Efficient Enrichment of Gene-Modified Primary T Cells via CCR5-Targeted Integration of Mutant Dihydrofolate Reductase

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Targeted gene therapy strategies utilizing homology-driven repair (HDR) allow for greater control over transgene integration site, copy number, and expression—significant advantages over traditional vector-mediated gene therapy with random genome integration. However, the relatively low efficiency of HDR-based strategies limits their clinical application. Here, we used HDR to knock in a mutant dihydrofolate reductase (mDHFR) selection gene at the gene-edited CCR5 locus in primary human CD4+ T cells and selected for mDHFR-modified cells in the presence of methotrexate (MTX). Cells were transfected with CCR5-megaTAL nuclease mRNA and transduced with adeno-associated virus containing an mDHFR donor template flanked by CCR5 homology arms, leading to up to 40% targeted gene insertion. Clinically relevant concentrations of MTX led to a greater than 5-fold enrichment for mDHFR-modified cells, which maintained a diverse TCR repertoire and/or for ex vivo selection of gene-edited T cells for the treatment of cancer.

INTRODUCTION

Gene therapy approaches display enormous promise in a broad array of genetic, infectious, and malignant diseases. Gene editing-based strategies utilize targeted nucleases to induce DNA double-stranded breaks (DSBs) at specific sites in the genome, which are preferentially repaired by the nonhomologous end joining (NHEJ) pathway, resulting in therapeutic gene disruption. Previous studies have demonstrated how gene disruption/NHEJ at the CCR5 locus can be applied for HIV cure (Perez et al.;1 Holt et al.;2 Tebas et al.;3 Peterson et al.;4 and Peterson et al.5). Alternatively, homology-driven repair (HDR) can be utilized to insert specified sequences at the nuclease-targeted locus. Like lentiviral vector gene therapy, HDR-dependent gene insertion restores or augments gene function by generating modified cells, which subsequently engraft in peripheral blood and traffic to tissues. To facilitate HDR, a donor DNA molecule encoding a transgene of interest is flanked by homology arms that are matched to sequences on either side of the nuclease cut site. Gene insertion occurs when the nuclease-induced DSB is repaired by the endogenous HDR pathway, using the donor DNA sequence as a template. HDR-based approaches are superior to lentivirus-based strategies in several regards, including exquisite control over the location, copy number, and expression of the introduced transgene. Several recent publications demonstrate the power of this approach for diseases such as HIV.6–9

The potency and safety of gene-modified cell therapy products is dependent on the dose of gene-modified cells. Currently, both gene editing and lentiviral vector-mediated gene modification strategies are limited by the proportion of gene-modified cells that can be generated from a given cell product; frequently, the number of these cells is insufficient to impact the disease in question. For example, we have shown in a nonhuman primate model of HIV infection that lentivirus-modified cells and CCR5 gene-edited cells engraft, persist, and undergo virus-dependent positive selection in vivo but are not present in sufficient quantities to durably reduce plasma viremia (Younan et al.;10 Peterson et al.;4 and Peterson et al.5). We are interested in strategies to select for gene-modified cells, in order to increase the dose of gene-modified cell products to therapeutically relevant levels. So-called in vivo chemoselection strategies utilize modified human proteins with engineered point mutations that confer...
resistance to cognate small molecules. For example, we have previously used the P140K mutant of methylguanine methyltransferase (MGMT^{P140K}) to select for MGMT^{P140K}-modified hematopoietic stem and progenitor cells (HSPCs) following treatment with O6-benzylguanine and temozolomide; this strategy has shown clinical benefit in glioblastoma patients.11–13 Furthermore, since these approaches utilize human genes with conservative point mutations, transgenic proteins’ immunogenicity should be minimal, relative to an exogenous chemoselection marker.

Importantly, different chemoselection platforms may be required for different cell types; previous studies suggest that the MGMT^{P140K} system may be suboptimal in T cells.14 Because T cells are intrinsically more proliferative than HSPCs, chemoselection with methotrexate (MTX) is an ideal strategy to increase the proportion of gene-modified T cells in order to reach a minimal threshold for therapeutic efficacy. MTX is an antimetabolite used to treat neoplasias, severe psoriasis, and adult rheumatoid arthritis.15–18 MTX inhibits dihydrofolate reductase (DHFR), which converts dihydrofolate to tetrahydrofolate during the synthesis of purine nucleotides and thymidylate. By allosterically inhibiting DHFR, MTX interferes with DNA synthesis, repair, and cellular replication and preferentially impairs growth in highly proliferative cells such as proliferating T cells.19 Mutant DHFR (mDHFR) constructs have been developed that confer resistance to lymphotoxic concentrations of MTX. Previous studies demonstrated that cells transduced with the L22Y DHFR variant can be enriched in vivo following treatment with antifolates.20–24 Subsequently, an L22F/F31S double mutant was developed that outperformed L22Y, maintaining catalytic activity while exhibiting a marked decrease in MTX-binding affinity.25 Another variant, F31R/Q36E, could withstand up to 1 μM MTX; murine bone marrow cells transduced with this mutant were enriched within 4 days.25 Previous clinical trials have characterized serum concentrations of MTX in order to better guide the selection of a relevant dose for chemoselection studies: 100 nM to 1,000 nM serum concentrations of MTX are observed in patients following low-dose treatment.26 Proliferation assays were performed in T cells in the presence of 100 nM MTX, and selection for mDHFR-modified cells was determined by measuring the expression of GFP on days 7, 14, and 21 post-cell activation. No-drug control cells were 15%–25% GFP+ at an MOI of 1, while MTX-treated cells were enriched 3-fold over a 2-week culture period, with populations that were more than 70% GFP positive (Figure 2). These data demonstrate that mDHFR-modified primary CD4+ T cells undergo robust positive selection in the presence of low-dose MTX.

**Targeted Gene Insertion of mDHFR Constructs in Primary CD4+ T Cells**

In contrast to lentivirus-based gene modification, HDR-based approaches enable greater control over the site, expression level, and copy number of a given transgene. We paired CCR5 gene editing megaTAL nucleases with a CCR5-targeted AAV donor template carrying mDHFR chemoselection cassettes (Figure 3A), a strategy that we have shown to be highly efficient in primary T cells.27 AAV donor constructs contained mDHFR variants F32R/Q36E (“DHFR1”), L22F/F32S (“DHFR2”), or L22Y (“DHFR3”).20,25 Each mDHFR variant was driven by an MND promoter and linked to GFP through a thymidine kinase (TK) sequence.28 The expression cassette was flanked by CCR5 homology sequences to direct recombination to the gene-edited CCR5 locus (Figure 3B). Cells were first transfected with CCR5 megaTAL mRNA and a control blue fluorescent protein (BFP) mRNA to measure transfection efficiency (>95% BFP+ cells with minimal cell death; data not shown), then transduced with AAV donor vectors. We first tested our strategy using AAV vectors that expressed GFP, but not mDHFR (Figure S1). In control cells that were only transduced with AAV, high levels of GFP (>30%) expression were measured 48 hr post-transduction, but this decreased to <1% within 2 weeks post-transduction. In contrast, GFP expression in cells that received the megaTAL nuclease and the AAV donor vector remained high throughout the duration of the experiment (Figure S1). In 10 total experiments with primary human CD4+ T cells isolated from nine healthy donors, we achieved mDHFR insertion efficiencies between 8% and 38% (Figures 3C and 3D). The presence of HDR-mediated insertion at CCR5 was confirmed by PCR, using genomic DNA extracted from gene-modified and control cells at days 14–16 in each experiment (Figure 3E). These results demonstrate that
HDR-based approaches generate similar proportions of mDHFR-expressing primary CD4+ T cells, as compared to lentivirus-based strategies.

**CCR5-Targeted mDHFR Alleles Are Efficiently Chemoselected by Low-Dose MTX**

Our data show efficient targeting of mDHFR to the gene-disrupted CCR5 locus. To test if these cells could be further enriched following chemoselection with MTX, we cultured mDHFR-modified T cells in 100 nM MTX and measured expansion of modified cells over 2 weeks in culture. After the targeted insertion protocol, cells were re-stimulated using a previously published rapid expansion protocol (REP) in the presence of MTX30 (Figure 4A). Across seven healthy donors, gene-modified CD4+ T cells were enriched from starting levels as low as 3% to as high as 45% following a 2-week selection process. At the physiologically relevant concentration of 100 nM, DHFR1 and DHFR2, as well as DHFR3, conferred comparable enrichment and survival (Figures 4B and 4C). Hence, CCR5-targeted mDHFR alleles are functional and facilitate chemoselection of CCR5-edited CD4+ T cells.

**Gene-Edited T Cells Maintain a Memory Phenotype and a Diverse TCR Repertoire**

We did not observe any significant change in growth kinetics of mDHFR-modified CD4+ T cells in selection culture. To determine the phenotype and clonality of our expanded, chemoselected products, we measured T cell subset markers and performed T cell receptor (TCR) spectratyping. Our culture conditions were designed to promote the growth of cells with a T central memory (TCM) phenotype, which have been shown to exhibit stem-cell-like properties and persist long-term in vivo.31,32 We observed that gene-modified cells retained robust expression of CD45RO throughout the culture period, with a slight decrease in expression of CD62L over >5 weeks in culture, suggesting retention of memory T cells, with a transition from central to effector memory (Figure 5A). We analyzed the T cell receptor repertoire using a PCR-based spectratyping assay.33

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Figure 1. Chemoselection of mDHFR-Modified Jurkat Cells with Low-Dose MTX
(A) Schematic of lentiviral construct. The L22Y-DHFR mutant is driven by an EF1α promoter and linked to GFP expression via an internal ribosome entry site (IRES). (B) Jurkat cells were transduced with the vector in (A) then incubated with MTX. Transgene selection was tracked using GFP flow cytometry. Representative flow plots show a 5-fold selection from day 1 (top row) to day 8 (bottom row) in 100 nM MTX. (C) Absolute numbers of gene-modified cells (cell count multiplied by %GFP+) at the indicated concentrations of MTX. Data shown represent the mean and SD of three experiments. *p ≤ 0.05 by paired Student’s two-tailed t test.
Similar to mDHFR lentivirus-modified T cells (Figure S2), CCR5 gene-edited/mDHFR-targeted T cells retained a comparable TCR repertoire throughout the chemoselection process (Figure 5B). Both modification strategies resulted in either a polyclonal Gaussian distribution or polyclonal skewed distribution of TCR clones at 16 days post-modification, which was maintained through a subsequent 2-week drug selection in MTX. These results demonstrate that our gene modification strategy generates a clonally diverse T cell product that is enriched for memory subsets, which is not impacted by drug selection.

DISCUSSION

We demonstrate safe and highly efficient ex vivo gene editing using a low-dose chemoselection strategy in primary human CD4+ T cells. Targeted gene insertion efficiency at the edited CCR5 locus is comparable to previously described lentivirus-based strategies. Most importantly, our approach not only facilitates close control over the location and expression level of the inserted transgene, but also titration of the number of modified cells in the final product. This approach could be easily modified to include therapeutic/corrective transgenes both in the setting of suppressed HIV infection and for T cell immunotherapies for cancer.

Our study is the first to report robust targeting of an MTX resistance cassette to the edited CCR5 locus in primary T cells, followed by MTX-dependent expansion. We used the hybrid megaTAL nuclease platform to target the CCR5 locus for gene editing in primary human CD4+ T cells. Previously, single-cell molecular analysis demonstrated that 82% of megaTAL-induced HDR events at CCR5 were biallelic, persisted long-term in vivo, and showed no evidence of insertional mutagenesis at off-target sites.6 Importantly, recent data suggest that our approach can be easily recapitulated with other gene-editing platforms such as zinc-finger nucleases (ZFNs) and CRISPR.7,34 Gene editing overcomes many safety concerns associated with lentiviral vector gene therapy, namely mutagenesis at random insertion sites and dysregulated transgene expression. However, in both gene modification schemes, the generation of a therapeutically relevant number of functional gene-modified cells presents a significant challenge for clinical applications. We optimized an ex vivo selection strategy that substantially enriches the proportion of gene-modified cells, which presents multiple advantages in terms of reduced expense and infrastructure for T cell manufacturing.

Drug-resistant DHFR and MGMT140K mutants are two methodologies that have been previously evaluated to enrich gene-modified hematopoietic cells (reviewed in Sorrentino35). These platforms contain key differences that must be considered for chemoselection of particular hematopoietic subsets. While the DHFR system utilizes antifolate metabolic pathways, the MGMT140K system uses an alkylating agent, which may induce genotoxic DNA mutations; the long-term risk of the latter therapy in patients is not well understood.36 In contrast, MTX has been widely used, and safety is well characterized; adverse

Figure 2. Expansion of Primary mDHFR-Modified CD4+ T Cells in MTX

Primary human CD4+ T cells were activated with anti-CD3/CD28 beads, transduced with mDHFR lentivirus, then incubated with varying concentrations of MTX. (A) Representative flow plots showing 3-fold selection for mDHFR-modified cells in no drug (top) versus 100 nM MTX (bottom). (B) Absolute number of gene-modified cells in the presence and absence of MTX, measured over the course of 2 weeks. MTX was added on day 4 post-transduction. Data shown is the mean and SD of five experiments across four healthy donors. All experiments contained two biological replicates.
reactions to MTX treatment can be easily reversed by a leucovorin-rescue regimen.37 The L22Y variant of DHFR was among the first examples of a drug-resistance chemoselection strategy that enabled enrichment of gene-modified hematopoietic cells without the risk of genotoxicity. The kinetics of MTX selection in primary T cells have been previously demonstrated in vitro.37 Previous studies in immunodeficient mice demonstrated the survival advantage and enrichment of lentivirus-modified, drug-selected T cells using L22Y-DHFR.27,28 mDHFR-modified T cells engrafted in these animals, demonstrated robust enrichment in vivo after administration of MTX, and expressed cell surface phenotypic markers that were highly similar to unmodified cells.28 Central memory T cells expressed comparable levels of CD4, CD8, CD28, CD45, TCRαβ, and CD127 surface expression before and after treatment with MTX drug over a short-term 2-week culture period.28 Our results are consistent with multiple past findings, which suggest that mDHFR/MTX-mediated chemoselection is a safe and efficient method of enrichment for gene-modified T cells.

Targeted gene insertion followed by low-dose chemoselection can be incorporated into the manufacture of clinical-scale therapeutic T cell products, namely autologous chimeric antigen receptor (CAR) T cells. The AAV genome can accommodate constructs less than 4.4 kb in size.38 As such, CAR constructs could easily be linked to mDHFR using a T2A sequence and flanked by donor homology arms; these vectors would be analogous to the CCR5-targeted mDHFR-2A-GFP transgenes, which were used to establish proof-of-concept in our study. This approach would facilitate not only the ex vivo enrichment of CAR+ T cells during clinical-grade manufacturing, but also could be used for in vivo selection of modified cells following low-dose MTX in transplanted patients. We have carefully selected a concentration of MTX in our ex vivo experiments to match serum concentrations that are known to be safe in vivo. Intriguingly, low-dose MTX in vivo selection has the potential to synergistically enhance T cell-based therapies targeting a broad spectrum of cancers. For example, since MTX is used alone or in combination with other anticancer agents in the treatment of a variety of CD19+ hematologic malignancies and other neoplasias,39–41 MTX selection could act directly to restrict tumor cells, while simultaneously selecting for CAR-modified cells that will exert immune-based antitumor effects. Making use of suicide switches and/or reversible transgene expression systems such as the Tet-OFF system in CAR T cells has the potential to further provide exquisite temporal control over the long-term expression of our constructs.42,43 Whether used alone or in combination with other immunotherapy approaches, efficient chemoselection for mDHFR-modified cells represents a substantial step forward in the delivery of potent cellular immunotherapy products.

The T cell chemoselection strategy we present here is also applicable for HIV cure studies. Early clinical trials using CCR5-targeting ZFNs demonstrated transient control of HIV infection during antiretroviral treatment interruption.3 Coupling CCR5 knock out with drug selection should aid in achieving higher, therapeutically relevant levels of HIV protection by enabling efficient selection of CCR5-mutated T cells in patients. Increased numbers of persistent, HIV-resistant cells should also improve the broader anti-HIV immune response, consistent with our past findings in the nonhuman primate model.44 We have shown that gene-protected CD4+ T cells lead to decreased plasma viremia and enhanced virus-specific T cell responses both in modified and unmodified cells.45 Hence, an increased dose of HIV-protected, CCR5 mutant T cells, facilitated by mDHFR knock in and MTX chemoselection, should enhance both adaptive and innate immune responses against the virus.45 Furthermore, co-administration of MTX and combination antiretroviral therapy is safe, suggesting that in vivo selection strategies with low-dose MTX are feasible in stably suppressed HIV+ patients.46 mDHFR expression could also be linked to anti-HIV transgenes, such as broadly neutralizing antibodies, facilitating a titratable source of anti-HIV therapeutics. For example, low-dose MTX could be administered immediately upon detection of viral recrudescence, to induce expression of neutralizing anti-HIV antibodies.

In summary, we demonstrate highly efficient gene insertion of MTX-resistant variants of DHFR in CD4+ T cells using a CCR5-targeting megaTAL nuclease and an AAV-delivered donor template, resulting in stable gene insertion of mDHFR at the disrupted CCR5 locus. Subsequent selection with therapeutically relevant concentrations of MTX resulted in substantial enrichment of transgene expressing T cells ex vivo. The MTX platform is well suited for in vivo selection and holds promise for future in vivo selection studies. The application of our approach promises to increase the efficacy of cell therapy strategies to combat both malignant and infectious diseases.

MATeRIALS AND METHODS

**megaTAL Nuclease**

The CCR5-megaTAL nuclease was generously provided by bluebird bio (Boston, MA), and the megaTAL platform has been previously described.3 In brief, the CCR5-specific megaTAL is comprised of an engineered LAGLIDADG-motif homing endonuclease (LHE), also known as a meganuclease, fused to a 10.5-repeat variable di-residue transcription-activator like (TALE) domain via a linker. The CCR5 megaTAL recognizes and cleaves a site in the CCR5 locus located in the sixth transmembrane domain of the protein.

**Cell and Culture Conditions**

Jurkat cells (human T lymphoblast-like cell line) were purchased from the American Type Culture Collection (ATCC) and cultured in RPMI-1640 medium with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin. Cells were maintained at a concentration of between 1 × 10⁶ and 1 × 10⁷ viable cells/mL in T-75 flasks or 12-well plates. Media was replenished every 3–4 days. Primary human peripheral blood mononuclear cells (PBMCs) were isolated from blood of healthy human donors in accordance with institutional guidelines. Untouched CD4+ T cells were enriched using either CD4+ T cell Isolation kit (Miltenyi Biotec, Bergisch Gladbach, Germany) or EasySep Human CD4+ T Cell Enrichment Kit (StemCell Technologies, Vancouver, Canada). Cryopreserved stocks of CD4 cells were thawed, activated ex vivo for 48 hr using cell therapy systems (CTS).
A. Day: -3 -1 0 1 16
16 hr. 30°C Flow cytometry and/or DNA analyses
α-CD3/CD28 Beads megaTAL + AAV donor Return to 37°C
Bead Stim. Removed

B. 5' CCR5 Homology Arm
   MND
   5' CCR5 Homology Arm
   MND
   5' CCR5 Homology Arm
   MND
   DHFR1
   F32R/Q36E
   T2A
   eGFP
   3' CCR5 Homology Arm
   DHFR2
   L22F/F32S
   T2A
   eGFP
   3' CCR5 Homology Arm
   DHFR3
   L22Y
   T2A
   eGFP
   3' CCR5 Homology Arm

C. 

   Day 16
   AAV alone
   DHFR1 0.8%
   DHFR2 0.4%
   DHFR3 1.7%

   Day 16
   AAV + megaTAL
   DHFR1 29%
   DHFR2 24%
   DHFR3 37%

D. % GFP+ CD4+ T-Cells

E. mTAL: + - - + - +
   AAV: - DHFR1 DHFR1 DHFR2 DHFR2

(legend on next page)
Dynabeads CD3/CD28 (Life Technologies, Carlsbad, CA) at a bead-to-T cell ratio of 1:1, and cultured in T cell media. Media consisted of the following: RPMI-1640 Medium (Thermo Fisher Scientific, Hampton, NH), 20% FBS (Thermo Fisher Scientific Hyclone), 50 ng/mL interleukin-2 (IL-2) (Chiron, Emeryville, CA), 5 ng/mL IL-7 (Peprotech, Rocky Hill, NJ), 5 ng/mL IL-15 (Peprotech), and no antibiotics. T cells were seeded at 1–2 × 10⁶ per mL in 48-well plates, with a half media change every 2–3 days. Lentivirus gene-modified T cells were expanded long-term using G-Rex 10 flasks (Wilson Wolf Manufacturing, St. Paul, MN) using the same media formulation as described above. Activated T cells were seeded at between 5 and 10 × 10⁵ cells per flask. During expansion, 50% of the volume of the medium was changed every 5 days, with expansion and chemoselection being monitored by flow cytometry every 7 days. HDR gene-modified T cells were expanded according to a modified REP using pooled irradiated feeder cells, IL-2 and anti-CD3 antibody (clone OKT3, Ortho Biotech, Horsham, PA). 30 Between 3 × 10⁵ and 1 × 10⁶ T cells were seeded in T25 flasks, along with 2.5 × 10⁷ irradiated allogeneic PBMCs and 5 × 10⁶ irradiated CD19⁺ Epstein-Barr virus (EBV)-transformed lymphoblastoid cell line TM LCL. Cells were grown without antibiotics in 50 U/mL IL-2 and 30 ng/mL anti-CD3 OKT3 for 2–3 weeks, and fed with fresh media every 3–5 days as needed.

Homologous Recombination Assay

To target sequences for homologous recombination at the gene-edited CCR5 locus, flanking CCR5 sequences 0.8 kb in length were identified on each side of the megaTAL cleavage site and included in our viral vector donor constructs. The primers used in Figure 3E were designed to amplify CCR5 sequences on either side of the megaTAL cleavage site, allowing distinction of CCR5 with or without inserted exogenous sequences such as mDHFR or GFP. The right primer was located within the 3′ homology arm, while the left primer was located in the region upstream of the 5′ homology arm. The
primers used to amplify and distinguish between modified and unmodified CCR5 loci were 5′-CTGCCCTCATAAGGTGGCCCTAAGGGAT-3′ (sense) and 5′-TCCTCACCCTAGCTCAGTGGA-3′ (anti-sense).

**Cloning of DHFR Mutant Constructs**
Dual-expressing mDHFR/GFP gene-editing templates were created by modification of the previously described CCR5 gene-editing template,47 which introduced a GFP transgene under the control of the MND promoter, into the disrupted CCR5 loci. mDHFR transgenes were constructed as synthetic gBlocks (Integrated DNA Technologies, Coralville, IA).25,27 These cassettes were fused with CCR5 homology arms using Gibson assembly (InFusion HD EcoDry) and linked to GFP via a T2A peptide (Figure 3B).

**Production of Viral Vectors**
Lentivirus and AAV vectors were produced as previously described.18,49 Lentivirus particles were produced using a third-generation self-inactivating lentiviral vector that we have shown to be suitable for our previous gene-therapy studies and clinical trials. Third-generation lentiviral production plasmids were co-transfected into HEK293 cells along with a plasmid encoding the lentiviral genomic RNA encoding the modified DHFR gene. Lentiviral particles were pseudotyped with vesicular stomatitis virus G protein envelope, concentrated, and titered using flow cytometry. The constructs contained a human elongation factor-1alpha (hEF-1alpha) promoter driving the expression of the mDHFR variant, and a GFP reporter linked via a picornavirus internal ribosomal entry site (IRES). Similar to lentivirus production, AAV6 stocks were produced by co-transfecting HEK293T cells with plasmids for AAV vector (encoding the mDHFR donor sequence), serotype helper, and an adenoviral helper.23 These cells were harvested after 48 hr, lysed to release virions, treated with benzonase, and purified over an iodixanol density gradient. Viral titer was determined using qPCR analysis for detection of viral genomes. AAV6 stocks used in this study were titered at approximately 1 × 10^11 viral copies per mL.

**Genetic Modification of Cells**
Activated T cells were transduced with lentiviral vectors at an MOI of 1. Lentivirus was added directly to the T cell media seeded at 2 × 10^5 cells/mL in a 24-well plate. Excess virus was washed off after 24 hr, and efficiency of transduction was assessed at day 2 by flow cytometry. Targeted integration at the CCR5 locus was performed as described previously.17 In brief, activated T cells were transduced with 1 μg of CCR5 megaTAL mRNA in the Neon Transfection system (Invitrogen, Carlsbad, CA) using the settings 1,400 V, 10 ms, and 3 pulses. Two to three hours later, cells were transduced with AAV6 at 20% of the culture volume (independent of viral titer).

Excess virus was diluted by adding fresh media after 24 hr, and efficiency of transduction was assessed at day 2 by flow cytometry. After a day-long 30°C incubation, T cells were cultured in T cell media at 37°C, with fresh media added every 2–4 days during subsequent T cell expansion as described above.

**In Vitro Selection of Gene-Modified Cells**
Chemoselection in the presence of MTX (GeneraMedix, Bridgewater, NJ) was achieved by empirically determining the amount of drug and timing of addition into culture. MTX was diluted in PBS, and aliquots were stored at −20°C. During the REP stimulation protocol, 100 nM MTX was added once on day 4 (Figure 4A). Drug-induced selection was quantified by cell counting with trypan blue exclusion and by flow cytometry for transgene expression and viability.

**Flow Cytometry**
T cells were phenotyped with antibodies against surface markers CD4 (BD PharMingen, San Jose, CA, Clone L200), CD8 (BD PharMingen, Clone RPA-T8), CD195 (BD PharMingen, Clone 3A9), CD62L (BD Biosciences, Clone SK11), and CD45RA (BD Biosciences, Clone UCHL-1) according to manufacturers’ recommendations. Live/dead discrimination was performed using propidium iodide staining. Stained cells were processed on a FACScanto II (BD Biosciences) and analyzed using FlowJo software (Tree Star, Ashland, OR).

**TCR Spectratyping**
Spectratyping analysis of TCR Vβ CDR3 subfamilies 1–25 using multiplex transcription PCR was performed essentially as described previously.33

**SUPPLEMENTAL INFORMATION**
Supplemental Information includes two figures and can be found with this article online at https://doi.org/10.1016/j.omtm.2018.04.002.

**AUTHOR CONTRIBUTIONS**
H.-P.K. is the principal investigator of the study. B.P., C.W.P., D.J.R., and H.-P.K. designed the studies and experiments. B.P. and G.S.R.I. tested the AAV and megaTAL cellular delivery parameters, developed T cell optimization protocols, and generated cells for T cell-editing experiments. B.P., T.E., and G.S.R.I. designed and conducted cell culture and molecular analysis. B.P. and T.E. conducted all follow-up analysis and experiments. N.H. performed molecular cloning of the mutant constructs. B.P. assembled the figures. B.P., G.S.R.I., C.W.P., and H.-P.K. wrote the manuscript. A.A. contributed reagents and helped with protocol design. All authors reviewed and approved the manuscript.

Figure 5. mDHFR-Modified, MTX-Chemoselected Cells Maintain a Memory Phenotype and Diverse TCR Repertoire
(A) Phenotypic analysis of CD4+ T cells by surface marker expression of CD45RO and CD62L. Cells were analyzed on day 7 and day 20 in culture. (B) Following genetic modification with CCR5 megaTAL and CCR5-targeted AAV-mDHFR, TCR spectratyping was used to measure diversity at the CDR3-TCR beta-chain locus. Cells maintained their TCR repertoire following treatment with 100 nM of MTX.
CONFLICTS OF INTEREST
A.A. is a full-time employee of bluebird bio, Inc.

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