Engineered red blood cells (RBCs) expressing viral receptors could be used therapeutically as viral traps, as RBCs lack nuclei and other organelles required for viral replication. However, expression of viral receptors on RBCs is difficult to achieve since mature erythrocytes lack the cellular machinery to synthesize proteins. Herein, we show that the combination of a powerful erythroid-specific expression system and transgene codon optimization yields high expression levels of the HIV-1 receptors CD4 and CCR5, as well as a CD4-glycophorin A (CD4-GpA) fusion protein in erythroid progenitor cells, which efficiently differentiated into enucleated RBCs. HIV-1 efficiently entered RBCs that co-expressed CD4 and CCR5, but viral entry was not required for neutralization, as CD4 or CD4-GpA expression in the absence of CCR5 was sufficient to potently neutralize HIV-1 and prevent infection of CD4+ T cells in vitro due to the formation of high-avidity interactions with trimeric HIV-1 Env spikes on virions. To facilitate continuous large-scale production of RBC viral traps, we generated erythroblast cell lines stably expressing CD4-GpA or ACE2-GpA fusion proteins, which produced potent RBC viral traps against HIV-1 and SARS-CoV-2. Our in vitro results suggest that this approach warrants further investigation as a potential treatment against acute and chronic viral infections.

RESULTS

Enucleated RBCs express HIV-1 receptors
We used an in vitro differentiation protocol to differentiate human CD34+ hematopoietic stem cells (HSCs) into reticulocytes, an immature form of enucleated RBC that still contains ribosomal RNA.
At the end of the proliferation phase, erythroid progenitor cells were transduced using lentiviral vectors carrying CD4 or CCR5 transgenes by spinoculation (Figure 1A; Figure S1A). We also evaluated expression of a CD4-GpA fusion protein that contained the extracellular CD4 D1D2 domains fused to the N terminus of GpA, an abundantly expressed RBC protein. Three days post-transduction, transgene expression was evaluated by flow cytometry. Expression was low for all transgenes when the cytomegalovirus (CMV) promoter or alternative ubiquitous promoters were used (Figure 1B; Figure S1B). Surprisingly, CD4-GpA expressed only marginally better than CD4, suggesting that additional strategies are required to achieve robust expression of viral receptors on RBCs.

To evaluate whether transcriptional silencing can be prevented by using an erythroid-specific promoter, transgenes were subcloned into the CCL-βAS3-FB lentiviral vector, which contains regulatory elements that support the high expression levels of β-globin during erythroid development (vectors β-CD4, β-CD4-GpA, and β-CCR5) (Figure S1A). CD4 expression was greatly enhanced by this expression system, and CCR5 expression increased to a lesser extent, but CD4-GpA expression was not improved (Figure 1B).

We hypothesized that the limited availability of ribosomes and transfer RNAs potentially restricts transgene expression in differentiating erythroid cells. Transgene cDNA sequences were codon-optimized to generate β-CD4opt, β-CD4-GpAopt, and β-CCR5opt. For all transgenes, codon optimization drastically enhanced expression levels (Figure 1B). These results demonstrated that the combination of a powerful erythroid-specific promoter and transgene codon optimization yields high expression levels of HIV-1 receptors in erythroid cells.

Genetically engineered CD4+/CCR5+ erythroid progenitor cells differentiated efficiently into enucleated RBCs (Figure 1C). At differentiation, almost 90% of cells expressed GpA, of which >80% did not stain for the nuclear dye Hoechst, suggesting that the majority of cells were enucleated RBCs (Figure 1C). May-Grünwald-Giemsa staining confirmed that most cells had lost their nuclei (Figure 1D). Approximately one-third of the enucleated RBCs expressed CD4 and CCR5 on their surface (Figure 1E) at levels comparable to Rev-A3R5 CD4+ T cells (Figure S2). Similar CD4+ T cell lines have been shown to express ~105 copies of CD4 and ~103–104 copies of CCR5, providing a means to estimate receptor copy numbers on engineered RBCs.

HIV-1 enters RBC viral traps
To evaluate the efficacy of RBC viral traps against HIV-1, we generated RBCs that expressed CD4 with and without CCR5 or CD4-GpA with and without CCR5 (Figure 2A) and used the β-lactamase (BlaM) fusion assay to evaluate whether HIV-1 can enter RBC viral traps through attachment of HIV-1 Env spikes to the receptors presented on the RBC surface and subsequent fusion of the viral and RBC membranes. RBCs were incubated with a CCR5-tropic HIV-1 YU2 pseudovirus carrying a BlaM-Vpr fusion protein that enters cells upon infection. When infected cells are exposed to the fluorescence resonance energy transfer (FRET) substrate CCF2-AM, BlaM cleaves the β-lactam ring in CCF2-AM, resulting in a shift of its emission spectrum from green (520 nm) to blue (447 nm). Whereas viral entry events were ≤0.3% in control RBCs and CD4-RBCs, entry was detected in 6.1% of CD4-CCR5-RBCs, suggesting that RBC viral traps that present both receptors can entrap the virus (Figure 2D; Figure S3A). Since only one-third of these RBCs expressed both receptors (Figure 1E), this corresponds to infection of almost 20% of CD4-CCR5-RBCs. CCR5 expression on enucleated RBCs was slightly higher than on nucleated cells; thus, it is unlikely that...
HIV-1 preferentially entered the small number of remaining nucleated cells (Figure S3B). Higher rates of viral entry were observed for RBCs that co-expressed CD4 and the alternative HIV-1 co-receptor CXCR4 after incubation with a CXCR4-tropic HIV-1 HxBc2 pseudovirus (Figure 2C; Figure S4A). However, lower frequencies of viral entry were detected for RBCs that co-expressed the CD4-GpA fusion protein and CCR5 or CXCR4 (Figures 2B and 2C), and addition of the CD4 D3D4 domains to CD4-GpA did not improve viral entry efficiency (Figure S4B). Unlike CD4, GpA does not localize to lipid raft subdomains,18 and thus we speculate that these low rates of viral entry resulted from a lack of co-localization between CD4-GpA and the CCR5 and CXCR4 co-receptors.

RBC viral traps potently neutralize HIV-1 in vitro

We assessed the therapeutic potential of RBC viral traps using a modified version of the HIV-1 TZM-bl neutralization assay19 (Figure 3A). After incubating RBCs with HIV-1 YU2 pseudovirus, samples were centrifuged to remove RBCs and virions that attached to or infected RBCs. Supernatants containing free virions that had not been captured by RBCs were transferred to 96-well plates and TZM-bl cells were added to measure infectivity. In three independent assays, CD4-GpA-RBCs neutralized HIV-1 YU2 most potently at an average half-maximal inhibitory concentration (IC50) of 1.9 × 10^6 RBCs/mL (Figure 3B; Table 1). This concentration is equivalent to 0.04% of the RBC concentration of human blood (5.5 × 10^10 RBCs/mL), suggesting that it would be feasible to achieve therapeutic concentrations in vivo.

CD4-GpA-RBCs were 3-fold more potent than CD4-RBCs, likely due to higher expression levels (Figure 2A; Figure S5). While CCR5 co-expression had no impact on the potency of CD4-GpA-RBCs, co-expression of CCR5 lowered the neutralization activity of CD4-CCR5-RBCs by almost 3-fold in comparison to CD4-RBCs (Figure 3B; Table 1), implying that HIV-1 infection of RBC viral traps was not required for potent neutralization. CCR5 co-expression slightly lowered CD4 expression levels (Figure 2A), potentially explaining the observed drop in potency. However, these results do not exclude the possibility that CCR5 expression on RBC viral traps would have beneficial effects in vivo.

We previously showed that virus-like nanoparticles presenting clusters of CD4 (CD4-VLPs) that formed high-avidity interactions with
trimeric HIV-1 Env spikes on virions potently neutralized a diverse panel of HIV-1 strains and prevented viral escape in vitro. To confirm that RBC viral traps can also form high-avidity interactions with Env, we evaluated neutralization against a mutant HIV-1 YU2 Env G471R pseudovirus that was resistant to monomeric soluble CD4, but was sensitive to CD4-VLPs. CD4-GpA-RBCs potently neutralized the HIV-1 YU2 G471R pseudovirus (IC50 of 1.0/10^7 RBCs/mL) (Figure 3C), suggesting that RBC viral traps and CD4-VLPs would be similarly effective in preventing viral escape through formation of high-avidity interactions with HIV-1 Env spikes.

RBC viral traps prevent infection of CD4+ T cells in vitro

The ability of RBC viral traps to protect HIV-1 target cells from infection was evaluated by co-culturing control RBCs or CD4-GpA-RBCs with Rev-A3R5 CD4+ T cells, a reporter cell line that expresses luciferase upon HIV-1 infection (Figure 4A). RBCs, CD4+ T cells, and HIV-1 pseudovirus were co-incubated at RBC-to-T cell ratios of 2:1 and 5:1 overnight under shaking conditions. The pseudovirus was removed by centrifugation and the cells were re-suspended in Rev-A3R5 CD4+ T cell media to permit outgrowth of CD4+ T cells. After 36 h, luminescence was measured to determine whether the presence of RBC viral traps prevented infection of CD4+ T cells. While control RBCs had no effect, CD4-GpA-RBCs lowered infection rates by 50% and 70%, respectively, demonstrating that RBC viral traps can effectively prevent infection of HIV-1 target cells at RBC/T cell ratios that are ~1,000-fold lower than typically found in human blood (~5,000:1) (Figure 4B). Since HIV-1 did not efficiently enter CD4-GpA-RBCs (Figure 2B), these findings also suggest that high-avidity binding of HIV-1 virions to RBC viral traps is sufficient to prevent attached virions from infecting target cells.

Erythroblast cell lines stably express viral receptors and continuously produce RBC viral traps against HIV-1 and SARS-CoV-2

To generate a renewable and cost-effective source of RBC viral traps, we engineered the immortalized BEL-A erythroblast cell line to stably express high levels of CD4-GpA (Figure 5A). The BEL-A/CD4-GpA cells efficiently differentiated into enucleated RBCs, as >50% of CD71-expressing cells did not stain for the nuclear marker DRAQ5 (Figure 5B). After differentiation, CD71+/DRAQ5 RBCs were purified using fluorescence-activated cell sorting (FACS). Most RBCs still expressed CD4-GpA (Figure 5C) and potently neutralized HIV-1 YU2 in vitro (IC50 of 2.1/10^7 RBCs/mL) (Figure 5D). Independent replicates of in vitro differentiation of BEL-A/CD4-GpA cells achieved comparable yields of RBC viral traps (Figures 5B and 5C; Figure S6), suggesting that engineered erythroblast cell lines could be used to continuously produce potent RBC viral traps against HIV-1. However, overall production yields would also depend on the quality of the RBCs, as the viability of BEL-A cells decreases to ~80% at the end of differentiation and cells could also get damaged during the purification process. To ensure complete removal of nucleated cells for in vivo studies, the RBC viral traps could be further purified using leukoreduction filters and/or gamma irradiation.

To evaluate whether RBC viral traps could be effective against other viruses, we generated a BEL-A cell line that continuously produces RBC viral traps against SARS-CoV-2, the virus that caused the ongoing COVID-19 pandemic. BEL-A cells were transduced to
stably express a chimeric ACE2-GpA protein containing the extracellular domain of the SARS-CoV-2 receptor ACE2 fused to GpA (Figure 6A). Differentiation efficiency and transgene expression on sorted CD71+/DRAQ5− RBCs were comparable to the BEL-A/CD4-GpA cell line (Figures 6B and 6C). Importantly, lentivirus-based SARS-CoV-2 pseudovirus was highly susceptible to ACE2-GpA-RBC neutralization (IC50 of 7 × 10^8 RBCs/mL) (Figure 6D), suggesting that RBC viral traps have the potential to be effective anti-viral agents against a range of viruses.

DISCUSSION

In summary, we described engineering strategies that facilitate efficient and continuous production of potent RBC viral traps against HIV-1 and SARS-CoV-2. HIV-1 efficiently entered engineered RBCs expressing HIV-1 receptors, and RBC viral traps potently neutralized the virus in vitro, thus demonstrating the desired properties of a viral trap.

A number of techniques have been developed to attach proteins to the RBC surface for therapeutic applications, including chemical conjugation and affinity targeting to RBC membrane proteins. However, genetic manipulation of RBCs has been challenging due to the loss of cellular organelles during erythroid maturation. RBC surface expression of chimeric proteins containing single-domain antibodies (VHHs) fused to RBC membrane proteins GpA and Kell has been achieved through lentiviral transduction of RBC precursor cells followed by in vitro differentiation into reticulocytes. While shown to be safe and effective in animal models, all of these methods are limited to RBC surface presentation of soluble or single-pass transmembrane proteins, and important properties of membrane proteins such as localization to specific plasma membrane subdomains and ligand-induced conformational changes and signal transduction activity may not be retained. Moreover, our results showed that fusing the extracellular domain of CD4 to GpA resulted in low surface expression levels in the absence of other optimization steps. However, the combination of an erythroid-specific promoter and transgene codon optimization greatly enhanced CD4-GpA expression and achieved similar expression of wild-type CD4. Importantly, this approach enabled RBC surface expression of the multi-pass transmembrane proteins CCR5 and CXCR4. To the best of our knowledge, this is the first demonstration of robust expression of unmodified non-erythroid transmembrane proteins on the surface of enucleated RBCs. The described engineering strategies could potentially be applied to any type of transmembrane protein and could be widely applicable to genetically engineering therapeutic RBCs.

HIV-1 pseudovirus entered engineered RBCs more efficiently when CCR5 and CXCR4 were co-expressed with wild-type CD4 rather than chimeric CD4-GpA, thus demonstrating that protein modifications that have been used to enhance RBC surface expression can affect the functionality of the therapeutic protein. A lack of co-localization of CD4-GpA and co-receptors could be the cause of the low entry rates, as CD4 and CCR5 have been shown to co-localize in lipid raft microdomains and GpA is not typically associated with lipid rafts. It is also possible that substitution of the membrane-proximal extracellular, transmembrane, or cytoplasmic domains of CD4 interfered with the ability of CD4-GpA to initiate the interaction between HIV-1 Env and co-receptors.

Expression of CD4 or the CD4-GpA fusion protein in the absence of CCR5 was sufficient to potently neutralize HIV-1 in vitro due to formation of high-avidity interactions between clusters of CD4 or CD4-GpA on the RBC surface and trimeric HIV-1 Env spikes on virions. RBC viral traps expressing CD4-GpA also reduced HIV-1 infection rates of CD4+ T cells, suggesting that viral attachment to RBC viral traps effectively prevents HIV-1 virions from infecting target cells. We previously showed that such high-avidity interactions enhanced the potency of CD4-VLPs by >10,000-fold in comparison to conventional CD4-based inhibitors such as soluble CD4 and CD4-immunoglobulin (Ig), and that HIV-1 was unable to escape against CD4-VLPs in vitro. In contrast to CD4-VLPs that have short in vivo half-lives, RBC viral traps could persist in vivo for months, implying the RBC approach has the potential to provide sustained control of HIV-1 infection. RBC viral traps neutralized HIV-1 in vitro at 2,500-fold lower concentrations than the concentration of total RBCs in human blood and reduced HIV-1 infection of CD4+ T cells by 70% at an RBC-to-T cell ratio of 5:1. Given that RBCs outnumber CD4+ T cells by ~5,000:1 in the blood and CD4+ T cell lines are more permissive than natural CD4+ T cells, these results suggest that therapeutic concentrations of RBC viral traps could be achieved in vivo.

Erythroblast cell lines that stably express therapeutic proteins represent a renewable and more cost-effective source for large-scale manufacturing of genetically engineered RBCs than CD34+ HSCs. BEL-A cell lines that stably expressed CD4-GpA and ACE2-GpA efficiently differentiated into potent RBC viral traps against HIV-1 and the pandemic SARS-CoV-2 virus, respectively, suggesting that RBC viral traps could be effective treatments against a diverse range of viruses. RBC viral traps could become a rapid-response treatment strategy for future viral outbreaks, as erythroblast cell lines could be

### Table 1. Neutralization potencies of RBC viral traps

| Engineered RBCs | IC50 (×10^8 RBCs/mL) |
|-----------------|----------------------|
| Control RBCs    | N/D                  |
| CD4-GpA-RBCs    | 7.8, 3.3, 6.9, 6.0, 2.2 |
| CD4-CCR5-RBCs   | 19.5, 5.9, 23, 16, 8.9 |
| CD4-GpA-CCR5-RBCs | 3.0, 0.9, 1.7, 1.9, 1.1 |
| Control RBCs    | N/D                  |

IC50 from three independent in vitro neutralization assays from three independent in vitro RBC differentiations are shown as ×10^8 RBCs/mL for control RBCs, CD4-RBCs, CD4-CCR5-RBCs, CD4-GpA-RBCs, and CD4-GpA-CCR5-RBCs. Arithmetic mean IC50 and standard deviations (SD) derived from the three experiments are shown. N/D, no data.
rapidly developed once a host receptor for a pandemic virus has been identified.

In vivo studies will be required to evaluate the safety and efficacy of RBC viral traps, and a number of potential issues need to be addressed. First, it has been shown that reticulocytes generated by in vitro differentiation mature in vivo into biconcave erythrocytes, but it needs to be determined whether surface expression of viral receptors is affected by this final maturation step in vivo. Second, the half-life of genetically modified RBCs expressing chimeric VHH-GpA/Kell proteins was comparable to control RBCs following intravenous injection in mice, but it is possible that surface expression of viral receptors would shorten the half-life of RBC viral traps. Third, in the case of CD4 presentation on RBCs, unintended interactions with antigen-presenting cells could have negative implications for the immune system. Fourth, surface presentation of antigens on RBCs has been shown to induce antigen-specific immune tolerance, so it needs to be investigated whether attachment of viruses to RBC viral traps has detrimental effects on anti-viral immune responses. In vivo experiments could address these questions and also elucidate whether entrapment of HIV-1 through co-expression of CCR5 has beneficial effects for viral control and whether entrapped viruses could still infect macrophages following phagocytosis of RBC viral traps. Finally, the ability of genetically engineered RBCs to remove circulating viruses and other pathological agents needs to be compared to other approaches such as nanoparticles coated with cellular membrane and RBCs modified through conventional techniques.

MATERIALS AND METHODS

**In vitro CD34+ HSC differentiation**

Human cord blood or mobilized peripheral blood CD34+ HSCs (STEMCELL Technologies) were differentiated into enucleated RBCs using a modified version of a previously described protocol. Briefly, CD34+ HSCs were cultured in expansion medium (100 ng/mL recombinant human Flt3 [rhFlt3], 100 ng/mL recombinant human stem cell factor [rhSCF], 20 ng/mL recombinant human interleukin [rhIL]-6, 20 ng/mL rhIL-3, and 100 nM dexamethasone in StemSpan II medium) at a density of 10^5 cells/mL for 4 days. Cells were then passaged into differentiation 1-2 medium (2% human AB plasma, 3% human AB serum, 3 U/mL heparin, 10 ng/mL rhSCF, and 1 U/mL erythropoietin in StemSpan II medium) at a density of 10^6 cells/mL for an additional 3 days. The cells were then passaged into differentiation 3 medium (2% human AB plasma, 3% human AB serum, 3 U/mL heparin, 10 ng/mL rhSCF, and 1 U/mL erythropoietin in StemSpan II medium) at a density of 2 × 10^5 cells/mL for 4 days. To induce RBC maturation, cells were cultured in differentiation 4 medium (2% human AB plasma, 3% human AB serum, 3 U/mL heparin, 0.1 U/mL erythropoietin, and 200 μg/mL holo-transferrin in StemSpan II medium) at a density of 10^6 cells/mL for 4 days, and in differentiation 5 medium (2% human AB plasma, 3% human AB serum, 3 U/mL heparin, and 200 μg/mL holo-transferrin in StemSpan II medium) at a density of 5 × 10^6 cells/mL for an additional 3 days.

**Transgenes and codon optimization**

Human CD4, CCR5, CXCR4, ACE2, and GpA cDNA sequences were obtained from the National Center for Biotechnology Information. The CD4-GpA fusion construct encoded the CD4 signal peptide and D1D2 domains fused to the N terminus of GpA. The ACE2-GpA fusion protein construct encoded the extracellular domain of human ACE2 (residues 1–614) fused to the N terminus of GpA with the nine-residue linker (Glu-Pro-Lys-Thr-Pro-Lys-Pro-Gln-Pro). The ACE2-GpA fusion protein construct encoded the extracellular domain of human ACE2 (residues 1–614) fused to the N terminus of GpA with the nine-residue linker. Transgenes were cloned into the lentiviral backbone plasmids pHAGE-IRES-ZsGreen (PlasmID Repository, Harvard Medical School) for expression under ubiquitous promoters (CMV, EF1α, UBC, and CASI promoters) and pCCL-FB (provided by Dr. Donald Kohn, UCLA) for erythropoietin-specific expression. Codon optimization of transgene cDNA sequences was performed using the GeneArt GeneOptimizer software (Thermo Fisher Scientific).

**Lentiviral transduction**

VSV-G-pseudotyped lentiviral vectors were produced by co-transfecting HEK293T cells with lentiviral backbone plasmids and packaging plasmids (pHDM-Hgpm2, pHDM-tat1b, pRC/CMV-rev1b, pHDM-G) using FuGENE HD (Promega) according to the manufacturer’s protocol. Supernatants were collected after 48 and 72 h, and lentiviral vectors were concentrated 50-fold using Lenti-X concentrator solution (Takara) according to the manufacturer’s protocol. On day 10 of the differentiation protocol, erythroid progenitor cells
were seeded at a density of 10^6 cells/mL in 12-well plates in the presence of 10 μg/mL Polybrene. 20 μL of concentrated lentiviral vector was added per well and plates were spun for 1.5 h at 850 x g at 30°C. Plates were then incubated for 3 h at 37°C before passing the transduced cells into differentiation 3 medium. For cells that were co-transduced to express two transgenes, 20 μL of each lentiviral vector was added per well. To generate large numbers of engineered RBCs for neutralization assays, two transduction steps were performed on days 10 and 14 of the differentiation protocol.

Flow cytometry
Transgene expression and RBC maturation efficiency were analyzed by flow cytometry (MACSQuant, Miltenyi Biotec). 2–3 x 10^5 cells were collected for each condition and samples were stained with the following antibodies: allophycocyanin (APC)-conjugated anti-human CD4 (BD Biosciences), fluorescein isothiocyanate (FITC)-conjugated anti-human CD71 (BD Biosciences), FITC-conjugated anti-human CXCR4 (Invitrogen), FITC-conjugated anti-human ACE2 (R&D Systems), APC-conjugated anti-CD235ab (BioLegend), and Brilliant Violet 421-conjugated anti-human CD4 (BioLegend), and Brilliant Violet 421-conjugated anti-human CD71 (BioLegend). The percentage of enucleated RBCs that expressed transgenes was measured by triple-staining cells with APC-conjugated anti-human CD4, FITC-conjugated anti-human CCR5, and Hoechst nuclear stain.

BlaM fusion assay
The ability of engineered RBCs to be infected by HIV-1 was evaluated using a modified version of the BlaM assay.15 R5-tropic HIV-1YU2 and X4-tropic HIV-1HxBc2 pseudovirus were produced by co-transfecting a confluent T75 flask of HEK293T cells with the PSG3ΔEnv backbone plasmid (8 μg), the YU2 or HxBc2 Env expression plasmid (4 μg), and a plasmid expressing a BlaM-Vpr fusion protein (4 μg; provided by Dr. Wesley Sundquist, University of Utah). The supernatant was collected after 72 h and concentrated by centrifugal filtration. 5 x 10^4 RBCs were seeded in 100 μL of differentiation 5 medium in 96-well plates in the presence of 10 μg/mL Polybrene. 20 μL of concentrated YU2-BlaM-Vpr or HxBc2-BlaM-Vpr pseudovirus was added and plates were spun at 1,000 x g for 1 h at 30°C. Plates were then incubated at 37°C overnight. On the next day, freshly prepared 6 x CCF2-AM labeling solution was added, and cells were stained for 2 h at room temperature in the dark. After two washes with PBS, the cells were analyzed by flow cytometry (MACSQuant, Miltenyi Biotec).

HIV-1 neutralization assays
The ability of engineered RBCs to inhibit HIV-1 infection of target cells was tested by using a modified version of the HIV-1 pseudovirus-based TZM-bl assay.19 Briefly, serial dilutions of control and engineered RBCs were seeded in 400 μL of TZM-bl media in 48-well plates and incubated with 0.4 μL of HIV-1YU2 pseudovirus (50% tissue culture infective dose [TCID50] of 3.2 x 10^5 IU/mL) for 4 h on an orbital shaker (400 rpm) at 37°C in the presence of 10 μg/mL Polybrene. Cells were then spun down at 500 x g for 10 min and 155 μL of the supernatants was transferred to 96-well plates. TZM-bl reporter cells (NIH AIDS Reagents Program) were added, and luminescence was measured after 48 h.

Rev-A3RS CD4+ T cell infection assay
To test whether RBC viral traps can prevent infection of HIV-1 target cells, 10^5 Rev-A3RS CD4+ T cells were incubated in 48-well plates with 0.4 μL of HIV-1YU2 pseudovirus (TCID50 of 3.2 x 10^5 IU/mL) in 400 μL of TZM-bl media in the presence of 10 μg/mL Polybrene. Control RBCs and CD4-GpA-RBCs were added at RBC/CD4+ T cell ratios of 2:1 (2 x 10^5 RBCs) or 5:1 (5 x 10^5 RBCs), respectively. Cultures were incubated for 12 h on an orbital shaker (400 rpm) at 37°C overnight. Cells were then spun down at 500 x g for 10 min, virus-containing supernatants were removed, and the cells were...
Luminescence was measured after 48 h using a plate reader (Tecan). Transduced cells were transferred to the 96-well plate with the seeded 293T-ACE2 cells. The average luminescence compared to the control infection of Rev- pRC/CMV-rev1b, and a plasmid encoding the SARS-CoV-2 spike protein with a 21-residue cytoplasmic tail deletion (Wuhan Hu-1 strain; GenBank: NC_045512). The neutralization activity of ACE2-GpA and BEL-A/ACE2-GpA cell lines were generated by growing the transduced cells in expansion media (50 ng/mL rhSCF, 3 U/mL erythropoietin, 1 µM dexamethasone, and 1 µg/mL doxycycline in StemSpan II medium) in the presence of 0.25 µg/mL puromycin for 3–4 weeks. Differentiation of BEL-A cells was initiated as described by transferring the cells into primary media (3% human AB serum, 2% FBS, 3 U/mL heparin, 10 ng/mL rhSCF, 1 ng/mL rhIL-3, 3 U/mL erythropoietin, 200 µg/mL holo-transferrin, and 1 µg/mL doxycycline in StemSpan II medium) for 3–4 days at a density of 2 × 10^5 cells/mL. To induce RBC maturation, cells were moved into tertiary media (3% human AB serum, 2% FBS, 3 U/mL heparin, 3 U/mL erythropoietin, 500 µg/mL holo-transferrin, and 1 U/mL Pen-Strep in StemSpan II medium) for 4 days at a density of 1 × 10^6 cells/mL.

**FACS**

Enucleated RBCs were purified by FACS on day 7 of the BEL-A differentiation protocol. Brilliant Violet 421-conjugated anti-human CD71 antibody (BioLegend) and the nuclear stain DRAQ5 (Abcam) were diluted 1:100 and 1:1,000 in PBS+. Cells were stained at a concentration of 2.5 × 10^7 cells/mL for 30 min at room temperature in the dark. After two washes in PBS+, cells were resuspended in PBS+ at a concentration of 1 × 10^7 cells/mL. Enucleated RBCs were defined as CD71+ /DRAQ5− and this cell population was purified using a SONY SH800 cell sorter (Sony Biotechnology).

**SUPPLEMENTAL INFORMATION**

Supplemental information can be found online at [https://doi.org/10.1016/j.omtm.2021.03.003](https://doi.org/10.1016/j.omtm.2021.03.003).
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AUTHOR CONTRIBUTIONS

M.A.G.H. and P.J.B. designed the research, M.A.G.H. and C.K. performed the research, M.A.G.H. and P.J.B. analyzed data, and M.A.G.H. and P.J.B. designed the research, M.A.G.H. and C.K. performed the research, M.A.G.H. and P.J.B. analyzed data, and M.A.G.H. and P.J.B. wrote the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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