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. Immunoregulatory interventions that act proactively 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, CD8+ 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.

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.


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generate red cells that have variable numbers of sortaggable 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 terminus to include an LPETGG motif, referred to here as Kell-LPETGGG mice, which were modified with an efficiency of ∼80%, likely an underestimate (Fig. 1B). 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 ∼80%, 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 μL 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-LPETGG RBCs). Sorting 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 ∼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 carboxylfluorescein 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-SIINFEKL complex; the CD4 TCR transgenic mouse (OT-II) recognizes the I-A*–ISQAVHAAHAEINEAGR complex; and the OVA-specific B-cell transnuclear mouse (OB1) recognizes the FGF-centered epitope contained in the 17-mer FDKLPGFDGDSIAQGGK (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. 2B). To further test the immunogenicity of RBC-OB1, we carried out repeated transfusions of sortagged 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 transfusions of RBC-OB1 did not elicit an antibody response against intact OVA protein (Fig. 2B and Fig. S2C). Finally, we transfused OB1-specific B cells that recognize and respond to the 17-amino acid OBI 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 transfused 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, transfused OT-I 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 posttransfusion, surviving T cells in RBC-OT-I recipients displayed characteristics of nonresponsive (tolerant) cells: they failed to down-regulate C6d2L, 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 transfused 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. S4 A–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). Abortive activation or deletion of T 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 glycoporphin 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 MOG35–55 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 sortagged with MOG35–55 peptide (RBC-MOG35–55) 7 d before induction of disease delayed onset, if not completely suppressed EAE (Fig. 4A). In contrast, all mice that received Kell-LPETGG RBCs sortagged with an irrelevant peptide...
transfused RBCs, analyzed by SDS PAGE followed by immunoblotting using streptavidin-biotinylated biotinylated substrates are boxed.

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. 6 A) (25).

To test this theory, we sortaged mouse C57BL/6 RBCs with various LPETGG-containing biotinylated peptides. Using monobiotinylated GFP as reference, we found that ~4,500 LPET-containing peptides could be attached to normal C57BL/6 mouse RBCs (Fig. 6 B). BALB/c RBCs behave similarly in this reaction (Fig. S8A). Moreover, transfusion with RBC-MOG35-55, C57BL/6 RBCs sortaged with MOG35-55-LPETGG reversed clinical signs of EAE to levels commensurate with RBCs genetically engineered to contain a sortase motif (Fig. 6 C).

Human RBCs likewise possess endogenous proteins that can serve as nucleophiles and resolve the LPET-sortase covalent intermediate. RBCs from unrelated donors sortaged with LPETGG-biotin yielded very similar labeling profiles; ~3,000 peptides were attached to each human RBC (Fig. 6 D and Fig. S8B). As assessed in an immunoblot, sortaging 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

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 insulinis and low pancreatic insulin content (20). Mice are considered diabetic when their plasma glucose levels rise to >250 mg/dL, **P < 0.01 (log-rank test). (B) Individual blood glucose level measurement in mice treated with RBC or RBC-InsB23, 23.

Unconjugated MOG35-55 peptide, or saline progressed to severe disease (Fig. 4 A). Histology of spinal cord sections confirmed the presence of inflammatory nodules and demyelination in mice treated with RBC-OVA233-399 (Fig. S5 A and B). Cells that infiltrate the cord of mice trans fused with RBC-OVA233-399 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. S5 C 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. 4 B). Although transfusion of RBC-MOG35-55 prevented EAE, its RBC-OVA233-399 counterpart did not (Fig. 4 B). 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. 4 C). 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).

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.
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, therapy is poorly effective for preclinical disease and may offer 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). Given the wriggling of elaborate signals that might drive antigen-specific 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 cell lines (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 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.

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