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Efficient manufacturing and engraftment of *CCR5* gene-edited HSPCs following busulfan conditioning in nonhuman primates

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Hematopoietic stem cell gene therapy has been successfully used for a number of genetic diseases and is also being explored for HIV. However, toxicity of the conditioning regimens has been a major concern. Here we compared current conditioning approaches in a clinically relevant nonhuman primate model. We first customized various aspects of the therapeutic approach, including mobilization and cell collection protocols, conditioning regimens that support engraftment with minimal collateral damage, and cell manufacturing and infusing schema that reflect and build on current clinical approaches. Through a series of iterative *in vivo* experiments in two macaque species, we show that busulfan conditioning significantly spares lymphocytes and maintains a superior immune response to mucosal challenge with simian/human immunodeficiency virus, compared to total body irradiation and melphalan regimens. Comparative mobilization experiments demonstrate higher cell yield relative to our historical standard, primed bone marrow and engraftment of CRISPR-edited hematopoietic stem and progenitor cells (HSPCs) after busulfan conditioning. Our findings establish a detailed workflow for preclinical HSPC gene therapy studies in the nonhuman primate model, which in turn will support testing of novel conditioning regimens and more advanced HSPC gene editing techniques tailored to any disease of interest.

INTRODUCTION

Although hematopoietic stem and progenitor cell (HSPC) gene therapies provide lifelong benefit in numerous disease settings, safety and toxicity remain a critical barrier. We and others have devoted substantial effort to optimizing large animal preclinical models of HSPC gene therapy, with a particular focus on clinically relevant methods of HSPC collection, efficient HSPC modification at the clinical scale, and comparison of reduced intensity conditioning regimens designed to enhance efficacy and simultaneously reduce toxicity. Our lab has led efforts to advance nonhuman primate (NHP) models of HSPC transplantation utilizing both rhesus (*Macaca mulatta*) and pigtail (*M. nemestrina*) macaques.[1–5] The NHP hematopoietic system shares many similarities with humans, including the kinetics of hematopoietic recovery following conditioning and HSPC engraftment, and common phenotypic markers that allow for tracking of HSPC subpopulations responsible for both rapid short-term recovery and true hematopoietic stem cells (HSCs) that facilitate long-term engraftment.[5] We have successfully performed autologous HSPC transplant of *CCR5*-edited cells in NHP models of HIV[6,7] and used CRISPR-Cas9 approaches to augment gamma-hemoglobin production in HSPC-derived red blood cells.[2] In both of these studies, primed HSPCs were isolated from G-CSF- and SCF-stimulated bone marrow after total body irradiation (TBI).[1] In contrast, current clinical paradigms for autologous HSPC transplantation most often utilize cells from G-CSF- and plerixafor-mobilized peripheral blood and reduced intensity chemotherapy conditioning. The primary goal of this study was to update and benchmark our model against current clinical paradigms, with a focus on conditioning regimens that are associated with efficient engraftment of autologous, CRISPR-edited HSPCs.[8] Because the choice of HSPC source and mobilization regimen may affect cell properties like gene editing efficiency and engraftment potential,[9–11] we also matched our source of cells for CRISPR-Cas9 editing to clinical methods.

No groups to date have conducted a head-to-head comparison of busulfan and TBI with other candidate conditioning regimens currently being used in gene therapy protocols (e.g., melphalan) in nonhuman primates.[12] Busulfan is an attractive candidate for conditioning because even at myeloablative doses, long-term engraftment of edited HSPCs is achieved with significantly reduced neutropenia, lymphopenia, and gut toxicity and has been shown to support engraftment of *CCR5*-edited HPSCs in mice.[13] Furthermore, busulfan is already being evaluated in ongoing HSPC gene

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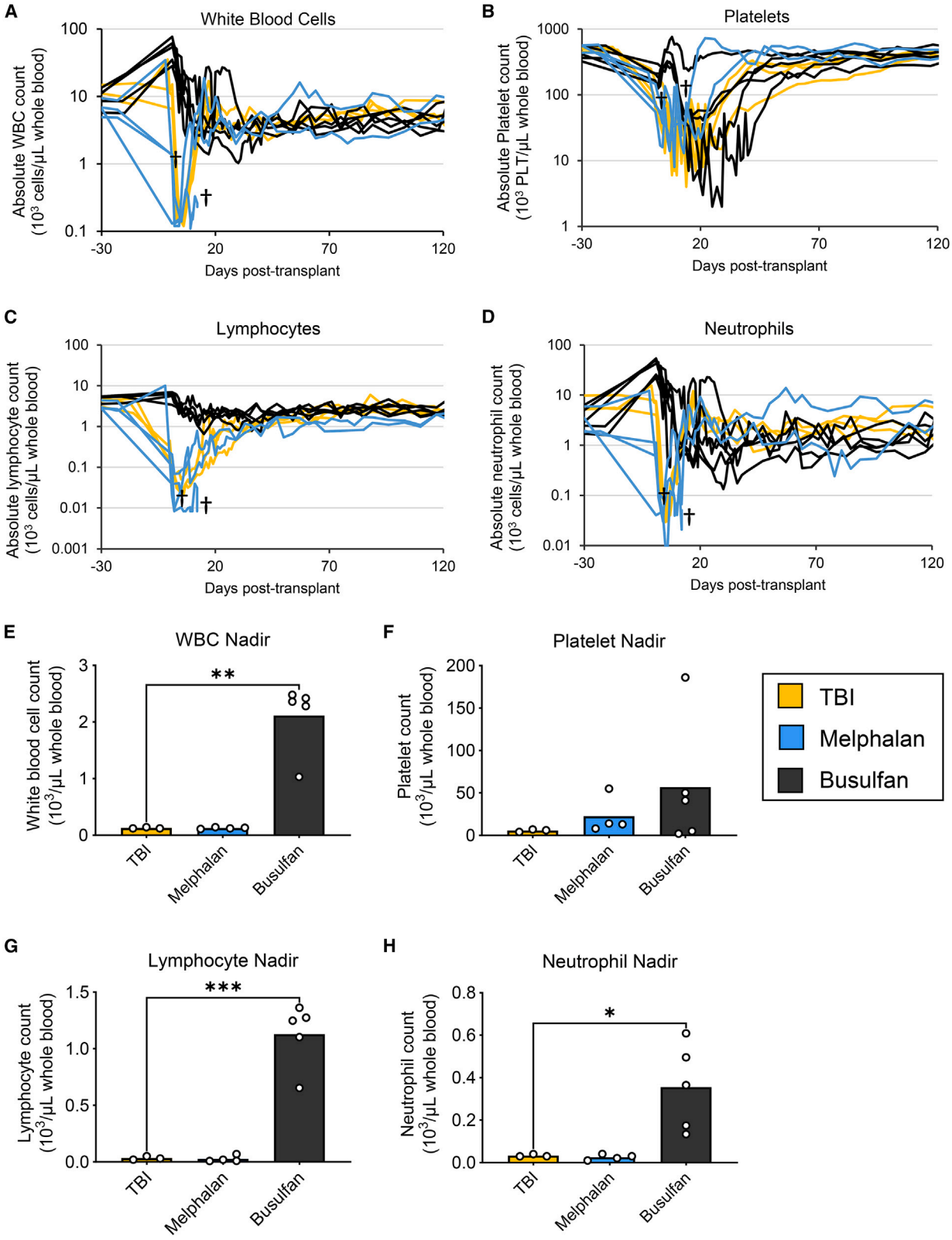

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therapy clinical trials in people living with HIV (ClinicalTrials.gov: NCT02500849). Here, we compared three different conditioning regimens for autologous HPSC transplantation in nonhuman primates and applied our model of CRISPR-induced *CCR5* knockout to understand how distinct HSPC sources and mobilization regimens impact the quality of HSPCs and efficiency of CRISPR editing.

RESULTS

Significant lymphocyte sparing following busulfan conditioning

We first compared the impact of two clinically relevant chemotherapy-based conditioning regimens against TBI on peripheral blood cell counts and the kinetics of recovery after autologous HSPC transplant in pigtail macaques (Table S1). Notably, these cells were transduced with a lentiviral vector encoding a CD4-based CAR,[14,15] which did not express *in vivo*; our data therefore focus only on hematopoietic recovery following distinct conditioning regimens. We started with a common clinical dose of melphalan (140 mg/m^2),[12] which induced extreme gut toxicity in the first animal. After lowering the dose to 70 mg/m^2, we observed additional adverse reactions in one animal and hematopoietic recovery in two others. We piloted an initial busulfan conditioning regimen based on our previous studies,[16] administering on 2 consecutive days at either 4 mg/kg/day (8 mg/kg total) or 2 mg/kg/day (4 mg/kg total). We observed complete hematopoietic recovery in the TBI and busulfan groups (Figure 1A). All three regimens depleted platelets (Figure 1B), but lymphocytes (Figure 1C) and neutrophils (Figure 1D) were largely spared with busulfan conditioning. Total white blood cell, neutrophil, and lymphocyte nadirs of animals in the melphalan and TBI groups were significantly lower than busulfan animals (Figures 1E–1H), with a similar degree of platelet depletion in all three groups (Figure 1F). These results suggest that busulfan conditioning is an attractive option for HSPC gene therapy settings where maintenance of adaptive immunity is critical, such as SCID or HIV infection. To confirm this, we next compared these animals' susceptibility to infection with an HIV-1 like virus (simian/human immunodeficiency virus, SHIV) following repeated low-dose mucosal challenge. Consistent with our previous studies,[17,18] we found that busulfan-conditioned animals more effectively resisted dose-escalating intrarectal SHIV challenges, relative to the TBI/melphalan cohort. Whereas all 5 TBI- or melphalan-conditioned animals were infected after 1 or 2 challenges, 2 out of 5 busulfan-conditioned animals resisted all 6 intrarectal challenges but were infected following a high-dose intravenous challenge (Figure S1). These results provide clear functional evidence that busulfan conditioning better maintains immunity following repeated mucosal exposure to SHIV, a key consideration in the design of effective HSPC gene therapies for infectious diseases such as HIV-1.

Consistent and reproducible CD34$^+$ HSPC yields following G-CSF + plerixafor mobilization

We next compared regimens to prime or mobilize NHP CD34$^+$ HSPCs for collection and gene engineering. Granulocyte colony stimulating factor (G-CSF) is the standard clinical agent for mobilizing CD34$^+$ HSPCs from the bone marrow into peripheral blood.[19,20] The CXCR4 antagonist plerixafor (a.k.a. AMD3100) also mobilizes HSPCs and is used in conjunction with G-CSF to augment HSPC yields relative to G-CSF alone.[21–24] Mobilized apheresis with G-CSF and plerixafor was previously optimized in nonhuman primates,[25] featuring consistently high yields of CD34$^+$ cells after mobilization superior to either drug alone. Whereas previous studies focused on the timing of G-CSF and plerixafor administration relative to bulk CD34$^+$ yields, we were also interested in enumerating mobilization kinetics of long-term engrafting CD34$^+$CD90$^+$CD45RA$^-$ HSC.[5] Although our initial conditioning comparisons were performed in pigtail macaques (Figure 1), we were also interested making a direct comparison to published data from Uchida et al. in rhesus macaques.[25] Therefore, we performed our mobilization regimen comparison in both macaque species. We administered G-CSF at a dose of 10 μg/kg/day for 5 days; on the fourth day, a single dose of 1 mg/kg AMD3100 was delivered 8 h before the final dose of G-CSF. Because the pharmacokinetics of plerixafor-mobilized HSPCs are dose dependent,[26–28] whole blood samples were collected hourly for up to 15 h post-plerixafor and analyzed for bulk CD34$^+$ HSPC and CD34$^+$CD90$^+$CD45RA$^-$ HSC populations by flow cytometry (Figure 2A). Total white blood cell counts were elevated in both species for the entire time period monitored, with higher mean HSPC and HSC counts in rhesus (Figure 2B) despite lower total white blood cell counts relative to pigtail (Figure 2C). These data show that peripheral HSPC and HSC counts are stably elevated in 2 NHP species after a 5-day course of G-CSF and at least 8 h after a single dose of plerixafor.

HSPC enrichment and CRISPR editing in mobilized apheresis versus primed bone marrow

After confirming that peripheral HSPC and HSC counts were stably elevated following G-CSF + plerixafor-mobilized apheresis, we compared this collection technique to our previous source of NHP HSPCs: G-CSF and SCF-primed bone marrow (Figure 3A). From our previous experiment (Figure 2), we chose to begin apheresis 10 h post AMD3100 on the fifth day of mobilization. Compared to primed marrow, mean yields of G-CSF + plerixafor-mobilized white blood cells per kilogram were more than 10-fold higher (Table 1). Although the fraction of CD34$^+$ HSPCs was much lower in peripheral blood cells even after mobilization, this still translated into a 3-fold increase in the number of CD34$^+$ cells enriched by immunomagnetic bead-based selection. Most importantly, a significantly higher

Figure 1. Preferential sparing of lymphoid and myeloid subsets following busulfan conditioning in nonhuman primates

Total peripheral white blood cell (A), platelet (B), lymphocyte (C), and neutrophil counts (D) from 12 pigtail macaques conditioned with total body irradiation (TBI, gold lines), melphalan (blue lines), or busulfan (black lines) prior to infusion of autologous HSPCs. (F–H) Nadir counts corresponding to the graphs in (A–D). *$p < 0.05$; **$p < 0.001$; ***$p < 0.0005$.

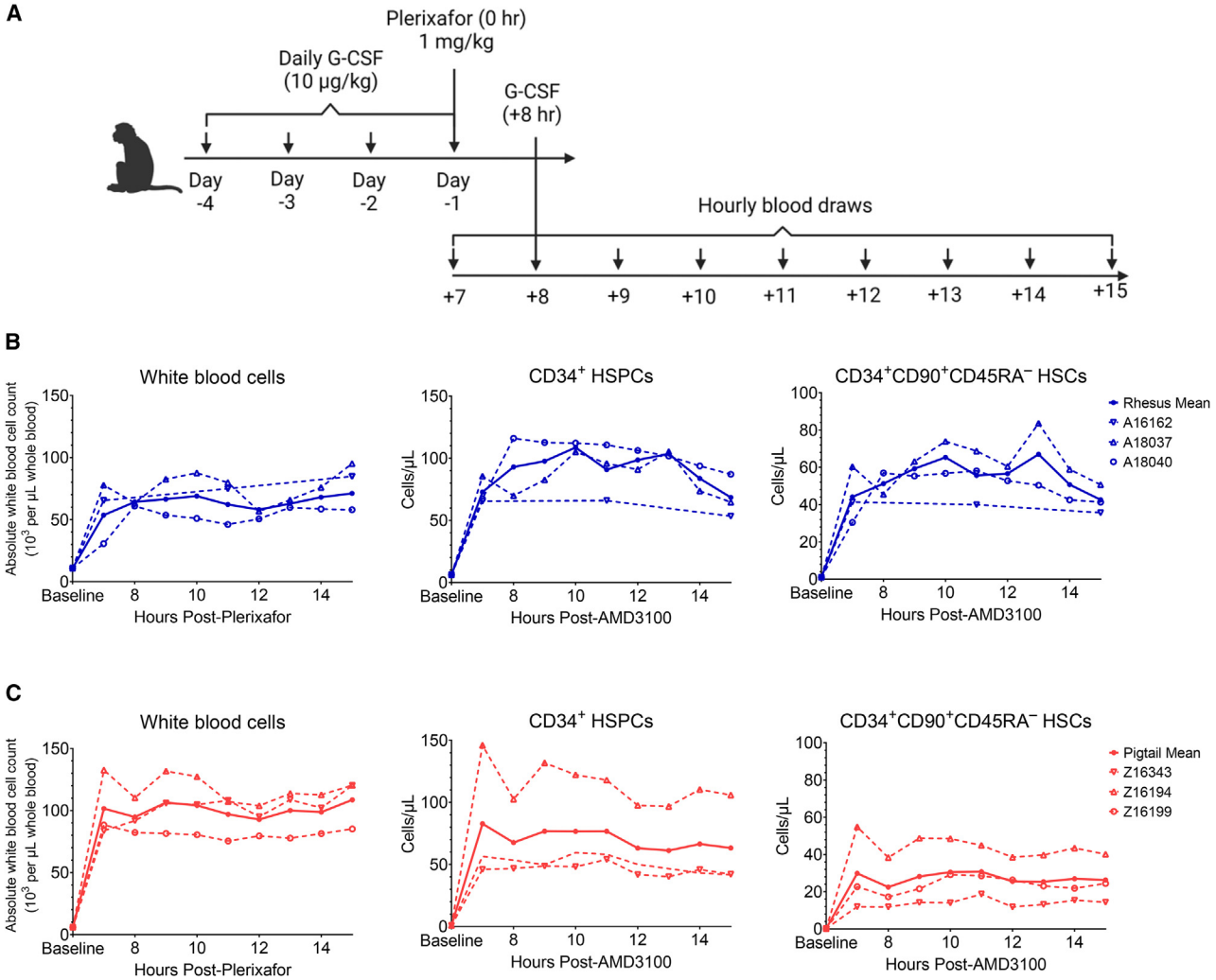


Figure 2. G-CSF + plerixafor efficiently mobilizes HSPCs into the periphery in rhesus and pigtail macaques

(A) Timeline of mobilization and sampling. (B) Hourly counts of total white blood cells, $CD34^+$ cells, and $CD34^+CD90^+CD45RA^-$ cells in rhesus macaques. Dashed lines indicate individuals. (C) Hourly counts of total white blood cells, $CD34^+$ cells, and $CD34^+CD90^+CD45RA^-$ cells in pigtail macaques. Solid lines: mean values.

fraction of $CD34^+$ HSPCS were $CD90^+CD45RA^-$ long-term engrafting HSCs (Figure 3B). Following overnight *ex vivo* culture, enriched HSPCs were electroporated with CRISPR ribonucleoprotein (RNP) specific for NHP *CCR5*. There was no appreciable difference in the efficiency of *CCR5* editing between mobilized apheresis and primed marrow cells, with maximum editing of approximately 90% observed on day 6 *ex vivo* (Figure 3C). A significantly higher percentage of mobilized apheresis cells maintained expression of CD34 (Figure 3D) and CD90 (Figure 3E) in culture after electroporation. In colony-forming assays, the proliferative potential of HSPCs from primed marrow versus mobilized apheresis was comparable after 12–14 days in culture (Figure 3F). Our findings demonstrate that despite comparable CRISPR editing efficiencies and colony-forming capacity, mobilized apheresis and primed marrow sources significantly differ in several key parameters, including HSC frequency and durable expression of key markers CD34 and CD90. There was no appreciable difference in CD34 expression, CD90 expression, or colony-forming potential between *CCR5*-edited cells and non-electroporated controls (Figures 3D–3F).

Edited cells successfully engraft after busulfan conditioning

After confirming that our NHP HSPC CRISPR RNP protocol enabled approximately 90% *CCR5* editing *ex vivo*, we next investigated the ability of G-CSF + plerixafor-mobilized, *CCR5*-edited HSPCs to engraft *in vivo* following busulfan conditioning (Figure 4A). Rhesus macaque HSPCs from 4 of the 6 donors in Figure 3 (Tables 1 and 2) were transplanted into autologous hosts. To match clinical paradigms (i.e., the need to complete post-manufacturing release assays prior to infusion into the patient), HSPCs were cryopreserved 1 day after CRISPR editing. Based on our initial findings (Figure 1), we

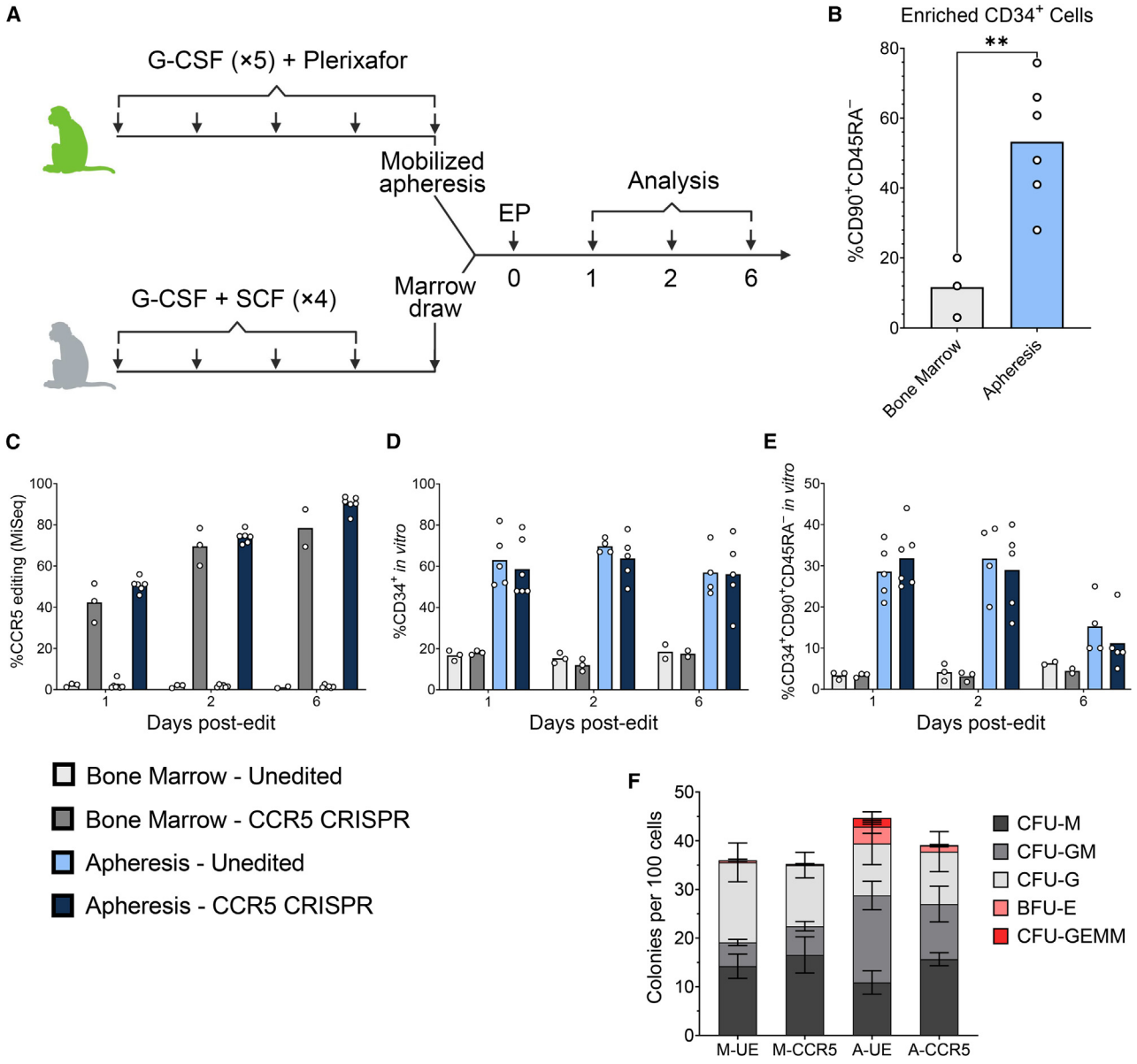


Figure 3. Comparison of *CCR5* gene editing efficiency in rhesus macaque HSPCs following distinct mobilization regimens

(A) Timeline of mobilization and sampling for 6 $CD34^+$ products collected from G-CSF + plerixafor-mobilized apheresis (green) vs. 3 products collected from G-CSF + SCF-primed bone marrow (gray). EP: electroporation with CRISPR-Cas9 RNP targeting the NHP *CCR5* locus. (B) Fraction of enriched $CD34^+$ cells that possess the $CD90^+CD45^-$ long-term engrafting HSC phenotype. (C) Illumina MiSeq quantification of *CCR5*-edited cells over up to 6 days post editing in *ex vivo* culture. Percent $CD34^+$ HSPCs (D) and $CD34^+CD90^+CD45RA^-$ HSCs (E) from editing experiments in (C). (F) Colony-forming unit (CFU) assays from non-electroporated control and *CCR5*-edited cultures. Error bars represent SEM. *$p < 0.05$; **$p < 0.001$; ***$p < 0.0005$; ****$p < 0001$. M-UE, bone marrow unedited; M-*CCR5*, bone marrow-*CCR5* CRISPR; A-UE, apheresis unedited; A-*CCR5*, apheresis-*CCR5* CRISPR.

conditioned each animal with busulfan but used a modified dosing scheme (5.5 mg/kg/day beginning 2–3 days after apheresis) in rhesus macaques to match recent findings that higher doses are well-tolerated in this species and support engraftment.[25] As a historical control in this experiment, we compared our 4 busulfan-conditioned, G-CSF + plerixafor-mobilized, *CCR5*-edited, HSPC animals to a previously published cohort utilizing HSPCs from primed marrow, edited within the gamma globin promoter (HBG) and conditioned with TBI[2] (Figure S2).

Consistent with the higher total busulfan dose in this cohort (5.5 mg/kg per day for 4 days vs. 2–4 mg/kg for 2 days), lymphocyte

Table 1. HSPC enrichment from mobilized rhesus macaque blood and bone marrow

Animal	Mobilization	Weight (kg)	Total cells	Total CD34+	Total CD90+	Cells/kg	CD34+/kg	CD90+/kg	CD90+ of CD34+ (%)
A18017	apheresis	11.1	3.7E10	5.7E7	3.8E7	3.3E9	5.1E6	3.4E6	67%
A19225	apheresis	10	2.2E10	4.3E7	1.2E7	2.2E9	4.4E6	1.3E6	29%
A21032[a]	apheresis	8.9	2.1E10	2.0E8	8.3E7	2.4E9	2.2E7	9.4E6	42%
A21042[a]	apheresis	6.7	2.1E10	3.4E7	2.5E7	3.1E9	5.0E6	3.8E6	73%
A21039[a]	apheresis	5.5	1.8E10	2.5E7	1.2E7	3.3E9	4.5E6	2.2E6	48%
A21040[a]	apheresis	6.0	1.6E10	2.2E7	1.4E7	2.7E9	3.7E6	2.3E6	60%
Apheresis average		8.0	2.3E10	6.4E7	3.1E7	2.8E9	7.5E6	3.7E6	52%
A18019	bone marrow	12.3	2.6E9	4.8E7	1.9E6	2.1E8	3.9E6	1.5E5	4%
A18025	bone marrow	17.1	1.2E9	2.3E7	2.8E6	7.1E7	1.3E6	1.7E5	12%
A20030	bone marrow	12.7	2.7E9	7.6E7	1.6E7	2.1E8	6.0E6	1.3E6	21%
Bone marrow average		14.0	2.2E9	4.9E7	6.9E6	1.6E8	3.8E6	5.3E5	12%

[a]Autologous transplantation recipients; details in Table 2.

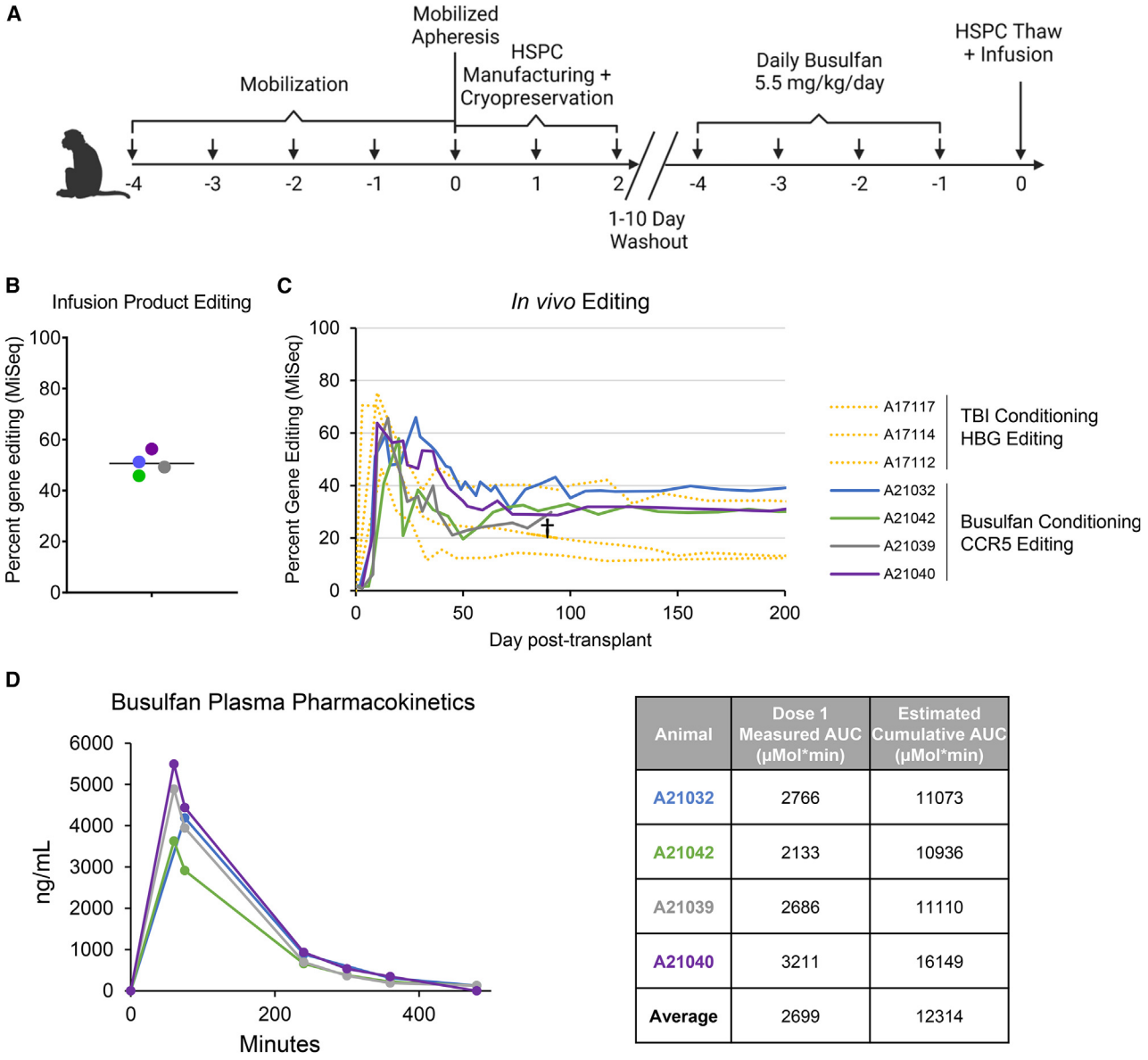
and neutrophil nadirs were lower than in our initial experiment (compare Figures 1A–1D and S2A–S2D). However, the lymphocyte compartment was still significantly maintained even with a higher busulfan dose, relative to historical TBI controls (Figures S2E–S2H). To infuse the 4 donors with *CCR5*-edited, cryopreserved autologous HSPCs, we adapted a "bedside thaw" procedure in which cryopreserved cells were warmed to 37°C immediately prior to infusion into the autologous host, without washing steps (Figure 4A). Post-thaw infusion products from mobilized apheresis contained half as many $CD34^+$ cells normalized to weight as primed marrow controls (7.3×10^6 vs. 1.3×10^7 $CD34^+$ cells/kg) but significantly more $CD34^+CD90^+CD45RA^-$ HSC (3.7×10^6 vs. 7.1×10^5) (Table 2) and approximately 50% *CCR5* editing (Figure 4B). Because we continue to see an increase in editing *ex vivo* over time consistent with previous reports in human HSPCs[29–32] (Figure 3C) and peak editing *in vivo* meets or exceeds our infusion product (Figures 4C and S4), we suspect this value may underestimate the true editing efficiency of our infusion product.

After 90 days post transplant, *CCR5* editing in peripheral white blood cells ranged from 20% to 40% of alleles, which trended higher than HBG editing in the primed marrow/TBI cohort (Figure 4C) but did not reach statistical significance. Serum busulfan pharmacokinetic/pharmacodynamic measurements showed that this dose washes out within 8 h (Figure 4D). Similarly to Uchida et al.,[25] we euthanized 1 busulfan-conditioned animal (A21039) 90 days post transplant due to sustained thrombocytopenia. In summary, our comparison of engraftment of CRISPR-edited NHP HSPCs in autologous hosts showed that myelosuppression and immunosuppression following busulfan conditioning are highly dose dependent and are capable of supporting incrementally increased efficiencies of edited HSPC engraftment *in vivo*.

DISCUSSION

Gene-edited HSPC therapies are currently in clinical trials for the treatment of numerous infectious and genetic diseases, including persistent HIV-1 infection (ClinicalTrials.gov: NCT02500849) and hemoglobinopathies.[20] We have advanced our NHP model of CRISPR-edited HSPC gene therapy to inform and augment clinical standards at each key stage, including mobilization, conditioning, and engraftment. We consistently observed yields of $CD34^+$ cells greater than the recommended minimum[33,34] 2×10^6/kg following a clinically relevant mobilized apheresis regimen in rhesus macaques, with similar kinetics observed in a second relevant NHP species. Crucially, the $CD34^+CD90^+CD45RA^-$ subset is more than twice as prevalent in our enrichment product, and normalized cell yields of $CD34^+CD90^+CD45RA^-$/kg were over 7-fold higher from apheresis products than bone marrow aspirates. This difference translates to a 5-fold increase in infused $CD90^+$ cells/kg compared to historical controls despite halving the $CD34^+$/kg dose. While it has long been known that HSPCs enriched from peripheral blood are suitable for transplant, our direct comparison suggests that mobilized apheresis may contain more long-term engrafting HSCs than primed marrow. Interestingly, we found that editing efficiencies were comparable in mobilized apheresis- vs. primed marrow-derived HSPCs, although kinetics of CD34 and CD90 expression markedly differed. We conclude that mobilized apheresis, which in the clinic can be performed in the outpatient setting and also contains higher numbers of long-term HSCs, is a superior collection route, relative to primed bone marrow.

Our findings suggest dose-dependent toxicity following busulfan conditioning *in vivo*. Our initial experiments tested a dose of 2–4 mg/kg that was administered on 2 consecutive days. As our intended chimeric antigen receptor (CAR) construct was not detected *in vivo,* our analysis of these data focus on hematopoietic recovery after conditioning. Consistent with previous studies in the macaque model,[35,36] we observed the greatest impact on the neutrophil and platelet compartments, although recovery occurred within approximately 2 months. Our subsequent experiments with an increased dose of 5.5 mg/kg busulfan in rhesus macaques administered on 4 consecutive days was associated with failed platelet recovery in 1 animal, again consistent with previous observations.[25] Busulfan-induced neutrophil



Animal	Dose 1 Measured AUC (μMol*min)	Estimated Cumulative AUC (μMol*min)
A21032	2766	11073
A21042	2133	10936
A21039	2686	11110
A21040	3211	16149
Average	2699	12314

Figure 4. Engraftment of *CCR5*-edited rhesus macaque HSPCS after myeloablative busulfan conditioning

(A) Timeline of mobilization and busulfan conditioning before autologous HSPC transplant. (B) CRISPR-mediated gene editing in infusion products or (C) total white blood cells following autologous HSPC transplant, measured by Miseq assay. (D) Pharmacokinetics of 5.5 mg/kg busulfan in NHP plasma.

cytotoxicity has been extensively modeled in clinical studies[37,38] and also triggers cell death in isolated platelets *ex vivo*.[39] As noted in historical literature,[36,40] busulfan may also exert an impact on HSPC-derived precursors that give rise to specific hematopoietic lineages. For example, despite only a transient impact of single-dose busulfan on white blood cell counts, this regimen was associated with a 60%–80% decrease in the number of HSPC clones contributing to granulocyte production,[36] and recent reports offer even more sophisticated techniques to track lineage-specific HSPC clones.[41,42] Future experiments focused on the impact of busulfan conditioning on the differentiation of HSPC-derived megakaryocytes as well as the function of terminally differentiated platelets will contextualize this primary source of toxicity that we observed in our study.

We found that CRISPR-Cas9 editing of *CCR5* did not impair stemness of mobilized cells compared to non-electroporated controls. After *CCR5* CRISPR editing, cryopreservation, and bedside thaw, G-CSF + plerixafor-mobilized HSPCs efficiently engrafted in busulfan-conditioned autologous hosts. Surprisingly, engraftment of *CCR5*-edited HSPCs rivaled our previous study using TBI

Table 2. CRISPR-Cas9-edited HSPC infusions in rhesus macaques

Animal ID	CRISPR edit	Infusion date	Weight	Live cells infused	CD34+ infused	CD90+ infused	Infused cells/kg	Infused CD34+/kg	Infused CD90+/kg	Peak editing *ex vivo*	Days cryopreserved	Reference
A21032	*CCR5*	12/21/21	7.8	1.1E8	7.7E7	2.6E7	1.3E7	9.8E6	3.4E6	93%	6	–
A21042	*CCR5*	2/16/22	6.7	6.4E7	4.3E7	2.5E7	9.6E6	6.5E6	3.8E6	94%	7	–
A21039	*CCR5*	3/7/22	5.5	4.8E7	3.1E7	2.1E7	8.6E6	5.7E6	3.9E6	90%	5	–
A21040	*CCR5*	7/25/22	6.0	3.2E7	2.4E7	1.4E7	6.2E6	5.0E6	3.5E6	90%	12	–
Average			6.5	6.2E7	4.4E7	2.2E7	9.3E6	6.5E6	3.3E6	92%	7.5	
A17117	*HBG*	7/14/17	5	1.3E8	5.3E7	3.5E6	2.6E7	1.1E7	7.1E5	71%	–	Humbert et al., 2019[2]
A17114	*HBG*	8/4/17	4.9	8.4E7	3.4E7	3.6E6	1.7E7	6.8E6	7.3E5	73%	–	
A17112	*HBG*	2/8/18	7.3	2.0E8	1.5E8	5E6	2.7E7	2.0E7	6.8E5	63%	–	
Average			5.7	1.3E8	7.9E7	4.1E6	2.3E7	1.3E7	7.1E5	69%	-	

Estimates of *CCR5*-edited infusion products are based on cell counts taken before cryopreservation. *HBG*-edited cells were not cryopreserved.

conditioning, which was associated with significantly lower nadirs of lymphocytes. Several variables in this comparison should be noted, including the use of non-contemporaneous TBI-conditioned controls that were infused with primed marrow-derived HSPCs edited with *HBG*-specific CRISPR RNP instead of *CCR5*. Additionally, most animals used in these experiments are male. While we are concerned with sex-based differences in immune response and strive to balance male and female macaques in our experiments, females are often not available due to higher demand.

The long-term persistence of up to 40% *CCR5*-edited HSPCs and progeny *in vivo* is unprecedented in a large animal preclinical model, demonstrating that migration to the bone marrow niche is not impaired by *CCR5* knockout. Consistent with previous reports, one of our busulfan-treated animals struggled to recover peripheral platelet counts and ended study earlier than the 3 others in the cohort. Nevertheless, hematopoietic recovery in both our low- and high-dose busulfan animals was superior to TBI and also melphalan. The increased susceptibility of TBI- or melphalan-conditioned animals to infection with SHIV further supports our conclusion that busulfan conditioning retains adaptive immune responses that are likely to be an essential component for curative endpoints for infectious diseases like HIV-1.

Our exciting findings pave the way for the evaluation of cutting-edge conditioning regimens and disease-specific HSPC gene therapies in the NHP model. Whereas previous reports focused on the engraftment of lentiviral vector-modified cells, we applied CRISPR-Cas9 RNP complexes to inactivate *CCR5* alleles in NHP HSPCs. The efficient engraftment of *CCR5*-edited HSCs and progeny provides proof of principle for *CCR5* editing strategies for people living with HIV and for *CCR5* as a safe harbor locus for other targeted editing approaches. To further improve on the safety of busulfan conditioning while maintaining or enhancing engraftment of autologous HSPCs, we are exploring antibody-based regimens that are designed to carve out a robust niche for HSC engraftment while minimizing collateral damage to other hematopoietic lineages.[43–46] We predict that iterative refinement of the minimal niche needed to maximize HSC engraftment will also maximize the retention of innate and adaptive immune responses that target the disease of interest (e.g., HIV-1) and also minimize the occurrence of opportunistic infections during hematopoietic recovery. Regarding the optimal HSPC gene therapy approach for a given disease, we have found that *CCR5* editing alone may be insufficient for an HIV cure.[47] We are therefore building on the NHP *CCR5* CRISPR platform described here to efficiently engraft cells that carry large therapeutic transgenes targeted to the edited *CCR5* locus, such as virus-specific CAR and broadly neutralizing antibody (bNAb) transgenes.[48,49] Our data further reinforce the notion that *CCR5* can serve as a safe harbor for Fanconi Anemia (FA), Severe Combined Immunodeficiency (SCID), and other diseases. These experiments will focus on maximizing the efficiency of targeted transgene insertion at the edited *CCR5* locus while maintaining a high percentage of $CD90^{+}CD45RA^{-}$ true HSCs that are fit for long-term engraftment.

MATERIALS AND METHODS

NHP studies

Healthy juvenile pigtail and rhesus macaques were housed at the University of Washington (UW) National Primate Research Center (WaNPRC) under conditions approved by the American Association for the Accreditation of Laboratory Animal Care. All experimental procedures performed were reviewed and approved by the Institutional Animal Care and Use Committee of the Fred Hutchinson Cancer Center (Fred Hutch) and UW (protocol no. 3235-01). This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health (*the Guide*) including at least twice-daily observation by animal technicians for basic husbandry parameters (for example, food intake, activity, stool consistency, and overall appearance), as well as daily observation by a veterinary technician and/or veterinarian. Animals were housed in cages approved by *the Guide* and in accordance with Animal Welfare Act regulations. Animals

were fed twice daily and were fasted for up to 14 h before sedation. Environmental enrichment included grouping in compound, large activity, or run through connected cages, perches, toys, food treats, and foraging activities. If a clinical abnormality was noted by WaNPRC personnel, standard WaNPRC procedures were followed to notify the veterinary staff for evaluation and determination for admission as a clinical case. Animals were sedated by administration of ketamine HCl and/or telazol and supportive agents for balanced anesthesia (such as diazepam and midazolam) before all procedures. After sedation, animals were monitored according to WaNPRC standard protocols. WaNPRC surgical support staff are trained and experienced in the administration of anesthetics and have monitoring equipment available to assist with electronic monitoring of heart rate, respiration, and blood oxygenation; audible alarms and digital readouts; monitoring of blood pressure, temperature, etc. For minor procedures, the presence or absence of deep pain was tested by the toe-pinch reflex, and the absence of response (leg flexion) to this test indicated adequate anesthesia. In cases of general anesthesia, similar monitoring parameters were used, and anesthesia was tested by the loss of palpebral reflexes (eye blink). Analgesics (generally buprenorphine with meloxicam or buprenorphine slow release) were provided as prescribed by the clinical veterinary staff for at least 48 h after the procedures and could be extended at the discretion of the clinical veterinarian based on clinical signs.

Pre-transplant conditioning

A preliminary cohort of 12 pigtail macaques was entered into study to screen a series of 3 conditioning regimens for engraftment of lentiviral vector-modified cells. Expression of the vector transgene was not detected in these animals, likely due to suboptimal vector titer. Hence, this preliminary cohort was studied exclusively to quantify the impact of TBI, melphalan, and busulfan conditioning on peripheral blood cell counts and the kinetics of recovery following infusion of autologous HSPC. TBI conditioning was administered to 3 animals (IDs Z15035, Z15017, Z16264) fractionated over 2 days prior to cell infusion with a total dose of 1,020 cGy. Melphalan was administered as a single intravenous dose of either 140 mg/m^2 (ID Z15055) or 70 mg/m^2 (IDs Z16272, Z17060, Z17052) 2 days prior to cell infusion. Busulfan was administered in 2 intravenous doses over 2 days prior to cell infusion. The total dose was 8 mg/kg (IDs Z16268, Z16243) or 4 mg/kg (IDs Z16047, Z16013, Z15368). The range of infused HSPC doses in each TBI, melphalan, or busulfan-conditioned animal ranged between 7.18×10^6 and 2.04×10^7 cells per kg body weight. In these animals, cell doses were not normalized to CD34$^+$ expression in the infused cell product.

CD34$^+$ HSPC mobilization and priming

For experiments utilizing mobilized peripheral blood and apheresis products, G-CSF was administered once daily at a dose of 50 μg/kg/day for 5 days to rhesus and pigtail macaques. On the fourth day, a single dose of 1 mg/kg AMD3100 was delivered 8 h before the final dose of G-CSF. Regular blood draws were performed 7–15 h post plerixafor, lysed twice in ammonium chloride lysis buffer, and analyzed by flow cytometry. Apheresis was begun 10 h following AMD3100. For experiments utilizing primed bone marrow, G-CSF was administered at 100 μg/kg/day and SCF for 50 μg/kg/day for 4 days before bone marrow aspiration.

CD34$^+$ HSPC enrichment and culture

Autologous NHP transplantation, priming (or mobilization), collection of cells, and genetic engineering were conducted consistent with our previously published protocols.[1] Before enrichment for CD34$^+$ cells, red cells were lysed twice in ammonium chloride lysis buffer and white blood cells incubated for 25 min with a custom-produced 12.8 IgM anti-CD34 antibody (Fred Hutch, Seattle, WA) and washed and incubated a further 25 min with magnetic-activated cell-sorting anti-IgM microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany). The cell suspension was run through magnetic LS Columns (Miltenyi 130-042-401) enriching for CD34$^+$ cell fractions with a maximum of 2 x 10^9 cells per column and an initial purity of 14%–52% confirmed by flow cytometry. Enriched CD34$^+$ cells were cultured in StemSpan (STEMCELL Technologies, Vancouver, BC) supplemented with penicillin-streptomycin (100 U/ml) (Gibco by Life Technologies, Waltham, MA), and 100 ng/mL each stem cell factor (PeproTech, Rocky Hill, NJ), TPO (thrombopoietin; PeproTech), and FLT3-L (FMS-related tyrosine kinase 3 ligand; Miltenyi Biotec) overnight prior to electroporation. *CCR5*-edited and non-electroporated control cells were maintained *in vitro* and further assayed for up to 6 days post editing.

Flow cytometry

Flow cytometric analysis was performed on a FACSymphony (BD Biosciences, Franklin Lakes, NJ). Antibodies used for analysis of NHP cells included CD34 PE-CF594 and CD34-APC clone 563 (BD Biosciences 561209 and 652449), CD90 PE clone 5E10 (BD Biosciences 555596), CD45 V450 clone D058-1283 (BD Biosciences 561291), and CD45RA APC-H7 clone 5H9 (BD Biosciences 561212). Dead cells and debris were excluded by forward/side scatter (FSC/SSC) gating.

CRISPR-Cas9 editing of CD34$^+$ cells

Purified Cas9 protein was acquired from Life Technologies (Cat no. A36499), and gRNAs were custom ordered from Synthego (Redwood City, CA; sequence: UCAUCCUCCUGACAAUCGAU). Lyophilized gRNAs were resuspended in nuclease-free water at a concentration of 100 pmol/μL and stored as frozen aliquots at −80°C. Enriched CD34$^+$ cells were cultured overnight after enrichment. CRISPR-Cas9 RNPs were formed by combining 180 pmol of Cas9 protein with 900 pmol of gRNA for 10 min and used for the electroporation of 9–10 million cells per cuvette in a Lonza 4D-Nucleofactor X Unit following the manufacturer's instructions.

Cryopreservation

Enriched, CD34$^+$ cells were cultured overnight after electroporation and cryopreserved at a density of 10 million cells/mL in CryoStor CS10 (STEMCELL). Cells were cooled to −80°C overnight in a Mr.

Frosty Freezing Container (Thermo Fisher Scientific, Waltham, MA) and stored at −80° for up to 2 weeks until infusion.

CFC assays

A total 1,000 to 1,200 enriched cells were seeded into 3.5-mL ColonyGEL 1402 (ReachBio, Seattle, WA) in 35-mm petri dishes. Hematopoietic colonies were scored after 12 to 14 days. Arising colonies were identified as colony-forming unit (CFU) granulocyte (CFU-G), CFU macrophage (CFU-M), CFU granulocyte-macrophage (CFU-GM), and burst-forming unit-erythrocyte (BFU-E). Colonies consisting of erythroid and myeloid cells were scored as CFU-GEMM.[1]

Quantifying indels with MiSeq

Editing at the *CCR5* locus was measured by next-generation sequencing using Illumina barcoded 2 × 150 base-pair-end MiSeq primers for complete sequencing; forward: TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGTGGCCATCTCTGACCTGCTT, reverse: GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGACCCCAAAGGTGACTGTCCT. Library primers used were Nextera XT Index 15055293 FC-131-1001, and bioinformatic processing of sequencing data was conducted using a custom-built pipeline.[50]

Busulfan dosing and cell infusions in rhesus macaques

The busulfan conditioning regimen was altered for transplant of CRISPR-edited cells. Busulfan was administered in 4 intravenous doses of 5.5 mg/kg/day (22 mg/kg total) beginning 3–4 days after mobilized apheresis. Cryopreserved cells were warmed to 37°C and infused into conditioned animals no sooner than 24 h after the final dose of busulfan. Complete blood counts were compared to TBI-conditioned animals published previously.[2]

Cell counts

Post-conditioning numbers of total white blood cells, platelets, neutrophils, and lymphocytes were determined by complete blood count integrated with flow cytometry-based quantification of HSPC markers as described above. Separately, cell counts for CD34+ enrichment and infusion products were determined using the Countess II Automated Cell Counter (Thermo Fisher) with trypan blue staining. $CD34^+$ cell purity was confirmed by flow cytometry.

Statistics

Statistical analyses were performed in GraphPad Prism 9. Cell count comparisons utilize two-tailed unpaired Welch's t test. *In vitro* comparisons of cell phenotype and gene editing utilize mixed-effects analysis.

DATA AND CODE AVAILABILITY

All data supporting the findings of this study are available within the paper and its supplemental information files or from the corresponding authors upon request.

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.omtm.2023.07.006.

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AUTHOR CONTRIBUTIONS

H.-P.K. and C.W.P. are the principal investigators of the study and designed and coordinated the overall execution of the project. J.M. and C.W.P. designed the animal experiments. JM., T.E., and R.V. performed the NHP HSPC transplantation and gene editing experiments with feedback from S.R. T.E. prepared all Illumina MiSeq libraries and performed CFU assays. J.M., T.E., and C.W.P. curated and analyzed the data. J.M. and C.W.P. wrote the manuscript, with feedback from all co-authors. A.Z., S.G.K., and M.A.C. contributed manuscript feedback.

DECLARATION OF INTERESTS

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