigens, because both natural and synthetic payloads can be attached simply by attaching the necessary sortase motifs. Antigen-decorated RBCs may thus provide a simple means to treat autoimmune disorders without compromising systemic immunity, and we suggest that such modified RBCs deserve further study as possible therapeutic agents. Nonetheless, the very existence of blood-group antigens, such as Kell, underscores the fact that RBCs are not always immunologically inert and that attempts at tolerance induction must be approached on a case-by-case basis.

Materials and Methods

Details of the mouse strains, RBC sortagging protocols, in vivo experimental setup, and other methods (flow cytometry, ELISA, and Western blotting) are provided in the *SI Materials and Methods*. All mice were maintained according to protocols approved by the Massachusetts Institute of Technology Committee on Animal Care.

ACKNOWLEDGMENTS. We thank members of the H.F.L. and H.L.P. laboratories, especially Lenka Kundrat and Jiahai Shi, for discussions; Jessica R. Ingram and Yushu Xie for critical reading of the manuscript; Marko Knoll and Leif S. Ludwig for providing reagents; John Jackson, Tony Chavarria, and Ferenc Reinhardt for mouse husbandry; Tom DiCesare for assistance with illustrations; and Prathapan Thiru and George W. Bell in Bioinformatics and Research Computing (Whitehead Institute) for assistance with statistical analyses. This work was supported by Defense Advanced Research Projects Agency Contract HR0011-12-2-0015 (to H.L.P. and H.F.L.) and grants from the Schlumberger Foundation Faculty for the Future, the Howard Hughes Medical Institute International Student Research Fellowship, and the Siebel Scholarship (to N.P.).

- Feldmann M, Steinman L (2005) Design of effective immunotherapy for human autoimmunity. *Nature* 435(7042):612–619.
- Liblau RS, et al. (1996) Intravenous injection of soluble antigen induces thymic and peripheral T-cells apoptosis. *Proc Natl Acad Sci USA* 93(7):3031–3036.
- Miller SD, Turley DM, Podojil JR (2007) Antigen-specific tolerance strategies for the prevention and treatment of autoimmune disease. *Nat Rev Immunol* 7(9):665–677.
- Luo X, Miller SD, Shea LD (2016) Immune tolerance for autoimmune disease and cell transplantation. *Annu Rev Biomed Eng* 18:181–205.
- Cremel M, Guérin N, Horand F, Banz A, Godfrin Y (2013) Red blood cells as innovative antigen carrier to induce specific immune tolerance. *Int J Pharm* 443(1–2):39–49.
- Lutterotti A, et al. (2013) Antigen-specific tolerance by autologous myelin peptide-coupled cells: A phase 1 trial in multiple sclerosis. *Sci Transl Med* 5(188):188ra75.
- Kontos S, Kouritis IC, Dane KY, Hubbell JA (2013) Engineering antigens for in situ erythrocyte binding induces T-cell deletion. *Proc Natl Acad Sci USA* 110(1):E60–E68.
- Lorentz KM, et al. (2015) Engineered binding to erythrocytes induces immunological tolerance to *E. coli* aspariginase. *Sci Adv* 1(6):e1500112.
- Griffith TS, Ferguson TA (2011) Cell death in the maintenance and abrogation of tolerance: The five Ws of dying cells. *Immunity* 35(4):456–466.
- Green DR, Ferguson T, Zitvogel L, Kroemer G (2009) Immunogenic and tolerogenic cell death. *Nat Rev Immunol* 9(5):353–363.
- 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.
- Shi J, et al. (2014) Engineered red blood cells as carriers for systemic delivery of a wide array of functional probes. *Proc Natl Acad Sci USA* 111(28):10131–10136.
- Srijbis K, Spooner E, Ploegh HL (2012) Protein ligation in living cells using sortase. *Traffic* 13(6):780–789.
- Sterry SJ, Kelly JM, Turner SJ, Carbone FR (1995) T cell receptor V alpha bias can be determined by TCR-contact residues within an MHC-bound peptide. *Immunol Cell Biol* 73(1):89–94.
- Barnden MJ, Allison J, Heath WR, Carbone FR (1998) Defective TCR expression in transgenic mice constructed using cDNA-based alpha- and beta-chain genes under the control of heterologous regulatory elements. *Immunol Cell Biol* 76(1):34–40.
- Dougan SK, et al. (2012) IgG1+ ovalbumin-specific B-cell transnuclear mice show class switch recombination in rare allelically included B cells. *Proc Natl Acad Sci USA* 109(34):13739–13744.
- Wherry EJ, Kurachi M (2015) Molecular and cellular insights into T cell exhaustion. *Nat Rev Immunol* 15(8):486–499.
- Grimm AJ, Kontos S, Diaceri G, Quaglia-Thermes X, Hubbell JA (2015) Memory of tolerance and induction of regulatory T cells by erythrocyte-targeted antigens. *Sci Rep* 5:15907.
- O'Connor RA, et al. (2008) Cutting edge: Th1 cells facilitate the entry of Th17 cells to the central nervous system during experimental autoimmune encephalomyelitis. *J Immunol* 181(6):3750–3754.
- Makino S, et al. (1980) Breeding of a non-obese, diabetic strain of mice. *Jikken Dobutsu* 29(1):1–13.
- Eisenbarth GS (2003) Insulin autoimmunity: immunogenetics/immunopathogenesis of type 1A diabetes. *Ann N Y Acad Sci* 1005:109–118.

22. Unanue ER (2014) Antigen presentation in the autoimmune diabetes of the NOD mouse. *Annu Rev Immunol* 32:579–608.
23. 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.
24. Giarratana MC, et al. (2011) Proof of principle for transfusion of in vitro-generated red blood cells. *Blood* 118(19):5071–5079.
25. Swee LK, Lourido S, Bell GW, Ingram JR, Ploegh HL (2015) One-step enzymatic modification of the cell surface redirects cellular cytotoxicity and parasite tropism. *ACS Chem Biol* 10(2):460–465.
26. Ruiz PJ, et al. (1999) Suppressive immunization with DNA encoding a self-peptide prevents autoimmune disease: Modulation of T cell costimulation. *J Immunol* 162(6):3336–3341.
27. Garren H, et al. (2001) Combination of gene delivery and DNA vaccination to protect from and reverse Th1 autoimmune disease via deviation to the Th2 pathway. *Immunity* 15(1):15–22.
28. Kang Y, et al. (2012) Treg cell resistance to apoptosis in DNA vaccination for experimental autoimmune encephalomyelitis treatment. *PLoS One* 7(11):e49994.
29. Ferrera F, et al. (2007) Gene vaccination for the induction of immune tolerance. *Ann N Y Acad Sci* 1110:99–111.
30. Fissolo N, et al. (2012) Treatment with MOG-DNA vaccines induces CD4+CD25+FoxP3+ regulatory T cells and up-regulates genes with neuroprotective functions in experimental autoimmune encephalomyelitis. *J Neuroinflammation* 9:139.
31. Baker D, Hankey DJ (2003) Gene therapy in autoimmune, demyelinating disease of the central nervous system. *Gene Ther* 10(10):844–853.
32. Ring S, Maas M, Nettelbeck DM, Enk AH, Mahnke K (2013) Targeting of autoantigens to DEC205+ dendritic cells in vivo suppresses experimental allergic encephalomyelitis in mice. *J Immunol* 191(6):2938–2947.
33. Wadwa M, Klopffleisch R, Buer J, Westendorf AM (2016) Targeting antigens to Dec-205 on dendritic cells induces immune protection in experimental colitis in mice. *Eur J Microbiol Immunol (Bp)* 6(1):1–8.
34. Aichele P, et al. (1994) Peptide-induced T-cell tolerance to prevent autoimmune diabetes in a transgenic mouse model. *Proc Natl Acad Sci USA* 91(2):444–448.
35. al-Sabbagh A, Miller A, Santos LM, Weiner HL (1994) Antigen-driven tissue-specific suppression following oral tolerance: Orally administered myelin basic protein suppresses proteolipid protein-induced experimental autoimmune encephalomyelitis in the SJL mouse. *Eur J Immunol* 24(9):2104–2109.
36. Park MJ, et al. (2008) Indoleamine 2,3-dioxygenase-expressing dendritic cells are involved in the generation of CD4+CD25+ regulatory T cells in Peyer's patches in an orally tolerized, collagen-induced arthritis mouse model. *Arthritis Res Ther* 10(1):R11.
37. Teitelbaum D, Arnon R, Sela M (1999) Immunomodulation of experimental autoimmune encephalomyelitis by oral administration of copolymer 1. *Proc Natl Acad Sci USA* 96(7):3842–3847.
38. Wang X, et al. (2015) Plant-based oral tolerance to hemophilia therapy employs a complex immune regulatory response including LAP+CD4+ T cells. *Blood* 125(15):2418–2427.
39. Weiner HL (2004) Current issues in the treatment of human diseases by mucosal tolerance. *Ann N Y Acad Sci* 1029:211–224.
40. Banz A, Cremel M, Rembert A, Godfrin Y (2010) In situ targeting of dendritic cells by antigen-loaded red blood cells: A novel approach to cancer immunotherapy. *Vaccine* 28(17):2965–2972.
41. Calabro S, et al. (2016) Bridging channel dendritic cells induce immunity to transfused red blood cells. *J Exp Med* 213(6):887–896.
42. Richards AL, Hendrickson JE, Zimring JC, Hudson KE (2016) Erythrophagocytosis by plasmacytoid dendritic cells and monocytes is enhanced during inflammation. *Transfusion* 56(4):905–916.
43. Turley DM, Miller SD (2007) Peripheral tolerance induction using ethylenecarbodiimide-fixed APCs uses both direct and indirect mechanisms of antigen presentation for prevention of experimental autoimmune encephalomyelitis. *J Immunol* 178(4):2212–2220.
44. Getts DR, et al. (2011) Tolerance induced by apoptotic antigen-coupled leukocytes is induced by PD-L1+ and IL-10-producing splenic macrophages and maintained by T regulatory cells. *J Immunol* 187(5):2405–2417.
45. Miyake Y, et al. (2007) Critical role of macrophages in the marginal zone in the suppression of immune responses to apoptotic cell-associated antigens. *J Clin Invest* 117(8):2268–2278.
46. Hardy CL, et al. (2013) Differential uptake of nanoparticles and microparticles by pulmonary APC subsets induces discrete immunological imprints. *J Immunol* 191(10):5278–5290.
47. Getts DR, et al. (2012) Microparticles bearing encephalitogenic peptides induce T-cell tolerance and ameliorate experimental autoimmune encephalomyelitis. *Nat Biotechnol* 30(12):1217–1224.
48. Cappellano G, et al. (2014) Subcutaneous inverse vaccination with PLGA particles loaded with a MOG peptide and IL-10 decreases the severity of experimental autoimmune encephalomyelitis. *Vaccine* 32(43):5681–5689.
49. Hunter Z, et al. (2014) A biodegradable nanoparticle platform for the induction of antigen-specific immune tolerance for treatment of autoimmune disease. *ACS Nano* 8(3):2148–2160.
50. Maruyama T, et al. (2015) Increasing the efficiency of precise genome editing with CRISPR-Cas9 by inhibition of nonhomologous end joining. *Nat Biotechnol* 33(5):538–542.
51. Akbarpour M, et al. (2015) Insulin B chain 9-23 gene transfer to hepatocytes protects from type 1 diabetes by inducing Ag-specific FoxP3+ Tregs. *Sci Transl Med* 7(289):289ra81.
52. 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.
53. Hirakawa H, Ishikawa S, Nagamune T (2012) Design of Ca2+-independent *Staphylococcus aureus* sortase A mutants. *Biotechnol Bioeng* 109(12):2955–2961.
54. Avalos AM, et al. (2014) Monovalent engagement of the BCR activates ovalbumin-specific transnuclear B cells. *J Exp Med* 211(2):365–379.
55. Mikel UV, ed (1994) *Advanced Laboratory Methods in Histology and Pathology* (Armed Forces Institute of Pathology, American Registry of Pathology, Washington, DC).