Correction

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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.

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

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Current therapies for autoimmune diseases rely on traditional immunosuppressive medications that expose patients to an increased risk of opportunistic infections and other complications. Immune-regulatory 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 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 transfected 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 (TID) 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 retrorval 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.
generate red cells that have variable numbers of sortaggable peptides 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 expression to the RBC compartment. Neither WT RBCs, nor white blood cells isolated from Kell-LPETGG mice could be labeled with (G)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 \( \mu L \) 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 biological properties (see Table S1 for the list of antigenic adducts synthesized and attached to Kell-LPETG 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. S2A). 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...
cells; the CD8 TCR transgenic mouse (OT-I) recognizes the H-2Kb-SINFEKL complex; the CD4 TCR transgenic mouse (OT-II) recognizes the I-A^b-ISQAVHAAHAEINEAGR complex; and the OVA-specific B-cell transmutational mouse (OBI1) recognizes the FGD-centered epitope contained in the 17-mer FDKLPG.

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 transusions of CFSE-stained RBC-OBI1 into a cohort of C57BL/6j recipients did not accelerate the rate of clearance of OBI-modified RBCs (Figs. 1D and 24). The multiple transusions did not elicit an antibody response against intact OVA protein (Fig. S2B). To further test the immunogenicity of RBC-OBI1, we carried out repeated transusions of sortaged RBCs into BALB/c mice, which show a Th2-skewed response, favoring IgG1 production in the presence of the adjuvants polyIC and anti-CD40, administered intraperitoneally (Fig. S2C). Once again, these multiple transusions of RBC-OBI1 did not elicit an antibody response against intact OVA protein (Fig. 2B and Fig. S2C). Finally, we transferred OBI1-specific B cells that recognize and respond to the 17-amino acid OBI1 peptide. Transferred OBI1 B cells disappeared at a faster rate in mice treated with RBC-OBI1 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. S3 B 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 posttransfusion, 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).

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 transfused OT-II T cells, followed by disappearance of the transfused 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. S4 D–F). Activative 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 glycofarin 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 MOG35-55 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 (residues 35–55) was shown to be effective in disease suppression (15). 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.

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When we transfused RBCs with MOG35-55, the disease score at day 10 was statistically similar to the score in untransfused mice (Fig. 4A). However, when RBCs with MOG35-55 were transfused 1 d after disease induction, we observed a marked suppression of disease severity (Fig. 4B). In contrast, mice transfused with RBCs with OVA or MOG35-55 alone did not show a similar reduction in disease severity (Fig. 4C).

Fig. 4. Engineered RBCs in EAE mouse models. (A) Mean EAE clinical scores of mice subjected to transfusion with RBC-MOG35-55, RBC-OVA32-33-39, unconjugated MOG35-55 peptide, or saline at 7 d before induction of EAE. Mean clinical scores of mice subjected to therapy by transfusion of RBC-MOG35-55 (or RBC-OVA32-33-39 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-MOG35-55 was shown to be significantly different from other treatment groups.
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. (B) Individual blood glucose level measurement in mice treated with RBC or RBC-InsB23.

RBCs Carrying InsB23 Confer Protection Against T1D. We investigated whether this strategy could also be applied to T1D in the NOD/ShiLtJ mouse model. These mice develop T1D as early as 12 wk of age, as manifested by insulitis 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 (InsB23) 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 InsB23 at 10 wk of age protected NOD/ShiLtJ 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 pancreases of mice treated with RBC-InsB23 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 translation of this approach to human RBCs could be achieved by using the Sortase modification strategy. This strategy allows the transfusion of enzymatically modified autologous RBCs from 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
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 a N-terminal (Glu) 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 sortaging 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 TID, 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, these approaches are poorly tolerated in the clinic (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). Signals for either triggering or suppressing signals that might either induce antigen-specific tolerance or an immune response (9, 10). Splenocytes or peripheral blood mononuclear cells chemically modified with peptides have been explored as tolerogenes, 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 glycoporphin 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 poly-clonal 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 controllable 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.

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