megaTAL-mediated Gene Editing at the CCR5 locus

Biswajit Paul

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Reading Committee:

Hans-Peter Kiem, Chair

Andrew M Scharenberg

Jihong Bai

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Abstract

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Biswajit Paul

Chair of the Supervisory Committee:
Dr. Hans-Peter Kiem
Professor of Medicine / Oncology and Pathology
School of Medicine

Engineered nucleases can be used to induce double stranded breaks at specific sites in the genome to facilitate therapeutic gene disruption and/or gene insertion. Here, we report the insertion of a selection marker following gene disruption, which allows us to specifically enrich for gene-edited CD4 T cells in the disease-context of Human Immunodeficiency Virus (HIV). HIV infection remains a substantial health problem worldwide. The C-C chemokine receptor 5 (CCR5) serves as a co-receptor for HIV entry into CD4+ T cells and therefore represents a potential therapeutic target for gene disruption. Our current work uses the CCR5-targeting megaTAL nuclease to achieve site specific DNA cleavage. We demonstrated highly efficient

CCR5 targeting in primary human CD4+ T cells *in vitro* (70-90% disruption). Subsequently, we tested the protective effects of megaTAL treatment of human CD4+ T cells in NOD/SCID/γc-null mice challenged with HIV-1. We observed a 100-fold increase of megaTAL-treated cells compared to untreated controls during an active *in vivo* infection demonstrating the functionality of this approach. Next we coupled CCR5-disruption with the insertion of a drug-resistance gene to enable efficient selection of only CCR5-modified T cells. The mutant human dihydrofolate reductase (mDHFR) chemoselection system has been used to render cells resistant to lymphotoxic concentrations of the drug methotrexate (MTX). We combined megaTAL-treatment with the delivery of a DNA donor template using Adeno-associated virus (AAV) to insert mDHFR constructs at the CCR5 locus. Primary human CD4+ T cells transfected with CCR5-megaTAL mRNA and transduced with AAV6 containing mDHFR flanked by 0.8kb CCR5 homology arms produced MTX-resistant CD4+ cells that also lack CCR5. These gene-modified cells exhibited between five to six-fold enrichment after chemoselection in 0.1uM MTX compared to unmodified controls *ex vivo*.

In conclusion, CCR5-megaTAL produces very high levels of gene-disrupted human CD4+ T-cells and protects these cells from subsequent HIV infection *in vivo*. Furthermore, targeted insertion of drug-resistance genes followed by selection results in the enrichment of gene-modified primary T cells. To our knowledge we are the first to report MTX-mediated chemoselection and expansion of CD4+ T cells following targeted insertion at the CCR5 locus.

TABLE OF CONTENTS

LIST OF FIGURES

Chapter 1. GENOME EDITING USING PROGRAMMABLE NUCLEASES

INTRODUCTION

The field of genome editing has seen tremendous advances in the past decade, with progress happening in incremental, and sometimes exponential steps. Researchers have long envisioned being able to specifically change the genetics of a person in order to treat hereditary disorders. However, the relationships between genes and diseases proved difficult to parse out due to technological limitations. As DNA sequencing became readily available, scientists desired to add or delete genetic sequences in order to determine phenotype. Mice and yeast emerged as dominant *model organisms* to investigate the role of genes in normal cell biology and in disease. This was primarily due to the discovery and development of techniques to manipulate their genomes by the addition or deletion of genetic information. With the completion of the sequencing of the human genome we got closer to being able to start targeting disease-causing genetic elements. Currently, with the advent of gene editing nucleases, every organism can be treated as a *model organism,* since we have the ability to precisely modify genetic sequences with ease. In this chapter, I summarize the current platforms available for gene editing, and describe the characteristics of a therapeutically relevant nuclease.

HISTORY OF GENOME EDITING

Eukaryotic genomes, with the exceptions of mice and yeast, are very difficult to manipulate to prove disease-gene causal relationships at a cellular level. Previously, targeting specific genes in the context of the genome and epigenome had seemed like an insurmountable challenge. The first studies in targeting genes and introducing foreign genetic elements into cells were done in

yeast (Scherer and Davis 1979). The next big leap in gene targeting was in mouse embryo-derived stem cells (Mansour 1988) in which researchers were able to use homologous recombination (HR) and a selection procedure to enrich for cells that contained targeted mutations. These pluripotent cells when injected back into mice gave rise to offspring carrying the mutations in all cell lineages. This technology enabled the creation of transgenic mice that revolutionized medical research by providing uniquely tailored model systems. The actual frequency of HR is around 1 in a million cells, but positive selection techniques help enrich for our desired phenotype. This technique did not translate well to other organisms for various factors, the most important being the incredibly low frequency of recombination. The next major breakthrough occurred when researchers discovered that introducing double strand breaks (DSB) into the DNA increase the frequency of HR by several magnitudes. Rouet et al, first inserted a recognition site for the rare-cutting endonuclease, I-SceI, and then used an assay to measure cleavage rates in the presence of the nuclease with and without a repair template (Rouet 1994). Another group demonstrated the ability to use I- SceI to design genome rearrangements at predetermined locations in the mouse genome (Choulika 1995). They used co-delivery of the I-SceI endonuclease and a donor molecule containing homologous regions flanking the breaks. The induction of a DSB increased the frequency of HR by 2-5 times. Also, these studies reached editing frequencies of about 5%. While this was useful, it had little clinical relevance unless the modified cells were endowed with some kind of a selective advantage.

The relatively recent discovery of programmable nucleases helped usher a new era in genome modification. The first enzyme that could be engineered to cause a targeted DSB was the zinc finger nuclease (ZFN) (Kim 1996, Smith 2000). ZFNs were quickly engineered to recognize a diverse array of targets in various different species (Durai 2005). Re-engineered meganucleases

were also able to introduce DSBs but the extent of targets they could be designed to identify was limited (Chevalier 2002). In the past six years two additional nuclease platforms have been discovered: Transcription activator-like effector nucleases (TALEN), and Clustered regularly interspaced short palindromic repeats (CRISPR)/ CRISPR-associated endonuclease (Cas9) (Christian 2010, Jinek 2013).

HOW GENOME EDITING WORKS: UTILIZING NHEJ VS HDR

DNA repair pathways

Genome editing occurs when DNA double strand breaks (DSB), introduced into the genome by the action of the nuclease, is repaired by the cell's endogenous DNA-repair pathways. DSBs are cytotoxic lesions that if unrepaired lead to genomic instability and cell death. DSBs can be repaired by four possible pathways: non-homologous end joining (NHEJ), homologous recombination (HR), single-strand annealing (SSA), and alternative end joining (alt-EJ). They are reviewed in detail in Ceccaldi 2016, and summarized here.

In NHEJ, breaks are repaired directly by blunt end ligation, in what is a relatively error-prone process. It can occur throughout the cell cycle but is dominant in G0/G1 and G2 phases (Karanam 2012). It also requires the presence of Ku70/80, DNA-PKcs, and DNA ligase IV.

DNA breaks can also be resected to reveal 3' single-stranded DNA overhangs, which are then repaired by HR, SSA or alt-EJ. HR repairs lesions with high fidelity whereas the other two pathways are intrinsically mutagenic. HR predominantly occurs during the mid-S and mid-G2 cycles when a homologous template – in this case a sister chromatid—is present. HR uses cellular factor RAD51 to initiate strand invasion and homology directed repair. SSA and alt-EJ less frequently repair the resected DSB, probably since these mutagenic pathways could

exacerbate the loss of genomic integrity. SSA results in the deletion of one copy of the repeated sequence and also any intervening sequence between the repeats. While the alt-EJ pathway has been found to work in yeast and mammalian cells, the mechanistic details of the pathway have still not been elucidated. alt-EJ has been shown to generate chromosomal translocations and mutagenic rearrangements of the genome because it preferentially joins DSBs on different chromosomes.

Utilizing endogenous cellular repair for gene editing

When a DSB is repaired using NHEJ insertions/ deletions are sometimes introduced at the site, thus disrupting the gene. The majority of DSBs are repaired in a non-mutagenic fashion by NHEJ in a cell. However, in the presence of a nuclease an iterative process of break and repair occurs until a mutation is introduced at the site wherein the nuclease can no longer identify and cleave it. When a DSB in the cell is repaired using HR in the presence of a donor sequence insertions of varying sizes can be introduced into the genome. The donor template needs to have significant homology to the targeted genetic locus for efficient HR. While homology arms as short as 400bp can result in repair, the frequency of insertion usually increases with longer regions of homology (Hendel 2014). For the purposes of this dissertation I will be focusing on gene disruption, facilitated by NHEJ, and gene insertion, facilitated by homology directed repair (HDR).

FOUR GENE EDITING PLATFORMS & HYBRID NUCLEASES

In this section, I briefly summarize the different categories of nucleases available for genome editing. Details on each of these technologies can be found in the following comprehensive

reviews: meganucleases (Stoddard 2011), ZFNs (Urnov 2010), TALENs (Scharenberg 2013), and CRISPR/Cas9 (Hsu 2014).

Homing endonucleases

Homing endonucleases (HE) are naturally occurring selfish genetic elements which repeatedly replicate and insert copies of themselves into host genomes. Only a small fraction of the vast diversity of HEs found across fungal and archael kingdoms have been characterized, with HEs from the LAGLIDADG family being the most commonly used for genome engineering applications. HEs from the LAGLIDADG family are also called meganucleases. They are small ~300 amino acid proteins, naturally found in single celled eukaryotes. They have partially symmetrical N- and C-terminal domains that bind to 22bp DNA substrates and cleave them. Between 22-25 residues are in contact within one turn of the major grove of DNA (Heath 1997). Furthermore, cleavage occurs only when both domains can efficiently bind their respective 9bp half sites and if the central 4 bp adapt a cleavable conformation triggering DNA hydrolysis (Baxter 2012). Cleavage by HE results in a product with a 3' overhang—which has been shown to be unique amongst the available nuclease platforms—a property we can utilize for genome editing. A small minority of the HEs have been re-engineered, using techniques like yeast surface display, to recognize sites in the genome that are different than their natural target sites (Jarjour 2009).

Zinc finger nucleases

ZFNs are proteins in which a FokI restriction enzyme, with non-specific cleavage activity, is fused to a DNA-binding zinc finger domain. Two separate FokI domains need to dimerize in

order for cleavage to occur. Two ZFN monomers must bind to adjacent half-sites separated by spacers, that are 5-7bp long, to form an active nuclease. Each zinc-finger recognizes 3bp and a single nuclease subunit is usually composed of 3-6 zinc fingers. The binding sites, specificity, and protein interactions have been re-engineered extensively. Notably, ZFNs made by Sangamo have proceeded into several clinical trials and have been shown to be safe in vivo (Tebas 2014).

Transcription activator-like effector nucleases

TALENs are composed of DNA-binding TALE domains, which are fused to FokI, and like ZFNs, need to dimerize for cleavage. Each TALE comprises of tandem arrays of 33-35 amino acid repeats, each recognizing one single base pair of DNA. Each TALE repeat interacts with a single base using a two amino acid repeat variable di-residue (RVD). These two important amino acids, which they use for DNA base-recognition, are at positions 12 and 13. Engineering a TALEN is comparatively simpler than a ZFN because the TALEs have an easy to reproduce cipher, with a 1-1 relationship between the four RVD modules and the four bases. The most commonly used RVD recognition modules are: Asn-Asn for guanine, Asn-Ile for adenine, His-Asp for cytosine, and Asn-Gly for thymine.

Clustered regularly interspaced short palindromic repeats/ CRISPR-associated protein 9

In contrast to the three nucleases described above which use a protein-DNA recognition system, the CRISPR/Cas9 uses a RNA-DNA Watson Crick base pairing system to locate its target. Two homing-components of this bacterial immune system, crRNA and tracrRNA, were successfully combined into a single guide RNA (sgRNA) sequence that does not vary. Approximately 20bp of the sgRNA can be constructed to be homologous to the target DNA sequence. The Cas9

nuclease uses the sgRNA to home to the target site in the genome, unravel the DNA, and assay for sufficient homology before cleaving the site. A proto spacer-adjacent motif (PAM) sequence is required for cleavage to occur. This nuclease platform is by far the most easy to engineer towards new targets. However, up to 50% of the sites that the nucleases are designed for do not result in successful cleavage (the mechanism remains unclear).

Hybrid nucleases

Two hybrid-nuclease platforms have been created to add to the four described above. They are Cas9-Fn and megaTALs. The Cas9-Fn uses an inactive Cas9 nuclease fused to a FokI domain to produce DSBs similar to TALENs and ZFNs (Tsai 2014, Guilinger 2014). MegaTALs (which will be introduced in detail in chapter 3) are a hybrid nuclease platform that harness the benefits of HEs and TALE arrays, while simultaneously overcoming their limitations. Briefly, since the TALE array provides assistance in DNA-binding, we can use lower affinity HEs, thereby overcoming the problem of HE re-engineering to maintain binding affinity as well as cleavage activity. Also, since the TALE merely functions to assist in binding the TALE array itself can be shortened, thereby alleviating the difficulty of packaging long TALE repeat-domains.

SPECIFICITY OF NUCLEASES & OFF-TARGET EFFECTS

Decreasing off-target cleavage

One of the most important determinants of the therapeutic efficacy of a nuclease is its specificity. Ideally we require a nuclease with very high cleavage efficiency, but with none or negligible cutting at other areas of the genome. The various approaches, which have been used to increase specificity, are summarized below.

The FokI nuclease domain, which is the cleaving-module in ZFNs and TALENs, has been modified to successfully reduce off-target cuts (Szczepek 2007, Miller 2007, Guo 2010, Doyon 2011). This has been achieved by introducing the technology of obligate heterodimers in which FokI dimeric interface was artificially modifed; cleavage can only occur when two different versions of the FokI heterodimerize in a specific orientation and spacing. This concept has been extended to the CRISPR/Cas9 system too (Tsai 2014, Guilinger 2014). An inactive Cas9 nuclease has been engineered to use the FokI-cleavage domain to produce DSBs that require two gRNAs to bind to a locus, thereby increasing specificity. Alternatively, Cas9 nickase constructs retain the Cas9-cleavage activity, but use the dual-gRNA system to boost on-target cleavage by introducing specific distance and orientation requirements into the reaction (Ran 2013, Mali 2013). Two other methods to increase specificity in Cas9 have been: reducing the length of the complementary region shared by the gRNA and the target (Fu 2014), and structure-guided protein engineering to produce enhanced Cas9 variants (Slaymaker 2016, Kleinstiver 2016).

Evaluating Nuclease Specificity

Nuclease specificity has been assessed using three broad groups of tools: viral capture, biased bioinformatics approaches, and unbiased bioinformatics approaches. Viruses such as AAV or IDLV are often captured at the site of DSBs. This property has been used in the viral capture method to sequence for viral sequences and then detect the region of the genome they randomly integrated into (Gabriel 2011). In the biased bioinformatics method sequences that are similar to the target site are ranked in order of homology, and then deep sequencing is used to confirm whether actual cleavage occurs at the predicted sites (Cradick 2014, Fine 2014). The third method uses different algorithms that have been developed to detect DSBs in an unbiased

fashion: Guide-seq (Tsai 2015) HTGTS (Frock 2015) BLESS (Crosetto 2013) and Digenome-seq (Kim 2015).

So far there are no defined set of assays that can establish whether a nuclease is going to be safe for editing human cells. The off-target analyses need to be tested in the therapeutic cell type the researcher intends to use in the clinic. Cell lines have abnormal DNA-repair and unpredictable levels of background DSBs in their genomes. Therefore, testing nuclease off-target assays in transformed cell lines has limited utility because results cannot be easily extrapolated to normal human cells. Hence, in our study we characterized the specificity of our megaTAL nuclease in primary human T cells (Sather 2015). A general rule of thumb applied in the field is to reduce off-target gene editing by limiting the amount of time the nuclease is expressed in the cell. In our case, we use mRNA delivery to minimize the duration of megaTAL nuclease exposure in cells.

MEGATAL EXHIBITS THE CHARACTERISTICS OF A GOOD THERAPEUTIC NUCLEASE

A good therapeutic nuclease platform would be easily engineerable and highly specific. While naturally occurring nucleases have evolved over years to recognize their target sequence, in the clinic we need nucleases that can be re-engineered to novel (disease) targets. Genomic specificity is a necessary property in order to avoid cytotoxicity or tumorigenesis (Cornu 2008, Pierce 2001).

Additionally, the nuclease should be easily deliverable. The different methods available for delivery are: plasmid DNA, messenger RNA (mRNA), and viral vectors. Each of the delivery methods have their advantages and disadvantages. Plasmids are cheap and easy to manufacture, and are stable delivery reagents. However, they are usually toxic to primary cells due to the

induction of an immune response, or because of the delivery method. On the other hand, mRNA delivery has been shown to achieve high levels of gene expression with low toxicity. The limitations are the cost of manufacture, and the easily degradable nature of mRNA.

Here are factors to consider while evaluating a nuclease platform for use in the clinic:

1. Activity, specificity, and compactness of the nuclease
2. High levels of cleavage in primary cells
3. Efficient engraftment of gene-modified cells
4. Delivery system results in transient expression of nuclease
5. Compatibility with delivery of repair templates
6. Delivery of nuclease and template is economical and GMP-compatible

See Table for a comparison of these parameters across the existing nuclease platforms.

The CCR5-megaTAL is a modified HE re-engineered to target the CCR5 locus. It is a compact, single-protein enzyme, which makes it easy to package and deliver into cells via vectors. This actually opens up the possibility of multiplexing: multiple re-engineered HEs targeting different sites could be packaged inside the same vector.

In the following chapters I demonstrate how the CCR5-megaTAL nuclease platform – the subject of this dissertation – meets all of the criteria required for a nuclease to be therapeutically useful. CCR5-megaTAL produces a 3' overhang upon cleaving, thus leaving a 4bp "resected" DNA piece for DNA repair machinery to access. This unique biochemistry of the enzyme's DNA cleavage is a very important consideration, especially for gene insertion.

Table 1. Comparison of strengths and limitations of major gene editing nuclease platforms.

	ZFN	TALEN	CRISPR/Cas9	megaTAL
Recognition site	9-18bp per ZF 18-36bp per pair	14-20bp per TALE 28-40bp per pair	22bp for SpCas9 44bp for nickase pair	14-20bp upto 40 with TALE
Specificity	some off-targets	some off-targets	mismatches tolerated	highly specific
Ease of engineering	Difficult requires protein engineering	Moderate requires complex cloning	Easy quick oligo assembly	Difficult requires protein engineering
Ease of delivery	Moderate; Small size can be packaged into viruses; requires co-delivery	Difficult; Large size Repetitive sequence causes problems in viral packaging	Moderate; Large size requires co-delivery	Easy; Small size can be packaged into viruses; can be multiplexed
Type of DSB generated	5'	5'	blunt, 5'	3'
Targeting constraints	sequence needs to be G rich	5' base of target must be a T	PAM needs to precede target	Re-targeting can decrease activity
Immunogenicity	Low; human protein	Unknown; bacterial protein	Unknown; bacterial protein	Unknown; eukaryotic protein

Chapter 2. GENOME EDITING AS A THERAPY FOR HIV

INTRODUCTION

Acquired Immunodeficiency Syndrome (AIDS) is a fatal infectious disease characterized by the systemic collapse of the human immune system caused by a retrovirus named the Human Immunodeficiency Virus (HIV). HIV remains a significant global health problem with an estimated 33 million currently infected and 6300 new infections per day (amFAR report). Mortality rates have been drastically reduced by the use of daily doses of anti-retroviral drug therapy (ART). However, side effects from the drugs, drug interactions, and cytopathogenicity of the residual virus together contribute to an increase in morbidity over the lifetime of the patient (Burgoyne 2008). Hence, new treatments are needed to improve the quality of life of individuals living with HIV. Most strains of HIV require binding with both the CD4 and CCR5 T-cell receptors for entry into, and subsequent infection of the target cell. Importantly, over the last decade CCR5 has been efficiently disrupted by sequence-specific nucleases to render cells resistant to HIV-infection (Manjunath 2013).

In this chapter I summarize the various attempts to treat HIV over the last three decades. These range from the highly effective antiretroviral therapy, and cell modification by conventional gene therapy, to the more recent gene editing using nucleases. I subsequently highlight the limitations of current approaches and establish an argument for why my dissertation project addresses these.

HIV LIFE CYCLE & TROPISM

The key steps in HIV life cycle are: 1) binding, 2) fusion, 3) reverse transcription, 4) integration, 5) replication, 6) assembly, and 7) budding. During binding the virus attaches to the cell surface

using the CD4 receptor, in addition to a coreceptor: either CCR5 or CXCR4. After attachment, the cell membrane and HIV fuse and this completes viral entry into the cell. Once inside, HIV uses reverse transcriptase to convert its RNA-genome into DNA. This DNA is then exported into the cell nucleus and inserted into the host genome. HIV uses a specialized integrase enzyme to randomly integrate into the host DNA. Using cellular machinery, HIV starts replicating copies of its genome. These parts of the virus are assembled in the cytosol to form immature virus particles. These immature particles bud-off the cell membrane and use viral protease to cleave HIV proteins that are subsequently rearranged to yield infectious virus.

The viral preference for infecting one cell type versus another is called viral tropism. HIV's tropism can be divided into two broad categories based on cells infected: T-tropic, infecting T lymphocytes; and M-tropic, infecting macrophages, monocytes, peripheral blood mononuclear cells, and T lymphocytes. These two categories also differ in co-receptor usage and temporality during infection. While M-tropic viruses use CCR5, T-tropic viruses use CXCR4 co-receptor for successful infection. Also, M-tropic viruses are predominant in the early 'acute' stage of infection whereas T-tropic infection is established at later stages in chronically infected individuals (Hoffman 2007). HIV-1 Group M is the strain responsible for the current global epidemic contributing to roughly 90% of infections (Buonaguro 2007). In humans, HIV-1 mainly infects CD4 T lymphocytes, with monocytes, dendritic cells and macrophages being secondary targets.

ANTIRETROVIRAL THERAPY

Antiretroviral therapy (ART), a combination of antiviral drugs targeting stages of the virus life cycle, has been a very effective line of defense against progression of HIV into full-blown AIDS.

Clinicians have 24 FDA approved drugs at their disposal that specifically inhibit different steps in the virus replication and propagation cycle (reviewed by Arts 2012).

However there are several limitations of the therapy. Patient adherence to ART is significantly impacted by access to medication, drug related side effects, and social and mental stigma against treatment (DHHS- HIV/AIDS care continuum). ART drugs also contribute to long-term morbidity in patients, attributed to the low-level inflammation state of an immune system that is constantly 'under attack'. Even after long-term virologic control many patients cannot return to normal healthy CD4 T cell counts (Kelley 2009).

Curing HIV presents a significant challenge due to the characteristics of infection by the pathogen. The HIV-1 genome is composed of two identical copies of single-stranded RNA. During infection the virus inserts a copy of itself into the host cell's genome and irreversibly integrates into actively transcribed genomic material. Some of these integrated viruses remain dormant in what are known as the latently infected reservoir (Finzi 1997, Chun 1997). The latent viruses can be reactivated in response to activation of the cells. This is especially problematic since ART drugs target different stages of the replicating viral life cycle. Hence, even after years on ART, patients with undetectable viral loads will exhibit a rapid viral rebound if treatment is stopped or interrupted (Siliciano 2003).

GENE THERAPY STRATEGIES TO CONTROL HIV

Historically gene editing has been defined as the practice of adding genetic material to human cells. Conventional gene therapy approaches to treat HIV have focused on adding anti-HIV genes to CD4 T cells. The hope was that removing infectable cell populations would reduce viremia, while proliferation of the engineered cells would maintain immune function.

Some of the strategies targeting various stages of virus life cycle that have already been tested in clinical trails are: suppression of host or viral genes using RNA-based methods, viral RNA decoys, interfering peptides, and viral proteins (see Table).

TARGET	BIOLOGICAL PROCESS	MECHANISM OF ACTION	CELL TYPE USED
HIV-1 env protein	**Viral Entry**	**Fusion inhibitor peptide c46**	**Autologous CD4 T Autologous CD34**
HIV-1 tat-vpr RNA	**RNA transcription**	**Ribozyme**	**Autologous CD34**
HIV-1 Rev protein	**RNA nuclear export**	**RNA decoy**	**Autologous CD34**
HIV-1 TAR protein	**RNA nuclear export**	**Antisense RNA**	**CD4 T cells**

Here I summarize some of the key clinical studies demonstrating key principles used to target HIV. For example, a combinatorial lentiviral vector that targeted three RNA-based inhibitors was found to increase the suppression of HIV replication long term (Li 2005). The vector contained a short hairpin RNA targeting HIV-1 rev and tat mRNAs, a TAR RNA decoy, and an anti-CCR5 ribozyme. In another clinical trial, lentivirally transduced CD4 T cells harboring antisense trans-activation response [TAR] element and/or trans-dominant Rev-engineered constructs showed a survival advantage in infected patients over the course of three years, especially during a period of high viral load (Morgan 2005).

An HIV entry-inhibitory peptide called C46 introduced into CD4 T cells using a retrovirus was tolerated well and the ten patients showed sustained gene marking up to 12 months (van Lunzen 2007). Importantly, a majority of the patients experienced a significant decline in plasma viral load at their 1-year follow-up. When tested in a non-human primate model, mC46-gene modified autologous HSCs gave rise to gene modified T cells *in vivo* and showed a proliferative advantage after SHIV infection following bone marrow transplantation (Younan 2013). In a subsequent

study, researchers supplemented the same mC46-gene modified HSC transplantation system with a vaccinia virus-based vaccine expressing SIV-Gag Pol (Younan 2015). Peak and chronic levels of viremia were decreased by 1-log in the vaccine+C46 group demonstrating control of disease progression in these macaques. Importantly, these studies in the NHP model of HIV/AIDS demonstrated that genetically modified cells protected against infection in vivo (Younan 2013). More importantly, they contributed to the development of a heightened immune response against the virus. CD4 T cells showed a gradual recovery and macaques in the treatment group showed marked decrease in plasma viremia and an upregulation of immune response including antibody response and cytotoxic T cell response. The HIV-protected cells help in increasing both adaptive and innate immune responses against the virus.

CD4 cells retrovirally transduced with huM10, a humanized version of the REV protein that inhibits HIV-1 replication, showed a selective advantage over the course of 2 years, specifically during the time of increased HIV-1 load (Podsakoff 2005). In another study, CD34+ HPSCs expressing a tat-vpr-specific anti-HIV ribozyme OZ1 were tested in patients in a phase 2 clinical trial (Mitsuyasu 2009). Over the course of 100 weeks the patients who received OZ1 were found to have higher CD4 T cell counts. Taken together, these gene therapy trials have helped demonstrate safety and proof-of-concept. However, none were able to provide a sustained functional cure for HIV infection.

GENE EDITING STRATEGIES TO CONTROL HIV

Rationale (or why use gene editing)

Genome editing using engineered nucleases allows for very precise manipulation of cells. These technologies have significantly built upon the successes of conventional gene therapy approaches

that consist of gene delivery using semirandomly integrating viral vectors. When it comes to manipulating the sequence of the human genome, gene disruption is comparatively easier to accomplish than gene editing or insertion. First, disruption requires the delivery of only the nuclease, while the others require the added presence of a homologous DNA template. Second, the NHEJ pathway used during disruption is active during all stages of the cell cycle. In contrast, the HDR cellular repair machinery is active mainly in the S and G2 phases (Saleh-Gohari 2004; Karanam 2012). Hence, we have several anti-HIV gene disruption trials currently ongoing in the clinic.

A large number of gene editing approaches for HIV therapy have targeted viral entry into the cell using gene disruption. The CD4 receptor, which is absolutely essential for HIV entry, is also essential for the normal functioning of the immune system. Hence, only the viral co-receptors, namely CCR5 or CXCR4, can be targeted for disruption.

A naturally occurring, but rare, 32 base pair deletion in the CCR5 gene, called delta-32, confers resistance to HIV infection in certain individuals (Samson 1996, Huang 1996, Liu 1996). In European populations, heterozygote and homozygote carriers occur at a frequency of approximately ten and one percent, respectively. This deletion results in the expression of a truncated protein which is not expressed on the cell surface. Although studies have shown that this allele may be associated with an increased risk for symptomatic West Nile virus infection (Glass et al., 2006), it has not been associated with other significant health impacts. Several methods have tried to engineer cells with altered CCR5 expression or functionality, with varying success.

The functional cure of the 'Berlin patient', Timothy Ray Brown, energized the field of gene editing (GE) based HIV therapy in 2009 (Hutter 2009). Following a transplant of allogeneic stem cells from a donor homozygous for the CCR5 delta-32 deletion, Brown has remained virus free without ART since then (Allers 2011). Even though this approach is promising it has several limitations. One, the likelihood of finding a tissue-matched donor who is also homozygous for the rare delta-32 mutation is very small. Two, the treatment regimen for Brown was pretty invasive—including conditioning (total body irradiation), and two bone marrow transplants—and not suitable for widespread treatment. Third, he developed graft versus host disease, which probably helped reduce the size of his latent viral reservoir. Hence, mimicking the delta-32 phenotype by using GE-based CCR5 disruption to overcome these transplant-related limitations is a viable and logical next step.

Amongst the different nuclease platforms, the ZFN system has been the best characterized system amongst GE-based HIV therapies, primarily because it has been around for the longest duration of time. The first report on using ZFN to alter CCR5 was published in 2005 (Mani 2005). Most therapies have targeted CD4 T cells and CD34 stem cells for modification in humans. Primary cells have been modified ex vivo and subsequently either infused into humanized mouse models or, as in the case of the ZFN clinical trials, into patients (Perez 2008, Holt 2010, Tebas 2014).

Lastly, gene editing overcomes the complexities and invasiveness of allogeneic transplant. It also performs a permanent 'genetic surgery' and overcomes the short-term or incomplete treatment offered by previous RNA- and protein-based technologies (Yang 1997, Bai 2000, Cordelier 2004, Qin 2003, Anderson 2005).

Gene Editing of the CCR5 locus in CD4 T cells

A zinc finger pair designed to introduce a DSB at around 160bp inside the CCR5 ORF (corresponding to the first loop out of the seven that make up this transmembrane proteins) generates a non-functional protein (Perez 2008, Holt 2010). Pre-clinical studies using mouse models of HIV infection demonstrated the efficacy of protection when either CD4 T cells or CD34 HSCs are modified.

In the first pre-clinical study, Carl June's group delivered ZFNs using a chimeric Ad5/F35 adenoviral vector into primary human CD4 T cells (Perez 2008). They achieved between 30-60% CCR5 disruption, with 33% of these being biallelic disruption events. In a humanized mouse model—immunodeficient mice whose immune system has been reconstituted with human hematopoietic cells—these disrupted cells showed preferential survival during active HIV infection while the mice exhibited reduced plasma viremia levels. Off-target analysis of the ZFN pair showed a ~5% cleavage at the locus of the highly homologous CCR2 gene. If this off-target cleavage is found to be problematic in clinical trials it can be addressed in the future by re-engineering the enzyme if needed. Indeed, it is interesting to note that CCR2 modification might actually be beneficial to anti-HIV therapy since there are reports of patients with mutated CCR2 alleles exhibiting delayed progression to AIDS (Smith 1997).

Following the success of their model the group scaled up their manufacturing process in order to produce $>10^{10}$ CCR5 modified cells with the aim of making products that can be readily used in clinical trials (Maier 2013). Using their protocol CD4 T cells can be extracted from a patient, activated using CD3/28 beads, gene-modified using nucleases, expanded in a cytokine-enriched medium inside a bioreactor, and finally transplanted back into the same patient. They demonstrate that these expanded and modified cells still exhibit similar phenotype, cytokine

production, and clonal diversity as unmodified cells. They also showed that infusion into humanized NSG mice was not associated with toxicity or adverse events like T cell transformation.

The first human clinical trial applying the protocols described above, was started in 2009 with the aim to assess the safety and efficacy of transplanting gene modified cells and evaluating control of HIV. Twelve patients on ART who had undetectable viral loads were divided into two cohorts based on their CD4 T cell counts (Tebas 2014). Patients with counts >450/mm^3 were assigned to Cohort 1 while patients in Cohort 2 had between 200 and 500/mm^3. All patients were infused with 5-10 billion CD4 T cells, 10-20% of which were found to be CCR5 disrupted. The cells engrafted in all patients, trafficked to rectal mucosa, and persisted for >42 weeks post-infusion. 4 weeks after infusion patients in Cohort 1 were taken off ART for 12 weeks to assay viral rebound and potential protective effects of CCR5 disruption. Viral rebound was observed in the 4 patients who remained off ART for 12 weeks. CD4 T cell counts also decreased in these patients but at different rates. The decline of T cells was slower in the CCR5 modified experimental arm probably due to the protective effect of disruption. One of the patients in the trial was an especially interesting case study. The patient exhibited delayed viral rebound and a lower peak viremia level (when compared to their viremia before the transplant). This patient was later found to be a heterozygote for the delta-32 mutation. This meant that the transplant had effectively given them a population of biallelically modified CD4 T cells and therefore amplified the effects of treatment.

Since this initial trial, other clinical trials have been initiated to test different parameters to optimize the gene modification and transplant protocol. The parameters currently being evaluated are: improving engraftment of T cells using Cytoxan preconditioning; changing cell

dosage and number of infusions; and using RNA electroporation to deliver the ZFN (summarized in Wang 2016).

Gene Editing of the CCR5 locus in CD34 HSCs

Following the success of Carl June's group, Paula Cannon's research team edited CD34+ hematopoietic cells to disrupt CCR5 using the same ZFNs. The hope is that when these modified CD34s are infused in patients their progeny T cells and macrophages will provide a continuous source of HIV resistant cells. Initially, using plasmid transfection they were able to disrupt CCR5 at a mean frequency of 17% in hematopoietic stem/progenitor cells (HSPCs) (Holt 2010). CCR5-modified HSPCs engrafted in mice and differentiated equivalent to control group. Viremia post-HIV challenge was controlled to below the limits of detection in the ZFN-treated group and the treated cells selectively escaped HIV infection and death. More recently the Cannon group has optimized this system to be compatible with clinical trials by using CCR5-ZFN mRNA for better cell viability and a MaxCyte GT system that can modify up to 300 million cells at a time

Gene Editing of the CCR5 locus using other strategies

Other nuclease platforms TALENs and CRISPR/Cas9 have also targeted CCR5 with comparable levels of success in disruption (see Summary table for representative studies). These recently characterized nucleases have not made the transition into clinical trials yet since there are some technological hurdles for them to overcome.

Table 2. Summary of Gene Editing strategies targeting HIV using ZFN, TALEN, CRISPR and megaTAL.

REFERENCE	NUCLEASE PLATFORM & TARGET	CELL TYPE	DELIVERY
Yao 2012	ZFN CCR5-disruption	embryonic, induced pluripotent stem cells	expression vector
Lombardo 2007	ZFN CCR5-disruption	CD34 HSC	IDLV
Lei 2011	ZFN CCR5-disruption	hES	baculoviral vector
Li 2013	ZFN CCR5-disruption	HSPC	Ad5/35
Tebas 2014	ZFN CCR5-disruption	autologous CD4 T cells	Ad5/35
	ZFN CCR5-disruption	autologous CD4 T cells	Ad5/35 (+Cytoxan)
	ZFN CCR5-disruption	autologous CD4 T cells	mRNA (+Cytoxan)
	ZFN CCR5-disruption	autologous HSC	mRNA (+Busulfan)
Badia 2014	ZFN CCR5-disruption	TZM-bl cells	plasmid
Mussolino 2011	ZFN TALEN CCR5-disruption	293 T cells	
Ru 2013	TALEN CCR5	induced pluripotent stem cells	peptide
Liu 2014	TALEN CCR5	293 T cells	peptide
Mock 2015	TALEN CCR5	CD4 T cells	mRNA
Kang 2015	CRISPR CCR5	induced pluripotent stem cells	plasmid
Cho 2013	CRISPR/Cas9	293 T cells	
Ye 2014	CRISPR/Cas9	iPSCs	piggyBac transposon
Li 2015	CRISPR CCR5	CD4 T cells	Ad5/35
Didigu 2014	ZFN CCR5 CXCR4	CD4 T cells	Ad5/35
Wilen 2011	ZFN CXCR4-disruption	CD4 T cells	Ad5/35
Yuan 2012	ZFN CXCR4-disruption	CD4 T cells	Ad5/35
Hou 2015	CRISPR CXCR4	CD4 T cells	plasmid
Qu 2013	ZFN Provirus (LTR)	CD4 T cells	
Wayengera 2011	ZFN Provirus (Pol)	TZM-bl cells, HeLa-derived JC53-BL cells	
Ebina 2013	CRISPR Provirus (LTR)	Jurkat, 293T cells	plasmid
Hu 2014	CRISPR Provirus (LTR)	Microglial, promonocytic, and T cells	plasmid
Voit 2013	ZFN combination therapy	Jurkat cells, JLTRG-R5 cells	plasmid
Fadel 2014	LEDGF/p75 TALEN	293 T cells, Jurkat cells	plasmid
Zhou 2014	TSPO CRISPR	293 T cells	plasmid
Bogerd 2015	CRISPR combination therapy	CD4 T cells	
Sather 2015	megaTAL combination therapy	CD4 T cells	mRNA

Gene Editing of the *CXCR4* locus

Even though CXCR4-tropic viruses usually emerge during late stage infection in patients, a long-term cure for HIV-1 cannot be accomplished until viral entry from this receptor is also blocked.

CXCR4 is essential for normal hematopoietic stem cell homing and development (Peled 1999). Hence, targeted CXCR4 disruption with nucleases is clinically only feasible in terminally differentiated populations of CD4 T cells.

Ad5/35 vectors were used to deliver CXCR4-ZFNs into CD4 T cells and successfully edit the locus (Wilen 2011). These cells were preferentially selected for when transplanted into an NSG mouse model and infected with R4 virus. It is of note that this study ultimately failed due to the emergence of CCR5 tropic viruses in the mice.

In a similar study, ZFNs targeting CXCR4 were compared to an shRNA knockdown strategy in human CD4 T cells (Yuan 2012). The researchers found that efficient disruption of the CXCR4 locus by the ZFNs was superior in protecting cells when compared to shRNA in a humanized mouse model of infection.

Combinatorial Gene Editing approaches

Simultaneous disruption of both co-receptors used by HIV helped researchers establish protection from both X4- and R5-tropic viruses (Didigu 2014). These modified cells proliferated normally when introduced in NSG immunodeficient mice and offered a 200-fold protection from in vivo HIV infection for up to 55 days post.

In a different proof-of-concept study, resistance to both R5 and X4 tropic viruses was accomplished in T cell lines by using anti-HIV restriction factors APOBEC3G, TRIM5a, D128K that were knocked into the CCR5 locus using ZFN (Voit 2013). More recently, CRISPR/Cas9 was used to turn on the expression of APOBEC3B, an endogenous viral restriction factor, in T cells, rendering them resistant to infection by HIV-1 (Bogerd 2015).

Targeted recombination at CCR5 locus has been used to insert the C46 gene to help resist infection from CXCR4-tropic HIV (Sather 2015). These researchers also inserted an HIV-specific chimeric antigen receptor at the CCR5 locus. These protected T cells are then equipped with the ability to kill HIV-infected cells – a promising new form of anti-HIV immunotherapy.

Gene Editing targeting the Provirus

A major limitation of the above-described anti-HIV therapies is their inability to eliminate integrated HIV-1 genomes. Disruption of CCR5 and CXCR4 can lead to a functional cure by preventing spread of viral infection. However we need to eradicate viruses from already infected cells to be able to achieve a sterilizing cure. Hence, eliminating HIV proviral DNA from infected cells is another strategy being pursued for the purposes of a complete HIV Cure. ZFNs targeting the conserved 3' and 5' LTR sequences of HIV were able to successfully excise proviral genomes from CD4 T cells, even those that were latently infected (Qu 2013). Similarly, other groups have demonstrated proviral-genome disruption using HEs, CRISPR/Cas9, and TALENs (Aubert 2011, Ebina 2013, Hu 2014, Zhu 2015, Ebina 2015). A re-engineered Cre recombinase enzyme called uTre that recognizes conserved sequences within the HIV-1 long terminal repeat has also been used to excise integrated viral genomes from cells (Karpinski 2014).

While these proof-of-principle studies demonstrate this as a potential strategy for cure, the major limitation for clinical translation of this approach is the current inability of nuclease delivery technologies to target latently infected cells. These cells are very rare and sometimes inhabit regions of the body that are not easily accessible by current delivery.

SUMMARY

More recently, genome editing approaches to treat HIV have focused on precisely engineering hematopoietic cells. These one shot therapies rely on DSB induction by nucleases followed by repair using endogenous DNA-break repair pathways. Repair by the NHEJ pathway can be used for gene disruption of co-receptors or of integrated viral genomes. Repair by HDR can be used to add anti-HIV genes, chemoselectable markers, or chimeric antigen receptors targeting infected cells. Additionally, HDR could be used to edit host restriction factors or viral dependency factors in the genome.

In the next two chapters of this dissertation I shall demonstrate how the most recent advances in the application of genome editing use: (a) NHEJ to disrupt the CCR5 locus, and (b) highly efficient homology-driven repair to insert transgenes for targeted selection of modified human hematopoietic cells.

Chapter 3. EFFICIENT MODIFICATION OF THE CCR5 LOCUS IN PRIMARY HUMAN T CELLS WITH MEGATAL NUCLEASE ESTABLISHES HIV-1 RESISTANCE

INTRODUCTION

A naturally occurring 32 base-pair deletion of the HIV-1 co-receptor *CCR5* has demonstrated protection against HIV infection of human $CD4^+$ T cells. Recent genetic engineering approaches using engineered nucleases to disrupt the gene and mimic this mutation show promise for HIV therapy. By fusing a reprogrammed homing endonuclease (HE), also known as a meganuclease, to a TALE DNA binding domain, we have developed a hybrid nuclease platform, called a megaTAL, targeting the *CCR5* gene. Unlike the TALEN and zinc finger nucleases that require dimerization, and CRISPR/Cas9 nucleases that require co-delivery of a guide-RNA, megaTALs are monomeric and can achieve gene modification using a single cistron, a feature that may promote their application to clinical settings requiring scalability and ease of delivery.

In the present study, we evaluated the efficiency of this nuclease to disrupt *CCR5* and subsequently protect cells from HIV infection *in vitro*. We also investigated the ability of this nuclease to protect $CD4^+$ T cells during active HIV-1 infection *in vivo* using immune-deficient mice. Here we show that the CCR5 megaTAL nuclease disrupts the expression of *CCR5* and can prevent HIV infection in cell lines. The CCR5 megaTAL also disrupts *CCR5* in primary human $CD4^+$ T cells with a high efficiency, and we show that these gene-modified T cells stably engraft in immune-deficient mice. Furthermore, we demonstrate that CCR5 megaTAL-modified T cells are preferentially expanded during HIV-1 infection *in vivo*. Our study is an important step towards the ultimate goal of providing a population of immune cells that are resistant to HIV-1 infection, that could be used to reconstitute the patient's immune system.

SUCCESSFUL REPROGRAMMING OF THE I-ONUI HOMING ENDONUCLEASE TO TARGET *CCR5*

Reassembly of Homing Endonucleases

Customizable site-specific nucleases are quickly becoming an important tool for therapeutic gene editing applications. Homing endonucleases (HE) are well suited for this purpose since they are active and highly specific. However, they have been difficult to re-engineer to make them recognize non-native sequences.

Initial attempts at re-engineering I-AniI (a well-characterized HE) were energized with the discovery of a new LHE, I-OnuI which recognized target sites vastly different from previously known enzymes (Sethuraman 2009). I-OnuI was found to exhibit biochemical properties that made it a perfect choice for further engineering. When compared to I-AniI it showed 10-100 fold greater binding affinity, whilst retaining cleavage activity. The most common consequence of re-engineering nucleases is a loss of activity or binding. Starting the re-engineering process with a nuclease platform that displays high activity is hence advantageous. Hence, I-OnuI was used as a starting scaffold and re-targeted to cleave CCR5. We customized HEs for genome editing by using a method in which the native enzyme is split into 'modules' and its specificity towards substitutions within short pockets of the DNA target is redesigned. The resulting module mutations are combined to target the full desired sequence of interest.

Yeast surface display (YSD) technology can be used to reprogram the specificity of LAGLIDADG-motif homing endonucleases (LHE's), also known as meganucleases (**Figure 1a**).[20] Yeast surface display is a genome-engineering assembly line in which all steps-- from the generation of libraries, to the selection of putative enzymes and the final characterization of nucleases-- are integrated. Flow cytometry was used to rapidly assess the stability, binding and

cleavage activity of the re-egineered LHEs displayed on the surface of yeast. The synthesized LHE is fused to an inducible surface displayed protein Aga2p by means of two disulfide bonds (Jarjour 2009). YSD is extremely useful as a screening mechanism to screen libraries comprising tens of millions for three reasons. First, only properly folded and functional proteins are presented because they are passaged through the yeast's ER and Golgi secretory system. Second, binding affinity for different DNA sequences can be easily assayed by incubating fluorescently labeled target oligonucleotides, washing, and looking for increased signal by flow cytometry. Third, cleavage activity of the enzymes is quantified by recording the cleavage-associated loss in fluorescence when labeled putative target oligos are incubated with the YSD. Flow cytometry allows high throughput analysis of several nucleases and eliminates the need for recloning at each subsequent step.

Design and construction of the CCR5-megaTAL nuclease

A CCR5-targeting meganuclease was engineered from the native I-OnuI meganuclease by Dr. Jordan Jarjour of bluebirdbio, Inc. (previously Pregenen, Inc). A target sequence in *CCR5* was identified which comprised the central-4 binding motif of the LHE, I-OnuI, a sequence required for efficient DNA hydrolysis and double-stranded break (DSB) formation. The enzyme's C-terminal domain (CTD) and N-terminal domains (NTD) were reprogrammed separately by screening degenerate libraries harboring mutations in the DNA recognition interface of each domain (**Figure 1b**). We assembled customized HEs by first engineering overlapping subregions of the DNA-nuclease interface, followed by screens to enhance catalytic activity and specificity. First, we divided the entire HE-DNA interface ($20^{40\text{-}50}$ HE variants, 4^{22} substrate variants) into smaller overlapping units that could be sequentially screened for nuclease re-engineering. At the end of the modularization process we created a database of ~27,000 modular HE variants

targeting ~79% of the 704 triplets queried within the I-OnuI scaffold. The HE re-engineering process first separates DNA targets into their component triplets, references these against our modular variant database, and identifies ones that have a high probability of being successfully merged. We were able to readdress each domain, i.e. change their half site recognition, and subsequently fuse these domains.

Following domain reprograming, pools of successfully reprogrammed domains were fused and screened to arrive at a fully reprogrammed homing endonuclease that could recognize the target *CCR5* sequence (**Figure 1c**). The initial versions of the enzymes assembled demonstrated low activity in reporter cell lines inspite of sharing comparable biophysical properties to wild type I-OnuI. The enzymes went through a process of iterative mutagenesis to select for variants that exhibited enhanced catalytic activity and binding.

Specificity of the CCR5-megaTAL nuclease

The reprogrammed LHE was subsequently assembled to a TALE DNA binding domain via a flexible linker; this megaTAL architecture was utilized to increase the binding constant (K_{on}) of the meganuclease. This approach has been successful in generating similar reagents which exhibit desirable characteristics for translatable applications.[20-22]

Previous work has shown that the hybrid megaTAL architecture results in very efficient targeted cleavage because it combines an elongated target site (provided by fusion of the TAL effector binding domain and meganuclease catalytic domain), with the inherent high cleavage specificity of the HE domain. High specificity is the ultimate goal for a therapeutic nuclease. The auxiliary TAL effector DNA binding domain adds to the specificity of active meganucleases by increasing the level of cleavage activity at the 'on-target' site only. The re-engineered CCR5-megaTAL utilizes this unique property of this particular nuclease architecture. It uses a meganuclease configuration

that exhibited no *in vivo* activity as a standalone enzyme, and cleaved its target when tethered to the adjoining DNA by its TAL domain. The resulting nuclease has essentially no off target activity in vivo.

We also tested the re-engineered enzymes for specificity by probing a library of DNA substrates that had all possible single base pair substitutions represented in it. The enzymes with the lowest profiles for off-target activity were selected for further development. A full human genome scan revealed two sites with potential off target binding and cleavage: the highly homologous CCR2 sequence and a previously uncharacterized gene KIAA1257. We randomized three key residues in the HE in order to reduce off target activity against KIAA1257. This specificity refined HE was confirmed to have reduced cleavage by in vitro analysis.

Figure 1

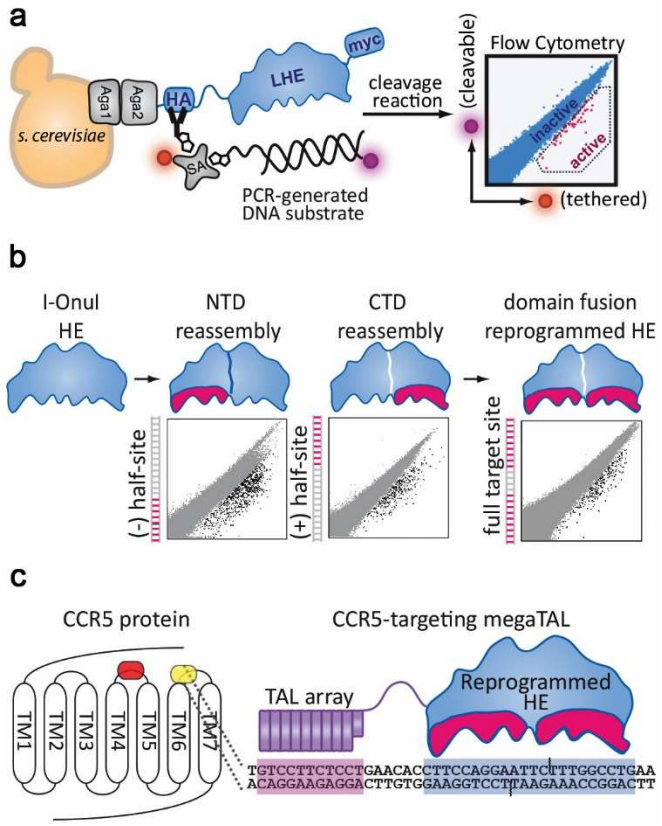

Figure 1. **Assembly of a reprogrammed megaTAL targeting the CCR5 gene.** (**a**) Schematic depiction of yeast surfaced display technology used to reprogram the specificity of LAGLIDADG-motif homing endonuclease (LHE) I-OnuI. (**b**) The N-terminal (NTD) and C-

terminal domains (CTD) of the LHE component of the megaTAL were reprogrammed separately to achieve activity against 9 base pair 'half-sites' that were subsequently assembled to produce the fully reprogrammed enzyme recognizing the complete CCR5 target site. **(c)** CCR5 is a G protein-coupled receptor (GPCR) with seven transmembrane (TM) domains whose extracellular facing loops comprise, in part, the binding interface used by HIV-1 to gain entry to CD4+ T cells. We targeted the third extracellular loop (ECL3) located between the sixth (TM6) and seventh (TM7) transmembrane domains (yellow shading), downstream of the *CCR5Δ32* deletion (orange shading). This region was selected for disruption, as this loop contains a cysteine residue that contributes to the structural integrity of CCR5 and its ability to function as an HIV-1 coreceptor.[46] The megaTAL targeting the CCR5 ECL3 loop is shown schematically, with a modular TALE array (purple) linked to the reprogrammed I-OnuI LHE (HE; blue) and their corresponding target sequences separated by 6 base pairs of non-contacted DNA. The amino acid sequence for the CCR5 megaTAL has been previously published.[19] Aga1 and Aga2, subunits of a-agglutinin heterodimer; HA, hemagglutinin; SA, streptavidin; Myc, c-myc tag.

MEGATAL DISRUPTION OF *CCR5* IN A REPORTER CELL LINE PREVENTS CCR5 EXPRESSION AND HIV ENTRY

We chose to deliver the megaTAL coding sequence by transient transfection of mRNA for several reasons. mRNA can be efficiently introduced into both primary cells and cell lines using electroporation, and achieves high levels of reporter mRNA expression for short periods of time with little loss of cell viability [19] Unlike many viral delivery methods, mRNA transfection avoids safety risks due to chromatin integration, as well as long-term expression of the nuclease, which could increase the risk of off-target cutting and cell toxicity. Other methods of transferring a coding sequence that avoid chromatin integration include integration-deficient lentivirus (IDLV) and adenovirus, which would be more expensive to produce for clinical scale applications[29] and may elicit immune responses in patients.[30, 31] Finally, mRNA production and cell delivery technology has already been established under large-scale good manufacturing practice (GMP) conditions.[32]

To determine whether CCR5 megaTAL disruption of *CCR5* confers HIV resistance, we simultaneously tested its abilities to disrupt cell surface expression of the protein and to provide

protection from live HIV infection using the GHOST(3) CCR5^{+} (GHOST Hi-5) cell line, a human osteosarcoma cell line stably transduced with human CD4 and CCR5 expression cassettes to allow HIV infection, as well as an HIV-2 LTR-GFP reporter (activated by TAT expression) to indicate HIV infection[23]. We used electroporation to introduce capped and poly-A tailed mRNA encoding the CCR5 megaTAL or blue fluorescent protein (BFP; negative control) in these cells. Untransfected control cells and cells receiving BFP mRNA retained equivalently high levels of surface CCR5 expression by flow cytometry one week following electroporation (98.8% and 98.3%, respectively). In contrast, cells electroporated with CCR5 megaTAL mRNA had a mean *CCR5* expression of 13.5% (**Figure 2a**), consistent with disrupted expression in 87% of engineered cells.

To verify that this CCR5 disruption was protective against HIV infection, we transfected GHOST Hi-5 cells with BFP, CCR5 megaTAL, or CCR5 megaTAL and Trex2 mRNA. Trex2 is a DNA end-processing enzyme that increases mutagenic NHEJ vs. precise end-joining following nuclease cleavage.24 Transfection of Ghost Hi-5 cells with mRNA resulted in approximately 90% efficiency in BFP marker gene expression with >80% viability. One week following mRNA transfection, we added an R5 tropic virus, HIV-1Ba-L, to the cell culture media; R5 viruses were previously shown to induce peak LTR-driven GFP expression in GHOST Hi-5 cells 48-hours post-primary infection.23 A high proportion of untreated and BFP mRNA-treated control cells retained surface expression of CCR5, totaling 89 and 92% respectively (Figure 2b-c, summing top right and top left quadrants), and 23-27% of these cells expressed the GFP reporter indicative of HIV infection at the 48-hour time point (Figure 2b-c, summing top right and bottom right quadrants). Alternatively, cells transfected with the CCR5 megaTAL alone or with Trex2 mRNA were only 22% and 2.5% CCR5+ respectively (top right and top left quadrants), with fewer HIV

infected (GFP+) cells (6.1% and 2.2%, respectively in top right and bottom right quadrants). Overall, the CCR5 megaTAL disrupted CCR5 surface expression in approximately 85% of GHOST Hi-5 cells, reducing HIV infection by approximately 80%; a process that was further enhanced by the addition of Trex2. HIV infection was restricted to the unmodified, CCR5hi populations. While the presence of synthetic DNA or RNA in the cytosol could activate anti-viral effects that might interfere with HIV infection, no statistically significant difference was seen in infection rates in control mRNA treated cells. This suggests that the reduction of GFP expression (and therefore HIV infection) seen in the megaTAL and megaTAL+Trex2 samples is not a result of mRNA transfection. These results are consistent with the hypothesis that the CCR5 megaTAL disrupts CCR5 in a manner that will mimic the CCR5Δ32 allele, providing protection from HIV infection by preventing CCR5 expression on the cell surface.

Figure 2

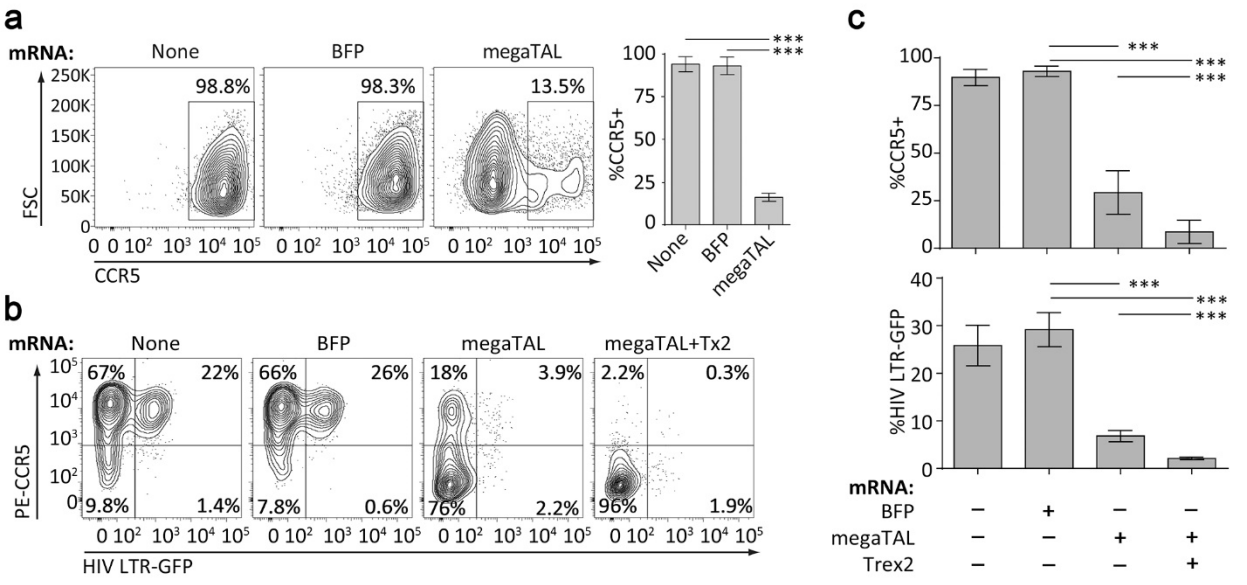

Figure 2. megaTAL efficiently disrupts *CCR5* in Ghost Hi-5 cells and protects against HIV *in vitro*. (a) Representative flow cytometry data (left) and statistical representations (bar graphs; right) of CCR5 surface expression in an untransfected sample or samples electroporated with either BFP or CCR5 megaTAL mRNA (n=3). **(b)** Representative flow cytometry data showing

surface CCR5 expression vs. intracellular GFP expression. PE-labeled anti-CCR5 antibody was used to assess CCR5 surface expression (vertical axis) and HIV infection was tracked by HIV-2 LTR-driven GFP expression (horizontal axis) in Ghost Hi-5 cells infected 1 week following mRNA transfection using the CCR5-tropic virus HIV BaL. HIV infection preferentially occurs in CCR5+ positive cell populations (TR). **(c)** Statistical representations (n=6) of Ghost Hi-5 cells shown in (**b**), showing CCR5 surface staining (upper panel) and GFP expression (lower panel). All data represent mean ± SD. *P* values calculated using Student's *t* test. Results of statistical analyses in this and subsequent figures as follows: * = $p < 0.05$; ** = $p < 0.01$; *** = $P < 0.001$; n.s. = not significant.

CCR5 MEGATAL EFFICIENTLY DISRUPTS *CCR5* IN PRIMARY HUMAN CD4$^+$ T CELLS

Having demonstrated that megaTAL-induced *CCR5* disruption can prevent *in vitro* HIV infection in the GhostHi-5 cell line, we wanted to demonstrate the efficacy of this approach in primary human T cells. We previously reported gene modification (8 to 60%) at *CCR5* in primary human T cells using this megaTAL alone or in association with adeno-associated virus (AAV)-delivered donor DNA templates.[19] Using the procedure shown (**Figure 3a**), we found that we could transfect primary human CD4$^+$ T cells with mRNA with high efficiency without compromising viability. Transfection of primary human T cells with CCR5 megaTAL mRNA resulted in ~80% fewer CCR5$^+$ cells by surface staining 4 days after transfection in comparison with either untransfected or BFP mRNA transfected cells (**Figure 3b**). Note that CCR5 expression is highly variable among T cell developmental subsets and in resting versus stimulated cells.[25] Therefore, genomic DNA was collected from transfected cells for determination of cutting rates (measured as indels) using two alterative molecular assays. First, we used the T7 endonuclease I (T7E1) assay, and identified 63% of alleles with indels in megaTAL mRNA treated cells compared with a background level of 5-10% in untreated and BFP mRNA treated cells. Of note, the uniform spectrum of indels generated by

megaTAL cleavage (consisting primarily of 1 to 4 base pair deletions)[19] is predicted to lower the frequency of DNA-mismatch bubble formation, the substrate for T7EI. Thus this assay is likely to underrepresent the indel readout. Deletions at this site are be predicted to abolish HE cleavage due to the critical importance of the central four (C4) nucleotide positions.[26] Hence, we also utilized a re-cleavage assay (RCA) to assess NHEJ frequency. As opposed to measuring heteroduplex formation (as in the T7E1 assay), the RCA assay quantifies in vitro nuclease digestion of a genomic PCR amplicon that contains the megaTAL target site, using a recombinant LHE protein identical to that used to create the CCR5 megaTAL. Because LHE's have binding-dependent activity, a mutation at the *CCR5* site arising from an NHEJ event *in cellulo* would abolish LHE activity *in vitro*. By RCA, we observed background NHEJ rates of <2% in untreated and BFP mRNA transfected cells and 60-80% NHEJ at *CCR5* in CCR5 megaTAL mRNA treated cells (**Figure 3c-d**). Thus, based upon both assays, the CCR5 megaTAL lead to 60-80% *CCR5* gene disruption in primary human T cells.

Figure 3

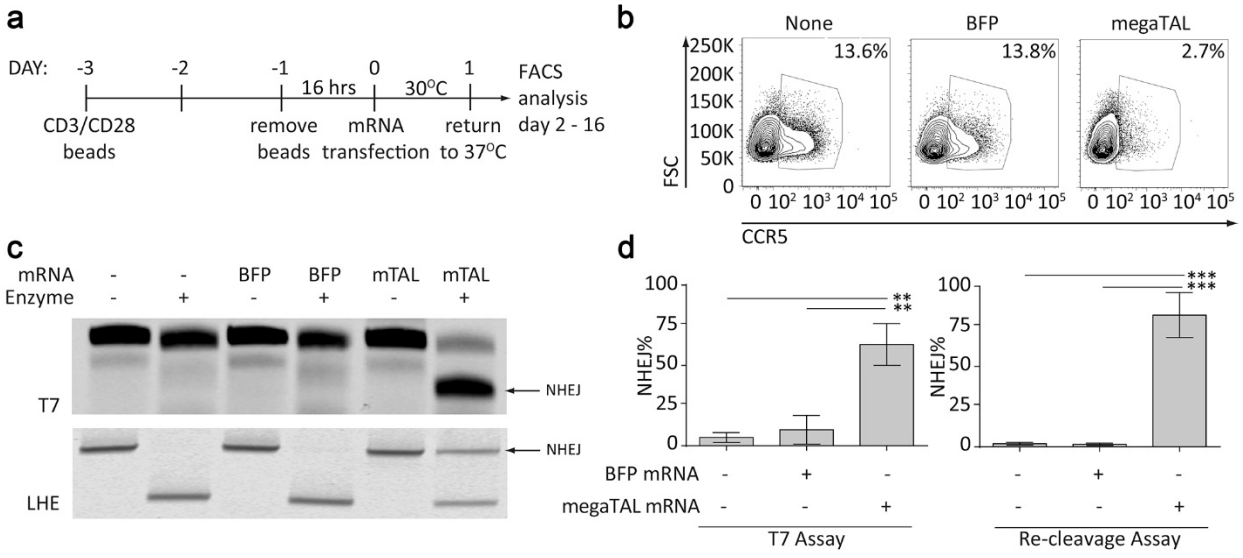

Figure 3. megaTAL efficiently disrupts *CCR5* in primary CD4$^+$ cells. (a) Timeline representing workflow in primary CD4$^+$ cells relative to time of transfection (t=0), beginning with the addition of anti-CD3/CD28 beads on cryo-preserved CD4+ T cells. **(b)** Surface staining of CCR5 in primary CD4$^+$ cells comparing expression in untreated cells or cells transfected with BFP or CCR5 megaTAL mRNA. **(c)** Representative agarose gels quantifying *CCR5* modification by T7 endonuclease assay (surveyor assay; top panel) or by re-cleavage assay (RCA) using a fluorescently labeled forward primer (lower panel). **(d)** Molecular quantification (n=3) by T7 (left) or RCA (right) of *CCR5* disruption in primary CD4+ cells. Values are calculated from fluorescent densitometry (%NHEJ = NHEJ band/sum of NHEJ+undisrupted bands).

STABLE ENGRAFTMENT OF GENE MODIFIED PRIMARY T CELLS *IN VIVO*.

We next tested the engraftment and subsequent stability of megaTAL-treated cells in immune-deficient, NOD-SCID-IL-2Rγ^{null} (NSG) mice (**Figure 4a**). Mice were transplanted with primary human CD4$^+$ T cells that were treated with either CCR5 megaTAL or control BFP mRNA. CCR5 megaTAL treated input cells were modified at 71% of CCR5 alleles by RCA, with an assay background of 5% in BFP mRNA treated cells (**Figure 4b**). Six weeks post-transplant, we found that some of the mice did not meet our criteria of engraftment (>1% of splenic cells hCD45$^+$CD4$^+$) and results from these mice were excluded from additional analyses (including in 6 animals in the megaTAL-treated and 3 in BFP control cohorts, respectively). We identified hCD45$^+$CD4$^+$ cells in peripheral blood samples throughout the experiment in both treatment groups, and found no significant differences in the frequency of circulating hCD45$^+$CD4$^+$ (**Figure 4c**). Similarly, we found equivalent levels of splenic engraftment as measured by hCD45$^+$CD4$^+$ expression in BFP and CCR5 megaTAL mRNA treated cells (11.3% and 11.4% respectively; **Figure 4d**). As predicted, engrafted hCD45$^+$CD4$^+$ showed significantly lower levels of CCR5 surface expression in megaTAL treated cells relative to control BFP mRNA treated cells (21.2% and 80.4% respectively, **Figure 4e**). To determine if reduced CCR5 expression was a result of megaTAL-mediated gene

modification at *CCR5*, we interrogated splenic gDNA using the RCA assay. Control animals exhibited a mean *CCR5* disruption rate of 10.2%, while animals transplanted with megaTAL mRNA treated cells exhibited a mean disruption rate of 56.3% (**Figure 4f**), confirming the stability of cells with megaTAL-mediated *CCR5* disruption *in vivo*.

In uninfected NSG mice, CCR5 megaTAL-edited T cells had equivalent engraftment and stability relative to control cells. While a slightly higher number of mice transplanted with CCR5 megaTAL treated cells failed to engraft (6 mice in the CCR5 megaTAL treated vs. 3 in the control cohort), the overall level of engraftment in both groups was equivalent. In previously published data, our group has shown that CCR5 megaTAL-edited T cells engraft equivalently to unmodified T cells.[19] In the absence of HIV, the fraction of engrafted human T cells that were $CCR5^{hi}$ in peripheral blood was significantly decreased in the CCR5 megaTAL cohort relative to mice receiving control cells, evidence that the gene editing strategy produces a viable cell product with the expected phenotype.

Figure 4

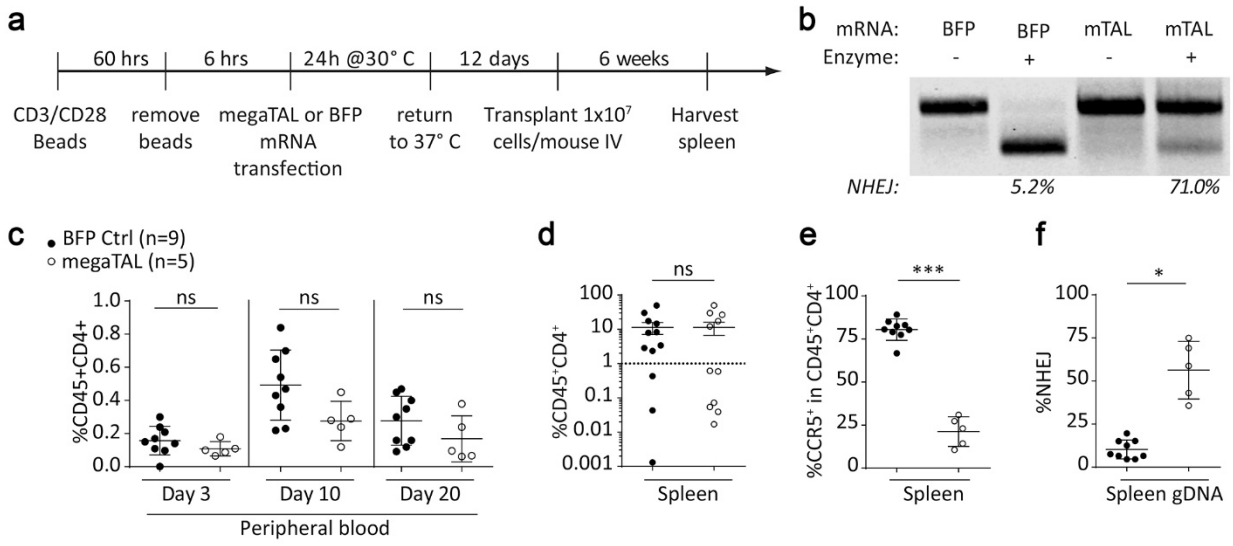

Figure 4. Primary $CD4^+$ cells modified by CCR5 megaTAL engraft in the immunodeficient NSG murine model and show stable modification of CCR5. (a) Timeline representing

workflow for NSG mouse experiment. **(b)** Re-cleavage assay (RCA) of input cells shows 71% modification at *CCR5* in cells treated with CCR5 megaTAL mRNA and 5.2% background modification in cells transfected with BFP. **(c)** Mice were bled on days 3, 10, and 20 post transplant. Surface staining of hCD45 and hCD4 was used to track engraftment in peripheral blood over time. **(d)** Six weeks post-transplant, mice were sacrificed and spleens were harvested. Splenic engraftment of human T cells was tracked through surface staining of hCD45 and hCD4. **(e-f)** Samples with >1% splenic T engraftment were subsequently analyzed for: **(e)** surface expression of CCR5 by flow cytometry and **(g)** molecular quantification of *CCR5* disruption by RCA analysis of genomic DNA (gDNA).

GENE MODIFICATION PROTECTS CD4$^+$ T CELLS FROM *IN VIVO* HIV INFECTION.

Having established the stability of *CCR5* modified cells *in vivo* in the absence of HIV, we wanted to challenge these engineered T cell populations using the same *in vivo* system in the context of an HIV infection. As above, we modified primary human CD4$^+$ T cells by transfection with either BFP control mRNA or CCR5 megaTAL mRNA. By surface staining, BFP and CCR5 megaTAL mRNA treated CD4$^+$ cells were 34% and 2.7% CCR5$^+$, corresponding to a relative disruption level >90% in megaTAL treated T cells (**Figure 5a**). We transplanted each mouse with 7x10^6 gene edited or control primary CD4$^+$ T cells, 1x10^6 infected CD4-depleted PBMC's, and 1x10^6 freshly thawed PBMC's (see workflow, **Figure 5b**) from the same donor to prevent toxicity resulting from mixed lymphocyte reactions. Throughout the transplant, human CD4$^+$ T cells and HIV levels in peripheral blood were monitored over time as shown (**Figure 5b**).

The frequency of hCD45$^+$CD3$^+$CD4$^+$ cells in circulating blood was significantly higher in mice receiving megaTAL treated cells than those receiving control cells by days 21 and 35 post-transplant/infection. This difference was also observed in splenic cells, where human CD4$^+$ cells represented 31.8% and 3.4% of total cells in megaTAL or control recipient animals, respectively. By comparison, no statistical difference in hCD45$^+$CD3$^+$CD8$^+$ cells was observed at any time in

blood or spleens in either group (17.1% and 31.4%; megaTAL and BFP mRNA respectively; **Figure 5c**). This suggests that in the presence of HIV-BaL, megaTAL treated cells have a selective advantage relative to BFP mRNA control cells in the presence of HIV-$1_{Ba\text{-}L}$. Within $hCD45^+$ T cells, surface expression of CCR5 was indistinguishable between megaTAL and BFP treated cells in either $CD4^+$ or $CD8^+$ cells (**Figure 5d**). In this experiment, all $CD4^+$ T cells are expected to have low levels of CCR5 expression due to either HIV infection of CCR5 positive cells or due to megaTAL disruption of the locus. Consistent with this idea, we noted that the low levels of CCR5 observed in $CD4^+$ cells in HIV infection experiments were not observed in the absence of HIV (**Figure 4e**). By comparison, $CD8^+$ cells maintained a CCR5 expression level similar to those seen by untreated cells in the absence of HIV. On day 35, we observed a 100-fold increase in the proportion of CD4 cells present in recipients of megaTAL-treated compared control treated T cells in the presence of HIV. Together, these data show that HIV-$1_{Ba\text{-}L}$ exhibits cytotoxic properties on wild-type $CCR5^+CD4^+$ cells in this model; this negative selection is prevented by megaTAL disruption of CCR5.

In addition to analyzing cell populations in circulating blood, we collected serum at each time point for analysis by qPCR. On day 7, when there were equivalent $CD4^+$ proportions recipients of both megaTAL and BFP control mRNA treated cells, recipients of megaTAL treated cells exhibited lower plasma viremia (**Figure 5e**). Interestingly, at day 35, the group receiving the megaTAL treated cells had a ten-fold greater proportion of circulating human $CD4^+$ T cells (**Figure 5c**), yet with statistically equivalent plasma viremia. Quantification of splenic gDNA by qPCR revealed fewer HIV integrants in mice receiving megaTAL treated cells vs. control cells (**Figure 5f**).

In the presence of HIV, mice that received megaTAL-treated cells had 100-fold more circulating human $CD4^+$ T cells and a 10-fold increase in splenic human $CD4^+$ cells at the 35-day endpoint than mice in the control cohort. Our approach successfully selected for $CD4^+CCR5^-$ cells and reduced the amount of integrated HIV DNA in edited splenic cells, reproducing HIV challenge data from other groups that were using a zinc finger nuclease targeted to ECL1 to disrupt CCR5 in human T cells cells or in $CD34^+$ HSCs (Cannon and June, 2011; Kiem et al., 2012; Li et al., 2013).[13-18, 33] However, while the CCR5 megaTAL cohort repressed blood viremia early on in the *in vivo* experiment, viremia equalized between experimental groups by the end of the study. We speculate this is likely due to the co-transfer of monocytes in PBMCs with the monocyte-tropic R5 virus as well as the delayed kinetics of HIV infection in megaTAL-treated cohort, resulting in the expansion and subsequent infection of unsuccessfully edited cells, and potentially leading to an increase in total viremia.

Figure 5

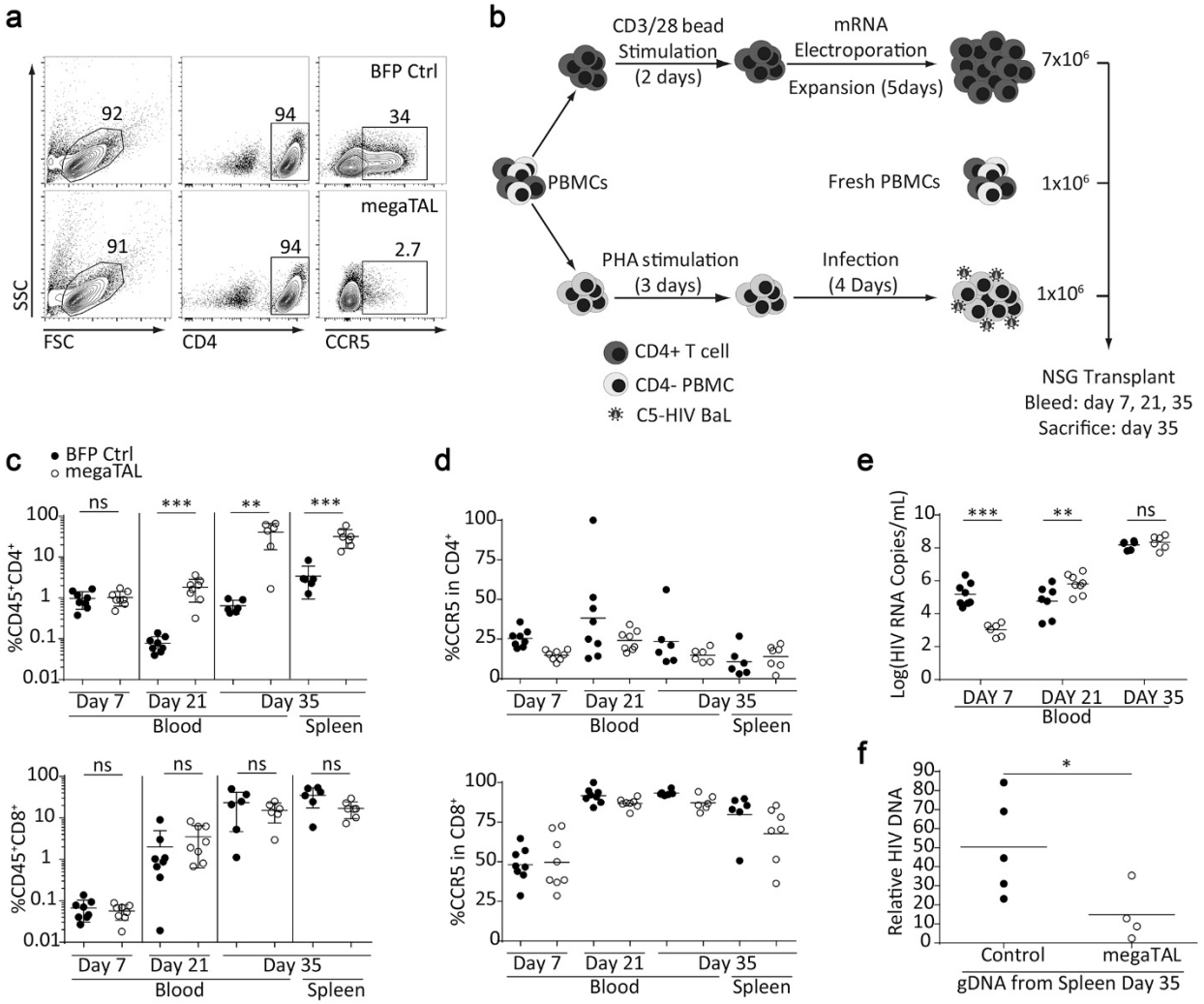

Figure 5. megaTAL editing of *CCR5* in primary CD4+ T cells protects against HIV *in vivo* in an NSG murine model. (a) Flow cytometry data of primary CD4 cells electroporated with either BFP or CCR5 megaTAL mRNA (Day 5 post electroporation). Data shows surface staining for hCD4 and CCR5 prior to transplant. **(b-f)** NSG mice were transplanted with $7x10^6$ modified or control CD4+ cells, $1x10^6$ HIV-BaL infected CD4-depleted PBMC's, and $1X10^6$ freshly thawed PBMC's from the same donor. **(b)** Experimental workflow detailing manipulation of transplanted cells represented**.** Mice were bled on days 7, 21, and 35 and subsequently sacrificed on day 35 for spleen harvesting. **(c)** Engraftment of $CD4^+CD45^+$ (top) and $CD8^+CD45^+$ (bottom) T cells tracked in blood and spleens. **(d)** Surface expression of CCR5 in $CD4^+$ cells (top) and

$CD8^+$ cells (bottom) tracked in blood and spleens. **(e)** HIV plasma viremia was tracked with qPCR throughout transplant. **(f)** HIV qPCR in spleen gDNA showing relative viral load in both experimental groups.

FUTURE DIRECTIONS

The disruption of *CCR5* in the context of HIV raises concerns of HIV tropism. In an attempt to mimic the "Berlin Patient" cure strategy, the "Essen Patient"[34] received a single bone-marrow transplant and was removed from ART, resulting in the emergence of CXCR4 (X4)-tropic viral variants. Recent studies using lentiviral vector delivered zinc finger nucleases have demonstrated the feasibility of stably disrupting both *CXCR4* and *CCR5* in CD4 T cells to prevent dual-tropic HIV infection.[13] A similar dual-receptor targeting approach could be implemented with the simple addition of a second mRNA, encoding a CXCR4 engineered megaTAL, to the electroporation reaction.

Although we were able to demonstrate that megaTAL-modified $CD4^+$ T cells preferentially expand in the context of HIV infection *in vivo*, the progression of HIV infection in humans is not replicated in these mice. Notably, the CCR5-megaTAL binding site is conserved and could be used to disrupt *CCR5* in pigtail macaques, a nonhuman primate species where the SHIV (a simian immunodeficiency virus envelope with HIV genetic core) has been used to model human HIV infection. Hence, future studies in our group will investigate CCR5 disruption and subsequent protection from SHIV in a macaque model.

The megaTAL nuclease platform has other potential applications for HIV therapies beyond CCR5 disruption in T cells (and mobilized $CD34^+$ cells)[19]. We have previously introduced several clinically relevant gene products into T cells at the *CCR5* locus, including expression cassettes for the C46 peptide[35], which acts as a fusion inhibitor for HIV, and an anti-

HIV envelope CAR. It as been shown that partial suppression of CCR5 and expression of the C46 peptide can have synergistic benefits to protect cells from R5-tropic HIV[36-38] or X4 and dual-tropic HIV infections.[39] Thus, surface expression of C46 at the *CCR5* locus as a result of homology-driven integration is likely to result in multi-tropic HIV protection.

Finally, a major challenge in HIV cure efforts is the development of a therapy that eliminates the reservoir of HIV-1 that persists in latently infected cells. Our group has successfully delivered several Chimeric Antigen Receptors (CARs), including an anti-HIV envelope CAR[19] that selectively activates modified T cells when co-cultured with HIV infected cells *in* vitro.[19] Concerns have been raised that the addition of an HIV-recognizing epitope on $CD8^+$ T cells will mediate HIV infection of effector cells[40-43]. By delivering the HIV-CAR into the CCR5 locus, effector cells might be protected from subsequent infection, increasing the persistence and potency of cellular therapies in the context of HIV. The ability to deliver these gene cassettes could contribute to a combinatorial gene therapy approach that could contribute to virus eradication.

MATERIALS AND METHODS

megaTAL nuclease design

The CCR5 megaTAL enzyme was designed by bluebirdbio using a proprietary method relying on directed evolution and its amino acid sequence has been previously described[19]. The enzyme recognizes a 38 base-pair stretch of sequence located in the sixth transmembrane domain of the CCR5 gene.

Primary $CD4^+$ T cell isolation and culture

Blood was drawn from consented adult healthy donors at Seattle Children's Research Institute in accordance to IBC protocols. Primary CD4 cells were isolated from either whole blood using RosetteSep Human CD4+ T Cell Enrichment Kit (StemCell Technologies), or from PBMCs using the Human CD4+ T Cell Isolation Kit (Miltenyi Biotec). Cells were grown in RPMI (Hyclone) supplemented with 20% fetal bovine serum, and human cytokines IL-2 (50 ng/mL), IL-7 (5 ng/mL) and IL-15 (5 ng/mL; all from PeproTech) and cultured at 0.5-3 × 10^6 cells/ml. Cells were activated using Dynabeads Human T-Activator CD3/CD28 (LifeTechnologies) for 48 hours at a 1:1 cell to bead ratio.

mRNA production and electroporation:

CCR5 megaTAL and BFP mRNA coding sequences were manufactured as previously described by our group and electroporated in a similar fashion.[19]

Isolation of genomic DNA and PCR amplification

Genomic DNA (gDNA) was extracted from 0.5 – 1.5 × 10^6 cells using a QiaCube (Qiagen) and following manufacturer's recommended settings and provided reagents. gDNA was eluted and stored in Qiagen Buffer AE. To amplify the CCR5 locus for NHEJ assessment, 2 µL of a 10 ng/µL gDNA dilution was mixed with 48 µL of Platinum HiFi master mix (1× Platinum HiFi buffer, 10 mM dNTPs, 50 mM $MgSO_4$, 10 mM each forward (TCATTACACCTGCAGCTCTC) and A.647 conjugated reverse (CAGTGGATCGGGTGTAAACTG) primers, 0.4 µL Platinum HiFi Taq (2.5 U/µL, LifeTechnologies). PCR was performed with denaturation at 94°C for 2 minutes followed by 35 cycles of 94°C for 20 s, 60°C for 20 s, 68°C for 1 minute, and a final extension at 68°C for 10 minutes. PCR products were purified using QiaQuick PCR Purification Kit (Qiagen).

Endonuclease Assays

Indel frequency at the megaTAL cleavage site was measured using a T7E1 assay as described,[19] and using a re-cleavage assay (RCA). For the T7E1 assay, 100 ng of PCR product were denatured and re-annealed in 1× New England Biolabs (NEB) Buffer 2 at a total volume of 10 µL. Samples were then incubated at 37°C for 1 hour in T7 endonuclease I (10,000 U/ml, New England Biolabs). The reactions were terminated by adding 500 mM EDTA. For the RCA, 100 µl of PCR product was incubated at 37°C for 16 hours in CCR5 HE and Cleavage buffer (400 mM Tris Acetate, 200 mM potassium acetate, 2 mM dithiothreitol (DTT), 20 mM $MgCl_2$). Reactions were terminated by addition of buffer with 50 mM Tris pH 8.0, 5 mM EDTA, 0.5% SDS, 0.5 mg/mL Proteinase K, 25% glycerol. Reaction products from both assays were run on an 0.8% agarose gel, imaged using Odyssey (LI-COR Biosciences), and bands containing the A.647 labeled reverse primer were quantified using the ImageStudioLite (LI-CORE Biosciences).

Flow cytometry

Cells were analyzed on an LSR II or Canto 2 (BD Biosciences) and analyzed using FlowJo X (Treestar). Cells were stained at 4°C for ten minutes in FACS buffer (phosphate buffered saline, 2% FBS). The following antibodies from BD Bioscience were used: CCR5 clone 3A9 , CD4 clone OKT4, CD3 clone OKT3. Dead cells were excluded from analysis using Near-IR Fixable Live/Dead stain (LifeTechnologies).

Virus production

Replication competent HIV-$1_{Ba\text{-}L}$ was from Suzanne Gartner, Mikulas Popovic and Robert Gallo[44, 45] obtained through the NIH AIDS Reagent Program, Division of AIDS, NIAID, NIH. HIV-$1_{Ba\text{-}L}$ was propagated in PHA-activated human PBMCs, the supernatant was collected and sterile filtered (.22 µm Steriflip, EMD Millipore) on days 3 and 5 post-inoculation. The virus was titered in GHOST Hi-5 cells against a virus standard.

HIV-1 assay in GHOST Hi-5 cells

GHOST Hi-5 cells expressing multiple copies of CCR5 were from Dr. Vineet N. KewalRamani and Dr. Dan R. Littman[23] and obtained through the NIH AIDS Reagent Program, Division of AIDS, NIAID, NIH. They were maintained in complete RPMI with 20% fetal bovine serum, and supplemented with 500 μg/ml G418, 100 μg/ml hygromycin, and 1 μg/ml puromycin. Cells were infected with HIV-$1_{Ba\text{-}L}$ at an MOI of 15 and harvested using Accutase buffer (LifeTechnologies) at 48 hours. HIV infected samples were fixed in 1% paraformaldehyde in neutral-buffered saline prior to flow cytometry.

T cell transfer and HIV infection mouse models

NOD.Cg-*Prkdc*^{scid} *Il2rg*^{tm1Wjl}/SzJ (NSG) mice obtained from Jackson laboratories were housed in specific pathogen free facilities at either the Seattle Children's Research Institute (SCRI) or the Fred Hutch Cancer Research Center (FHCRC; for experiments using the HIV infection model). Both facilities are accredited by the Association for Assessment and Accreditation of Laboratory Animal Care in accordance with National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals. All procedures using mice were approved by the Institutional Animal Care and Use Committee of either SCRI or FHCRC. To study engineered T cells in the absence of live HIV, 1×10^7 megaTAL-treated or control T cells in PBS were injected intravenously into unconditioned mice. Peripheral blood was obtained 3, 10, and 20 days after T cell injection to monitor engraftment, and mice were euthanized 6 weeks post-transplant to harvest spleens and assess engraftment.

For the in vivo HIV challenge, we infected PHA-activated PBMCs infected with HIV-$1_{Ba\text{-}L}$ (MOI ~0.4) for 72 hours. 1×10^6 autologous PBMCs were mixed with 7×10^6 CD4 T cells (either megaTAL or control BFP mRNA treated) and 1×10^6 infected PBMCs; this cell mixture was

suspended in 100 μL of PBS and injected intraperitoneally into unirradiated mice (n = 8 each group, megaTAL or control). Peripheral blood samples were collected on days 7, 21, and 35 post-cell transfer for flow cytometric analyses of cells, and for collection of serum for viral RNA quantitation. Mice were euthanized at 35 days upon developing symptoms of graft-versus-host disease, and spleens were collected. After erythrocyte lysis, spleen cells were passed through a 70 micron strainer then cells stained with antibodies for cell surface markers for flow analysis.

Plasma viremia

Total RNA was extracted from plasma samples using QiAmp viral RNA kit (Qiagen). Quantification of viral RNA was performed using TaqMan One-Step RT-PCR Master Mix Reagents Kit (LifeTechnologies 4309169). The following oligonucleotides were designed to amplify our target: Forward Primer 5'-GCC TCA ATA AAG CTT GCC TTG A-3', Reverse Primer 5'-GGC GCC ACT GCT AGA GAT TTT-3', Probe5'-6FAM-AAG TAG TGT GTG CCC GTC TGT TRT KTG ACT-TAMRA-3.' Samples values were calculated relative to a standard curve created by making serial dilutions of a stock virus titered using Acrometrix HIV-1 panel (Thermo Fisher Scientific). Each sample was run in duplicate on two separate plates (mean of four reactions plotted). Each plate had a no-template negative control and a PBS control to ensure the absence of contamination. The uninfected controls were run on a separate plate from the infected reactions to minimize aerosols or contamination. With the exception of day 35, all samples were run on the same day using the same standard curve to minimize run-to-run variability.

Statistical analysis

All statistical analyses were performed using GraphPad Prism software (GraphPad). A minimum of three data points was used for each analysis. *P* values were calculated using Student's *t* test.

SUMMARY

We developed a megaTAL nuclease targeting the third extracellular loop of *CCR5* that we delivered to primary human T cells by mRNA-transfection. Data from our gene disruption experiments is summarized in this chapter. The CCR5 megaTAL nuclease established resistance to HIV in cell lines and disrupted the expression of CCR5 on primary human $CD4^+$ T cells with a high efficiency, achieving up to 80% modification of the locus in primary cells as measured by molecular analysis. Gene-modified cells engrafted at levels equivalent to unmodified cells when transplanted into immune-deficient mice. Furthermore, genetically modified $CD4^+$ cells were preferentially expanded during HIV-1 infection *in vivo* in an immunodeficient mouse model. Our results demonstrate the feasibility of targeting *CCR5* in primary T cells using an engineered megaTAL nuclease, and the potential to use gene-modified cells to reconstitute a patient's immune system and provide protection from HIV infection.

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Guillermo S. Romano Ibarra[1*], Biswajit Paul[2*], Blythe D. Sather[1], Patrick M. Younan[2], Karen Sommer[1], John P. Kowalski[2], Malika Hale[1], Barry Stoddard[2], Jordan Jarjour[3], Alexander Astrakhan[3,^], Hans-Peter Kiem[2,4,^], and David J. Rawlings[1,5,6,^]

[1]Center for Immunity and Immunotherapies and Program for Cell and Gene Therapy, Seattle Children's Research Institute, Seattle, WA 98101, USA; [2]Clinical Research Division, Fred Hutchinson Cancer Research Center, Seattle, Washington, USA; [3]bluebird bio, Seattle, WA 98102, USA; Departments of [4]Medicine, [5]Pediatrics, and [6]Immunology University of Washington, Seattle, WA 98101, USA.

***Equal contribution**

Chapter 4. COMBINING TARGETED INTEGRATION OF A RESISTANCE-CASSETTE AT THE CCR5 LOCUS WITH CHEMOSELECTION

INTRODUCTION

In the previous chapter I demonstrated how gene disruption at the CCR5 locus has major implications in HIV cure. Gene disruption utilizes the cellular process of NHEJ during which iterative break and repair occurs until the target site can no longer be recognized by the nuclease. While NHEJ-repair causes insertions or deletions, HDR-repair can be modified to add specified sequences to the genome. In the current chapter I will demonstrate how the ability to utilize HDR pathways allows for even more sophisticated applications of gene editing through gene insertion.

Rationale

T cells are a great model system for proof-of-concept studies in primary human cells. They are easy to obtain, manipulate, expand, transduce and transfect. Recent protocols to accurately and efficiently re-engineer T cells using HDR have been successfully used for various disease applications (Sather, Wang 2015). Homology driven repair at a pre-defined site in the genome has tremendous potential. We used the CCR5 locus as a safe harbor site – a locus where the addition of a transgene does not result in unintended cellular transformation or dysfunction (Papapetrou 2016). Gene insertion is similar to lentiviral gene therapy, but with the added advantage of being able to exquisitely control the location, copy number and expression of the introduced transgene. Gene insertion has already been used to introduce an anti-HIV chimeric antigen receptor (CAR) at the CCR5 locus (Sather 2015). In future, gene insertion could easily be co-opted for use in anti-cancer treatments using CAR T cells to target certain leukemias (Maude 2014). In comparison traditional lentiviral gene therapy can result in multiple copies of the gene being inserted into the genome since we have limited control over the number of viruses that infect a cell. With targeted insertion we can be sure that there can only be a maximum of two

transgenic integrants per cell. Semi-random vector integration observed in lentiviral gene therapy, can also result in gene silencing, or trans-activation of neighboring genes and insertional mutagenesis (Cavazza 2013, HBA 2008).

In previous clinical trials, serum concentrations of MTX of between 100nM -1000nM have been achieved in patients who were on a low-dose (10-500 mg/m^2) regimen of the drug (Treon 1996). We aimed to develop a selection protocol, that would produce enrichment of gene-modified T cells at low concentrations of MTX over a short time in culture. In previous work, Jonnalagadda et al, have demonstrated the survival advantage and enrichment of mDHFR gene modified T cells upon drug selection *in vivo* in NSG mice (Jonnalagadda 2013). The mice both tolerated the engraftment of lentivirally-modified cells, and demonstrated robust enrichment after administration of MTX. Additionally they investigated the phenotype of the modified cells in detail and found them not to be significantly different from normal unmodified cells on the basis of surface markers CD4, CD8, CD28, CD62L, TCRab, CD127 and CD45.

The mDHFR-MTX system can be specifically used to improve the current T cell manufacturing protocol, which makes use of a truncated version of the epidermal growth factor receptor (EGFRt) to specifically enrich for modified cells (Jonnalagadda 2013). This method requires surface expression of the receptor, which can be variable, and an additional separation step. The use of MTX in culture medium, however, would obviate the need for secondary selection of gene-modified cells using magnetic beads.

Methotrexate as a lymphotoxic agent

Methotrexate (MTX) is an antimetabolite used to treat some neoplasias, severe psoriasis and adult rheumatoid arthritis. MTX inhibits dihydrofolate reductase (DHFR). Dihydrofolates must be converted to tetrahydrofolates by DHFR before they can perform their necessary cellular

function of transporting one-carbon groups during the synthesis of purine nucleotides and thymidylate. By allosterically inhibiting DHFR, MTX interferes with DNA synthesis, repair and cellular replication. Since cellular proliferation in malignant tissues is usually much greater than in normal tissues, MTX helps impair growth in malignant tissues with no irreversible harm to normal tissues (Walling 2006). In normal individuals, actively dividing tissues such as bone marrow, fetal cells, buccal and instestinal mucosa, and cells of the urinary bladder exhibit MTX-sensitivity. MTX is used alone or in combination with other anti-cancer agents in the treatment of a variety of neoplasia (Mennes 2005, Strojan 2005, Daw 2006, Slamon 2006).

Drug-resistant DHFR and Methylguanine methyltransferase (MGMT) mutants (reviewed here Sorrentino 2002) are two methodologies that have been previously evaluated for the enrichment of hematopoietic cells. However, the key differences in the mechanism of action between the two are important to keep in mind in a risk-benefit analysis. While the DHFR system utilizes the well-characterized antifolate therapy, the MGMT system uses an alkylating agent which makes acute and long term lesions in the cellular DNA. The long-term effects of alkylating agents in humans are poorly characterized and the effects the genotoxicity and chromosomal integrity of cells are not well understood (Sorg 2007). In contrast MTX has been widely used and the reversible effects of the drug are well characterized. Unlike in the MGMT-system, adverse reactions to MTX treatment can be easily reversed by a leucovorin-rescue regimen (Pinedo 1976, Frei 1980).

Mutant DHFR establishes MTX resistance

mDHFRs are modified human proteins – also referred to as muteins. Hence they are predicted to exhibit negligible immunogenicity when compared to other drug resistance transgenes when used in patients.

Previously, researchers have demonstrated that cells transduced with the L22Y variant can be enriched in vivo following treatment with antifolates (Zhao 1997, Allay 1998, Warlick 2002). The L22Y variant was the first example of a drug-resistance gene that enabled selection of gene-modified hematopoietic cells. However, this selection could not be replicated in a non-human primate model (Persons 2004). Only transient selection of HSCs with the selection cassette was achieved in rhesus macaques after antifolate-administration. This led the researchers to hypothesize a species-specific difference in HSC growth and cycling between murine and primate systems.

Subsequently, Volpato et al, found that a L22F/F31S double mutant they developed, performed better than the previously used L22Y mutant. This mutant maintained catalytic activity while exhibiting marked decrease in MTX-binding affinity (Volpato 2010). They showed that transduced myeloid progenitor cells were able to survive and preferentially expand in drug concentrations of up to 500nM. Additionally, murine bone marrow cells transduced with their F31R/Q35E mutants could be enriched within a 4 day culture and withstand up to 1uM MTX. In another study, Gori et al used a combination of MTX and Tyr22-DHFR mutants to increase the engraftment of CD45 and CD34 cells in the bone marrow and peripheral blood of NSG mice (Gori 2010).

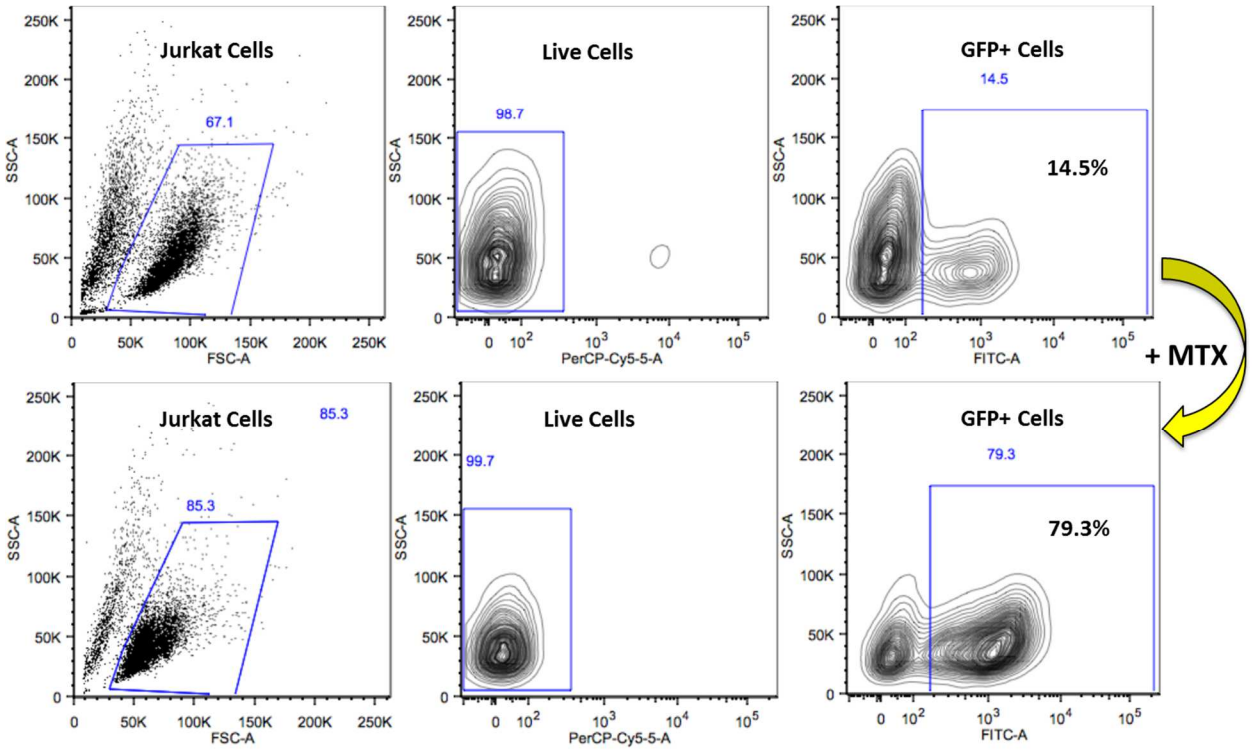
EXPRESSION OF MUTANT-DHFR IN JURKATS CONFERS RESISTANCE TO MTX

We first set out to test the levels of MTX-resistance using DHFR mutant constructs in a T cell line. In order to determine optimal growth and transduction conditions we cultured unmodified Jurkat cells. We tracked cell counts daily and viability using a Trypan-blue stain. We transduced

A.


EF1a
Y22-DHFR
IRES
eGFP

B.


Jurkat Cells
67.1
SSC-A
FSC-A
Live Cells
98.7
PerCP-Cy5-5-A
GFP+ Cells
14.5
14.5%
FITC-A
+ MTX
Jurkat Cells
85.3
85.3
Live Cells
99.7
GFP+ Cells
79.3
79.3%

C.

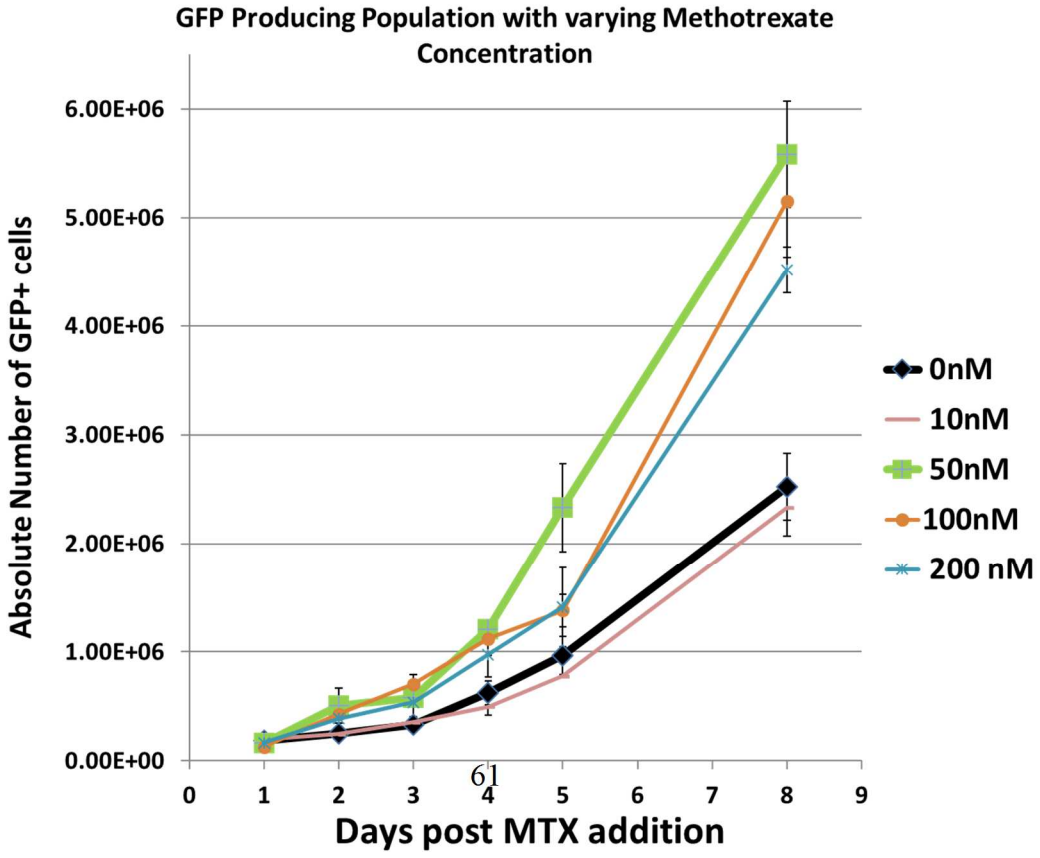

GFP Producing Population with varying Methotrexate Concentration
Absolute Number of GFP+ cells
6.00E+06
5.00E+06
4.00E+06
3.00E+06
2.00E+06
1.00E+06
0.00E+00
0
1
2
3
4
5
6
7
8
9
Days post MTX addition
0nM
10nM
50nM
100nM
200 nM

Figure 1: Enhanced survival of gene-modified Jurkat cells in methotrexate (MTX). Jurkat cells were gene-modified with a mutantDHFR lentivirus and incubated in varying concentrations of MTX three days post- transduction during peak transgene expression. Transgene selection was tracked over the course of a week by the coexpression of GFP. (A) Schematic of lentiviral construct containing a GFP reporter in addition to the L22Y-DHFR mutant driven by an EF1alpha promoter. (B) Representative flow plots showing gating strategy and five-fold selection from day 1 (top row) to day 8 (bottom row). (C) Absolute cell numbers of gene-modified cells (cell count multiplied by %GFP) at 10, 50, 100 and 200nM of MTX. The 50nM condition emerged as optimal for expansion of Jurkat cells. Data shown is the mean of three experiments.

the Jurkats with a biscistronic expression cassette expressing the Tyr22-DHFR mutant along with a GFP reporter **(Figure 1A)**. GFP expression was evaluated by flow cytometric analysis of cells. In order to characterize the expression of our transgene we transduced cells at various multiplicity of infection and evaluated %fluorescence at 48 hours post (data not shown). Cells transduced at multiplicity of infection 0.1 showed between 10-15% GFP positive cells. We used this virus and MOI for subsequent experiments.

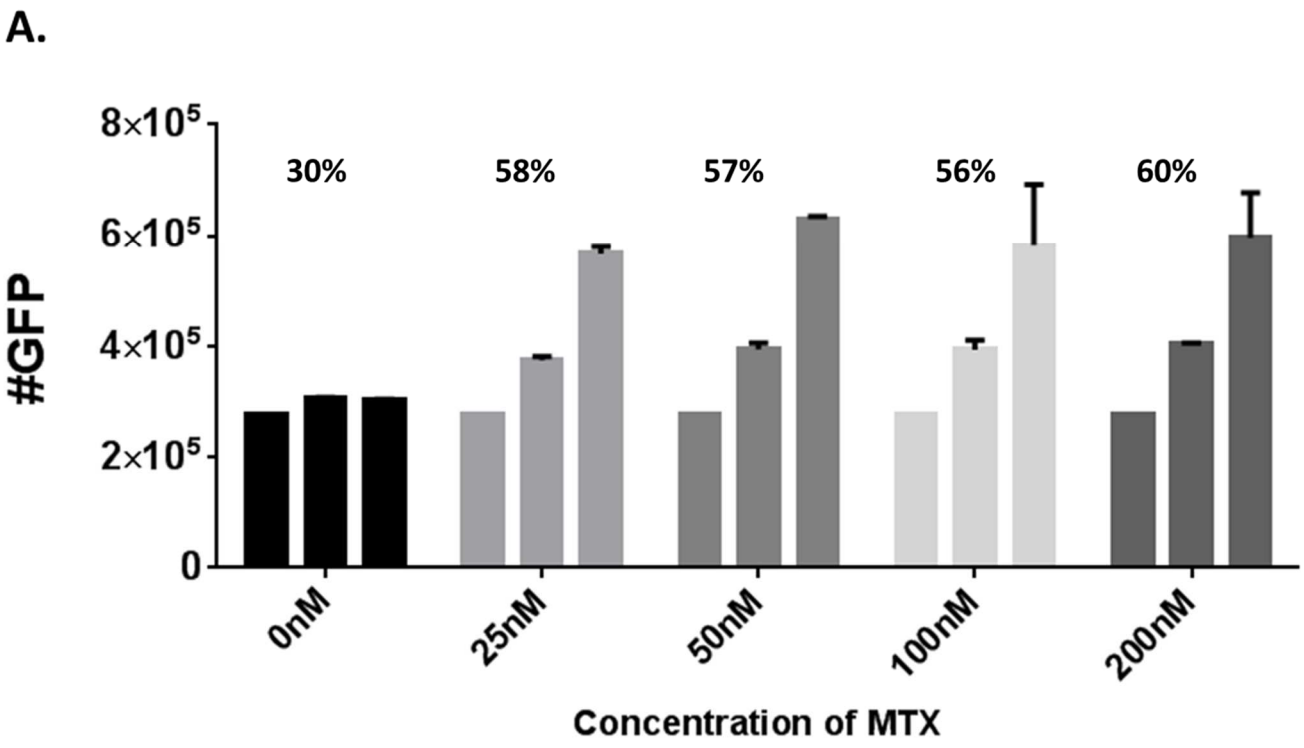
After having established that our transgene is stably expressed in our cells over 4 weeks in culture, we next used GFP to track the levels of transduced cells, and demonstrate post-drug enrichment. We demonstrated that Tyr22-DHFR greatly enhances the ability of Jurkat cells to survive at cytotoxic MTX concentrations **(Figure 1B)**. We tracked rapid and persistent selection of cells modified with the Tyr22-DHFR co-expressing a GFP reporter. Populations that started with 15% of cells expressing GFP could be enriched to >90% cells expressing GFP within 5 days in MTX. Hence, we observed a six-fold enrichment of mDHFR-modified Jurkat cells in the presence of drug **(Figure 1C)**. Additionally, we found that these cells did not exhibit any growth impairment and expanded to very large numbers.

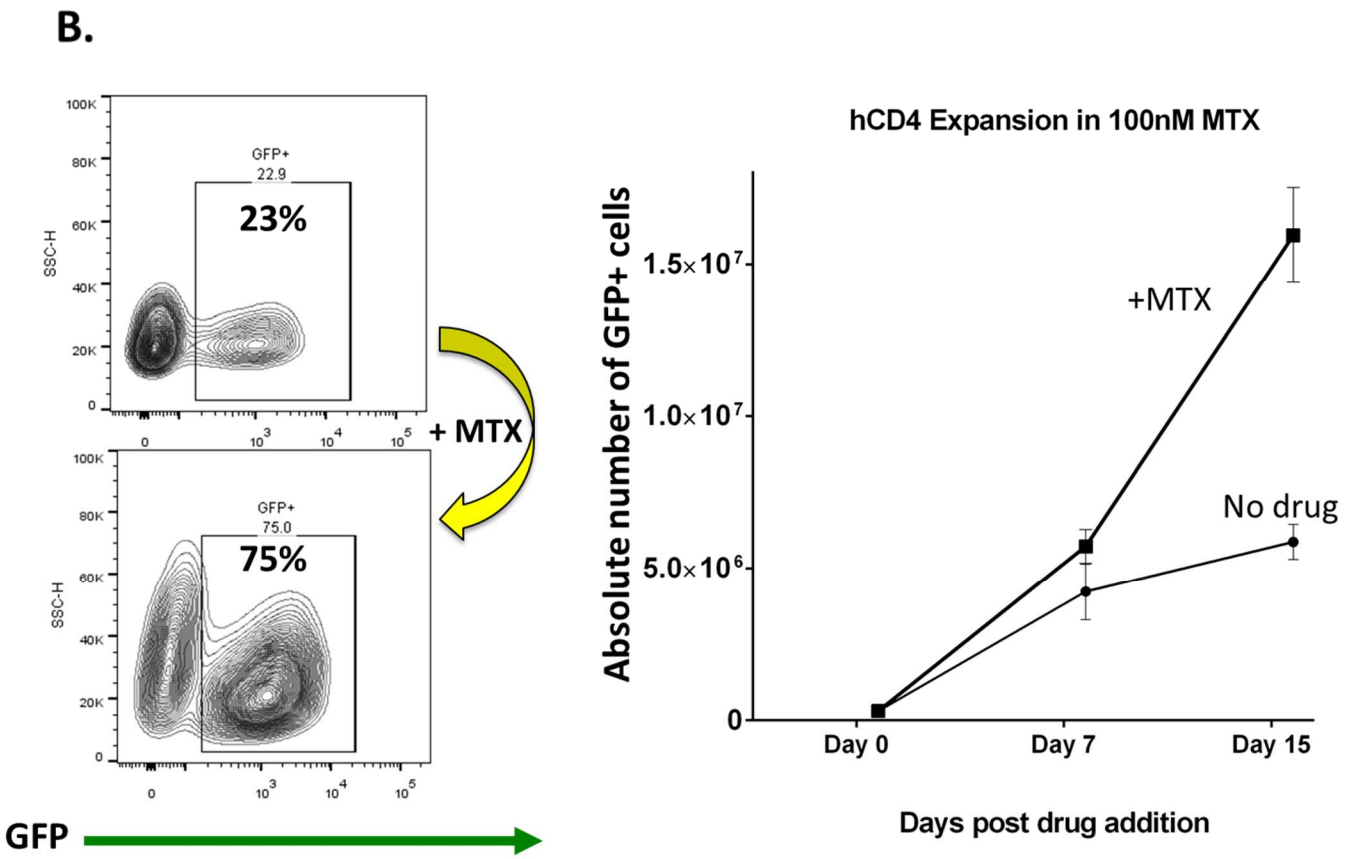
EXPRESSION OF MUTANT-DHFR IN PRIMARY CD4 T CELLS CONFERS RESISTANCE TO LYMPHOTOXIC CONCENTRATIONS OF MTX.

Next, we tested the effects of MTX in the selection of mDHFR modified CD4 T cells. We found that T cells with mDHFR constructs were resistant to MTX concentrations of 25nM to 200nM **(Figure 2A)**. We chose to use the 100nM concentration in subsequent experiments for two reasons: mDHFR conferred resistance at this concentration with minimal cell toxicity, and this level of drug is easily achievable in the blood of patients following very low-dose treatment (Treon 1996).

We performed selective outgrowth assays in the presence of MTX, and preferential survival was determined by assaying the expression of fluorescent proteins in our genetically modified CD4 T cells. Cells were collected on days 7, 14 and 21 post transduction. An average range of between 15-25 %GFP integration was observed in these cells at an MOI of 1. The transduced T cells enriched from <25% to >70% over a short-term two-week culture period **(Figure 2B)**. The selective enrichment of transgene observed in our CD4 T cells, was not seen in unmodified control-T cells which were also cultured in 100nM MTX (data not shown).

Figure 2: Enhanced survival and expansion of gene-modified CD4 T cells in MTX.
Activated human CD4 cells were transduced with mutantDHFR lentivirus and incubated in varying concentrations of MTX two days post- transduction during peak transgene expression. (A) The data shows absolute number of gene-modified (GFP expressing) cells following chemoselection collected at days 1, 5 and 7 post MTX-addition. The % of GFP positive cells is indicated above each condition. 100nM of drug was used for future experiments. (B) Representative flow plots showing gating strategy and three-fold selection in no drug (top row) versus MTX conditions (bottom row). Absolute number of gene-modified cells in the presence and absence of drug measured over the course of two weeks plotted on the right. 100nM of drug was added on day4 post cell re-activation and resulted in 3-fold expansion in the presence of drug. Data shown is the mean of five experiments, across four healthy donors. All experiments contained two biological replicates.


A.
#GFP
8×10^5
6×10^5
4×10^5
2×10^5
0
30%
58%
57%
56%
60%
0nM
25nM
50nM
100nM
200nM
Concentration of MTX


B.
GFP+
22.9
23%
SSC-H
+ MTX
GFP+
75.0
75%
GFP
hCD4 Expansion in 100nM MTX
Absolute number of GFP+ cells
1.5×10^7
1.0×10^7
5.0×10^6
0
+MTX
No drug
Day 0
Day 7
Day 15
Days post drug addition

TARGETED GENE INSERTION OF MUTANT-DHFR CONSTRUCTS IN HUMAN CD4 T CELLS

We paired RNA-based nuclease expression with the delivery of a CCR5-donor template containing a chemoselection cassette to achieve HR-mediated gene insertion in primary T cells. DSBs, engineered at pre-defined sites in the genome using nucleases, can be repaired in the presence of a homologous donor delivered by AAV. Hence, we cloned donor constructs such that they included several hundred base pairs of DNA sequence that were homologous to the region adjacent to the nuclease cleavage site. To accommodate the ~600bp mDHFR construct into the vector, we used 800bp homology arms on either side of the CCR5-megaTAL cleavage site.

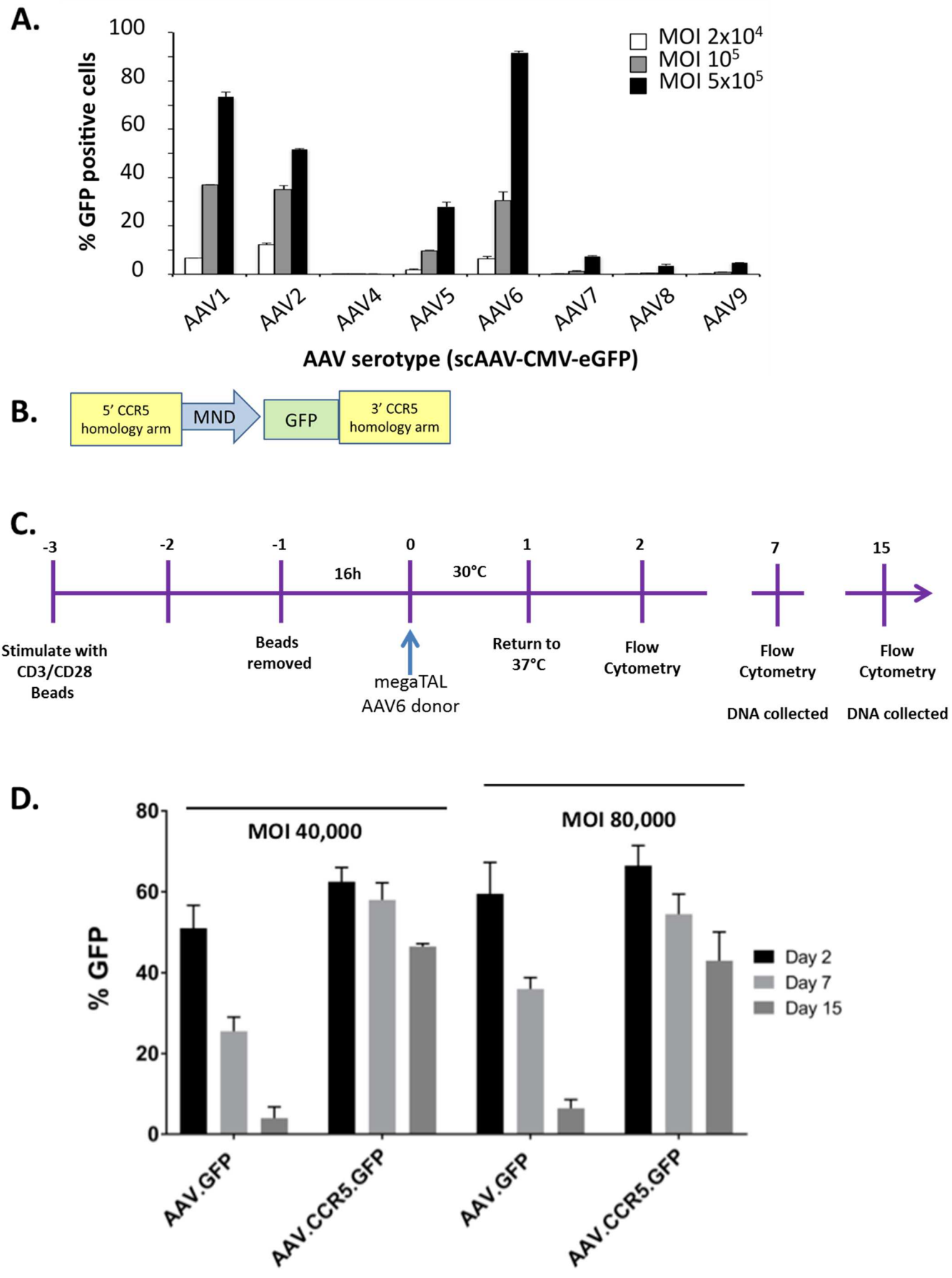
Nuclease delivery was performed by transient transfection of *in vitro* transcribed mRNA. We also transfected cells with control BFP mRNA to measure the efficiency of our procedure. At 48 hours we observed robust expression of fluorophore (>95%) with minimal cell death. Delivery of the repair template using double stranded DNA is problematic since it induces a Type I interferon response and results in a marked loss in cell viability. However, delivery of the homologous repair template using AAV is tolerated well because, like other viruses, AAV has evolved mechanisms to evade the innate intracellular immune response (Sather 2015, Wang 2015).

In order to test the AAV serotype with the best transduction efficiency we infected activated T cells with eight different serotypes: AAV1,2,4,5,6,7,8, and 9. These self-complementary AAVs express a GFP reporter driven by a CMV promoter. GFP expression was measured at 48 hours post transduction. AAV6 gave the highest rates, successfully transducing >90% of cells at a multiplicity of infection of 50,000 with >95% cell viability **(Figure 3A)**.

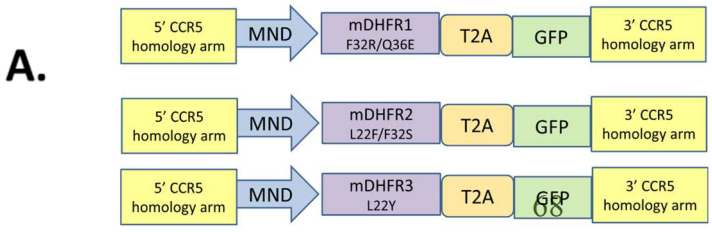
The preliminary experiment consisted of two treatment groups: one set of cells were transduced with AAV (the no HDR group); while the other set was transduced with AAV as well as transfected with megaTAL nuclease (the gene insertion group). Using activated T cells, we first introduced a targeted DSB using the CCR5-megaTAL, then delivered donor DNA by AAV-transduction after ~4 hours **(Figure 3C).**

In cells which were only transduced with the resulting "mDHFR.GFP" AAV high levels of GFP (>30%) expression were measured at 48h post, but this decreased to <1% within two weeks post. In contrast, cells that were concurrently transfected with the megaTAL nuclease showed consistent high levels of GFP which were maintained through the duration of the experiment **(Figure 3D)**. This observation is consistent with stable insertion of our transgene in the genome. It is particularly interesting that we found no evidence of non-specific integration of the AAV at the DSB sites (i.e. in the absence of homology arms).

Figure 3: Targeted gene insertion at the CCR5 locus. Primary human CD4 cells were permanently gene modified with GFP at the target CCR5 site at high levels (~50%). (A) Determination of transduction efficiency of different AAV serotypes in CD4 T cells at different concentrations of virus. AAV6 serotype transduced activated T cells at maximum efficiency with minimal loss in cell viability (Courtesy Daniel Stone and Keith Jerome). (B) Schematic of the viral construct used in validating targeted insertion experiments. (C) Schematic of the experimental overview of targeted insertion in CD4 T cells using a combination of megaTAL nuclease and AAV6 donor over a period of 18 days. (D) Sustained expression of GFP over 2 weeks demonstrates the stable insertion of transgene into the genome. In samples treated with AAV serotype 6 without homology arms (abbreviated AAV.GFP) GFP expression decreased rapidly in cells at both multiplicity of infection of 40,000 and 80,000 whereas expression remained stable in the AAV serotype 6 with homology arms (abbreviated AAV.CCR5.GFP) condition.


A.
% GFP positive cells
100
80
60
40
20
0
MOI 2x10^4
MOI 10^5
MOI 5x10^5
AAV1
AAV2
AAV4
AAV5
AAV6
AAV7
AAV8
AAV9
AAV serotype (scAAV-CMV-eGFP)
B.
5' CCR5 homology arm
MND
GFP
3' CCR5 homology arm
C.
-3
-2
-1
16h
0
30°C
1
2
7
15
Stimulate with CD3/CD28 Beads
Beads removed
megaTAL AAV6 donor
Return to 37°C
Flow Cytometry
Flow Cytometry
DNA collected
Flow Cytometry
DNA collected
D.
% GFP
80
60
40
20
0
MOI 40,000
MOI 80,000
Day 2
Day 7
Day 15
AAV.GFP
AAV.CCR5.GFP
AAV.GFP
AAV.CCR5.GFP

The mDHFR targeted insertion experiment knocked-in three different DHFR muteins that had been previously validated for MTX-resistance by the laboratories of Pelletier and Jensen. The transgenes are expressed from a single MND promoter with the two genes separated by a ribosomal skip T2A sequence, which enables the translation of two proteins from one transcribed message **(Figure 4A)** (Szymczak 2004). Additionally, the targeted insertion experiment included two control constructs that had been previously validated: a positive control "CCR5.GFP", and a negative control "WAS.GFP". The AAV6 CCR5.GFP donor contained a GFP reporter driven by an MND promoter, and flanked by 1.3 kb homology arms. The WAS.GFP was essentially the same construct except for it contained homology arms to the Wiskott-Aldrich syndrome protein gene locus. The CCR5.GFP positive control consistently resulted in >50% of the cells being modified long-term **(Figure 4B)**. This is in part due to the longer homology arms, and the smaller size of inserted sequence compared to our mDHFR.GFP constructs. As predicted, the WAS.GFP construct did not result in any detectable long-term modification of the genome since the nuclease cleavage was occurring at the CCR5 locus, and not at the WAS gene (data not shown). We consistently achieved sustained gene insertion of the mDHFR cassettes at efficiencies greater than 10% **(Figure 4B)**. Experiments with replicates for the DHFR3 construct are still in progress. We confirmed the presence of HR-mediated insertion at CCR5 using PCR. We extracted genomic DNA from gene-modified cells and controls at day 14-16 in all our experiments. We used primers that spanned our insert to amplify our target region and detected bands that matched the size of both the modified and unmodified loci **(Figure 4C)**.



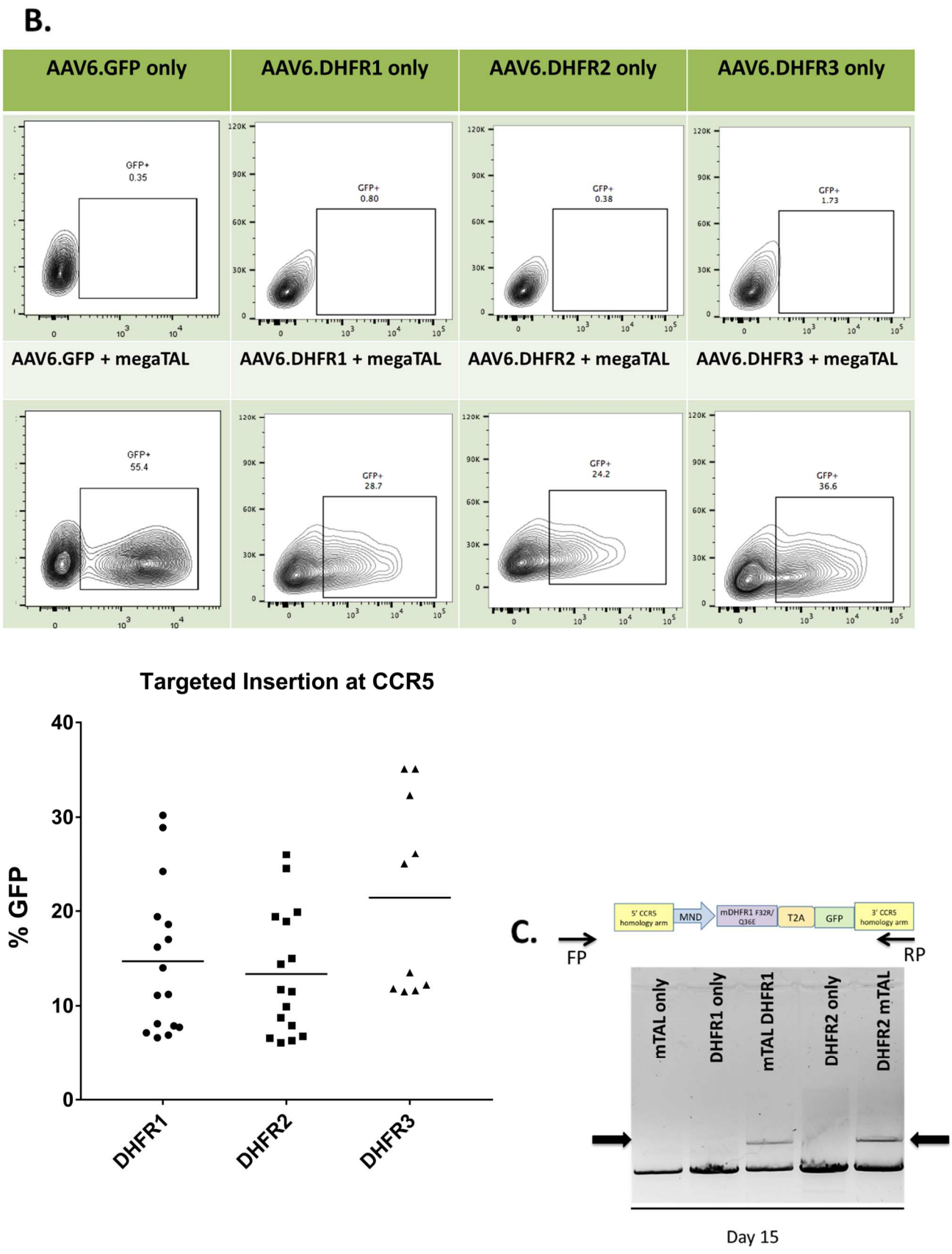


Figure 4: Targeted gene insertion of a chemoselectable mDHFR cassette at the CCR5 locus.

Primary human CD4 cells were permanently gene modified with three DHFR mutants at the target CCR5 site at high levels. The three DHFR mutants tested are F32R/Q36E DHFR (abbreviated as DHFR1), L22F/F32S DHFR (abbreviated DHFR2), and single-mutant Tyr-22 DHFR (abbreviated DHFR3). (A) Viral constructs used in targeted insertion experiments using different previously validated DHFR mutants. An MND promoter, engineered to overcome transcriptional silencing, was used to drive the expression of a GFP reporter, with an added mutant DHFR variant. (B) Representative flow plots showing successful Gene insertion using megaTAL nuclease on day 15 post- transfection and transduction. Data shown here is the mean of 10 experiments 9 donors: 6 male, 3 female. (C) PCR amplification of the gene modified CCR5 locus to demonstrate that megaTAL + AAV6 donor shows targeted integration in human CD4 cells. Arrows highlight the longer amplicon resulting from the insertion. Forward (FP) and reverse primer (RP) positions spanning the CCR5-insert are shown in schematic.

In a previous study, Sather and Romano et al, have used single-cell molecular analysis to show that 82% of their HDR events are biallelic using the same methodology as we have used in this work (Sather 2015). They also investigated the off target activity of the CCR5-megaTAL and found no evidence of gene insertion at the predicted off-target site, CCR2, by PCR analysis. Gene-modified cells harboring the GFP insertion at the CCR5 locus engrafted at comparable levels to normal untreated cells when transplanted into immunodeficient NSG mice. Modified cells also persisted in the mice when assayed for more than a month, and no signs of graft-versus-host disease were observed in the mice in the treatment group.

MUTANT-DHFR EXPRESSION FROM CCR5 LOCUS CAN BE USED FOR CHEMOSELECTING GENE MODIFIED CD4 T CELLS

To determine the degree of enrichment of gene modified T cells we could achieve by targeted insertion of mDHFR cassettes, we cultured T cells in 100nM MTX over two weeks and tracked cell selection and viability. As previously stated, we chose to use 100nM since this is a clinically relevant concentration of MTX that can be achieved in patients on low dose MTX-therapy. We found a four fold increase of gene modified cells from baseline levels **(Figure 5)**. Gene-modified CD4 T cells were enriched from starting levels of 10-25% to 40-75% at the end of the

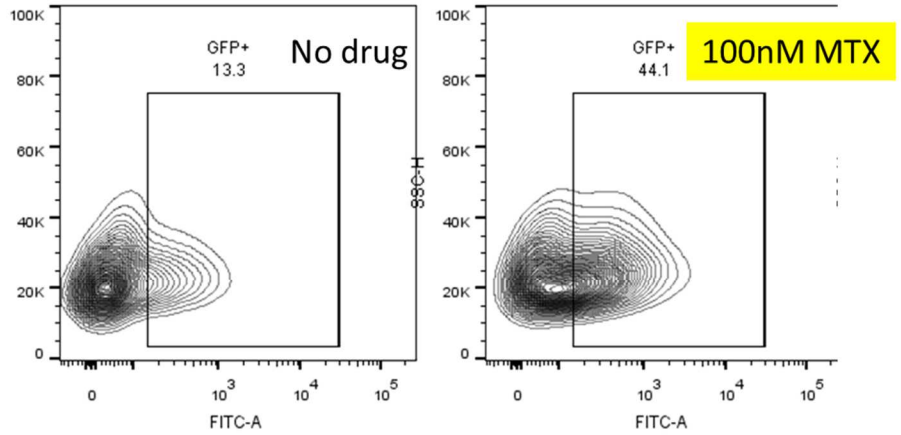
experiment, across five separate healthy donors. After the targeted insertion protocol, cells were re-stimulated using the rapid expansion protocol (REP) in the presence of MTX drug.

The rapid expansion protocol, first described by the Rosenberg group, uses anti-CD3 monoclonal antibody (OKT3), high dose interleukin-2 (IL-2) and irradiated allogenic feeder cells to expand T cells (Dudley 2003).

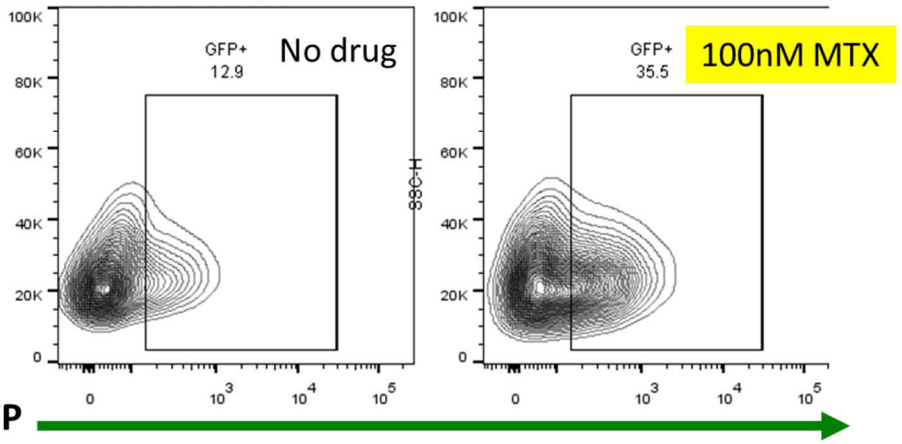
MTX selection resulted in progressive enrichment of chemoresistant transgene expressing T cells over a short time in culture. Currently cell manufacturing technologies use physical methods of enriching or sorting for target cell populations. Enrichment is achieved by flow cytometric cell sorting or immunomagnetic bead enrichment based on expression of cell surface markers. Both these methods require extra steps, which require expensive infrastructure and cGMP-compliant reagents. On the other hand, drug induced selection can be rapid, easy, and cheap. Here, we have demonstrated that MTX is a drug selection platform well suited for in vitro selection, and holds promise for in vivo selection. Importantly, a direct comparison between *in vitro* MTX-based drug selection and EGFRt-based bead selection found equivalent levels of enrichment for gene modified T cells between the two methods (Jonnalagadda 2013)

Figure 5: Preferential selection of gene-modified human CD4 T cells after targeted insertion of mutant DHFR. MTX conferred a selective advantage to cells that expressed mDHFR from permanently inserted cassettes at the CCR5 locus. DHFR1 and DHFR2 mutants showed roughly four-fold expansion in the presence of MTX compared to no-drug controls. Representative flow plots from experiments that contained two biological replicates, with each experiment repeated independently 10 times. There was no significant difference in fold enrichment between the DHFR mutant constructs tested at this low concentration of MTX.

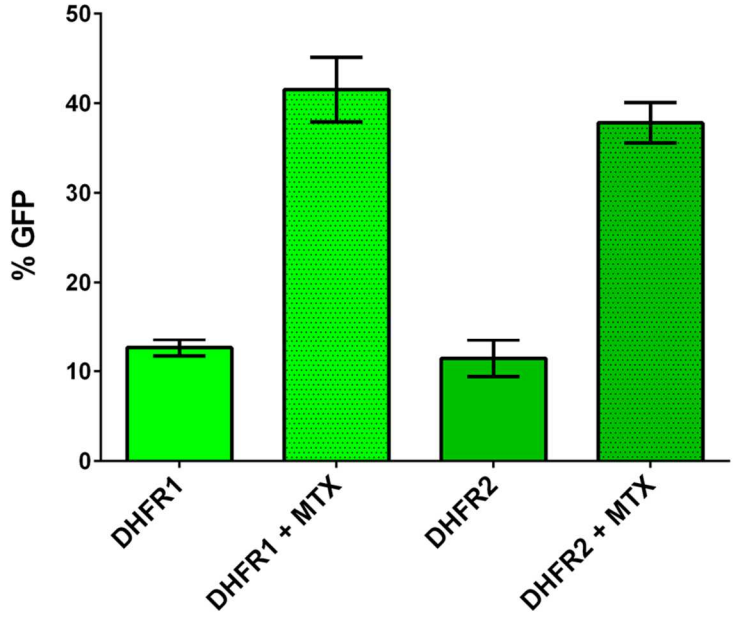
CCR5.MND.DHFR1.T2A.GFP



CCR5.MND.DHFR2.T2A.GFP



GFP



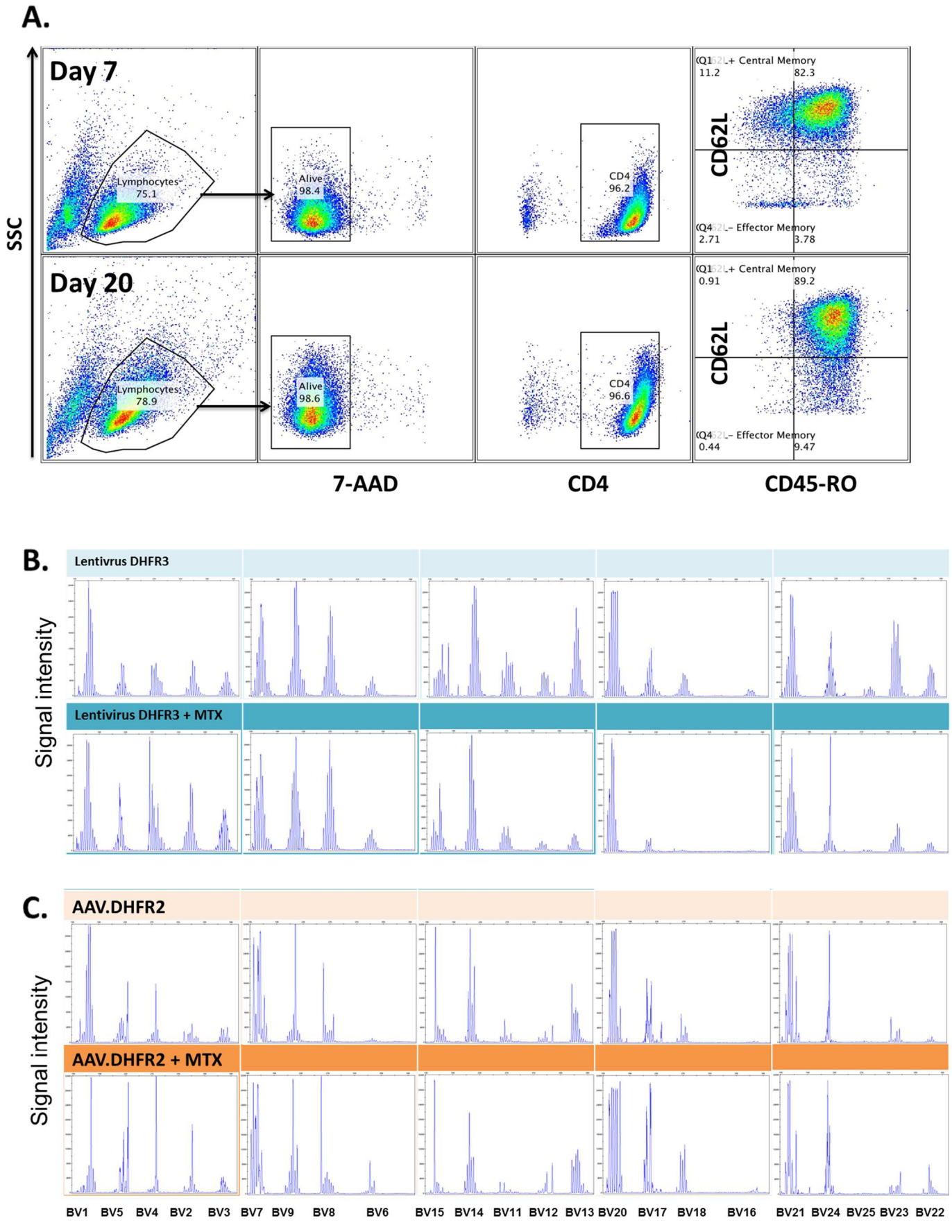
Mean absolute cell # 24x10e6 20.3x10e6 30x10e6 16.5x10e6

GENE EDITED T CELLS MAINTAIN SIMILAR PHENOTYPE AND TCR DIVERSITY

We optimized cell culture conditions to promote the growth of cells with a T central memory phenotype which have been shown to exhibit stem cell like properties (Graef 2014). T central memory cells persist in the body and give rise to progeny that survive for several years in non human primate models (Berger 2008). Cells were activated by CD3/28 bead stimulation and media supplemented with IL-2, IL-7 and IL-15 growth factors to skew cell growth towards this phenotype (Kaneko 2009). We did not observe any significant change in the growth phenotype of the cells or in the expression of cellular surface markers during the entirety of cell culture. Previous groups have used the expression of CD45RO and CD62L surface markers to describe a memory T cell phenotype. We observed that gene-modified cells retained high surface expression of both these markers for >20 days in culture **(Figure 6A)**.

CD62L+ CD45RO+ cells have been shown to engraft, persist, and re-populate T cell niches following adoptive transfer (Wang 2011). T cells, manufactured using a CD3/28 bead-stimulation protocol like we used, have been shown to maintain memory phenotype and persist in the body long-term (Kalos 2011). Recent results from long term follow up of T cell gene therapy trials indicate that the efficacy of treatment and clinical response is predicted by the proliferative phenotype of the cells infused (Biasco 2015). By tracking T cell clones over 12 years across two gene therapy trials researchers identified a population of T stem cells that survive, function, and proliferate long-term.

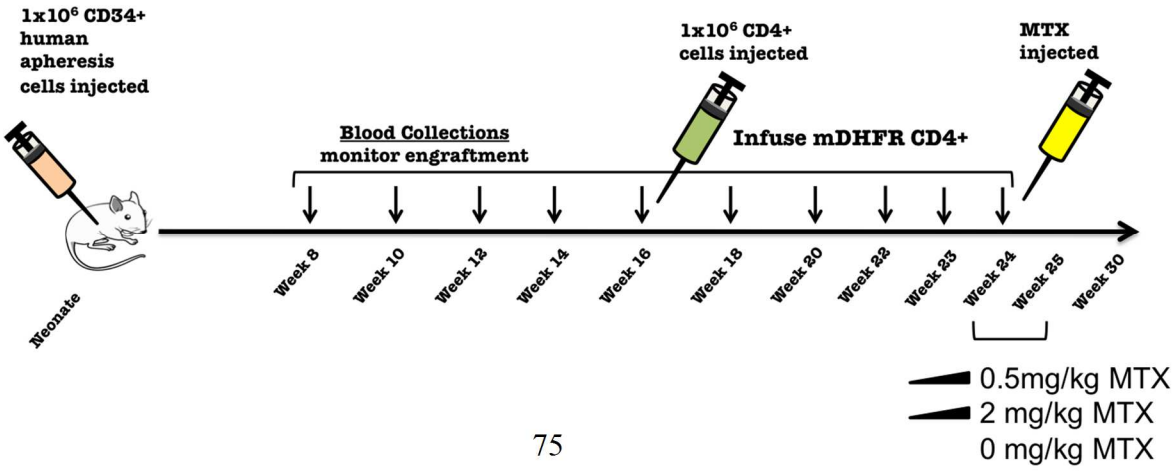
Figure 6: Gene modified and chemoselected cells maintained their phenotype and TCR diversity. (A) Phenotypic analysis of CD4 T cells by surface marker expression. Cells were analyzed on day 7 and day 20 in culture and demonstrated a predominantly memory T cell phenotype of CD45RO+ CCR7+ CD62L+. Naïve T cells do not express the CD45RO splice variant and constitute the CD45RO- fraction observed here. (B) TCR spectratype analysis of transduced cells shows no effect of MTX-selection on clonality in the case of both lentivirally modified cells as well as those modified by nuclease and AAV (C).


A.
Day 7
Lymphocytes
75.1
Alive
98.4
CD4
96.2
CD62L+ Central Memory
11.2
82.3
CD62L
CD62L- Effector Memory
2.71
3.78
Day 20
Lymphocytes
78.9
Alive
98.6
CD4
96.6
CD62L+ Central Memory
0.91
89.2
CD62L
CD62L- Effector Memory
0.44
9.47
SSC
7-AAD
CD4
CD45-RO
B.
Lentivrus DHFR3
Lentivirus DHFR3 + MTX
Signal intensity
C.
AAV.DHFR2
AAV.DHFR2 + MTX
Signal intensity
BV1 BV5 BV4 BV2 BV3 BV7 BV9 BV8 BV6 BV15 BV14 BV11 BV12 BV13 BV20 BV17 BV18 BV16 BV21 BV24 BV25 BV23 BV22

We also analyzed the T cell receptor repertoire using a PCR-based spectratyping assay (Akatsuka 1999). Gene edited T cells retained a broad TCR repertoire throughout gene-modification and subsequent chemoselection **(Figure 6B)**. This suggests that multiple cells were modified independently, and that there was no insertional mutagenesis resulting in any subsequent clonal dominance. Cells modified with both lentivirus and AAV showed either a polyclonal Gaussian distribution or polyclonal skewed distribution of TCR-clones at 16 days post-modification. Importantly, this distribution was maintained through a subsequent 2 week drug selection in MTX, demonstrating that drug selection has no detrimental impact on the expansion of multiple T cell clones.

IMPLICATIONS OF THIS WORK & FUTURE DIRECTIONS

In this study we have demonstrated the utility of using MTX to achieve five to six-fold enrichment after chemoselection in drug-concentrations as low as 0.1uM. Our study is the first report of the targeted gene-insertion of MTX-resistance at the CCR5 locus followed by chemoselection and enrichment of gene-modified cells. Future experiments will use NSG mice to determine the in vivo selection capabilities of modified cells containing mDHFR **(see schematic)**. NSG mice are immunodeficient mice that are xenografted with human cells and



support the engraftment and differentiation of human hematopoietic systems.

Another possible therapeutic use is in the attainment of a functional cure for HIV. The ultimate goal of gene editing for HIV/AIDS is to be able to engineer an immune system that is able to resist HIV infection, and control viral replication below limits of detection in the absence of ART. Early clinical trials using CCR5-targeting zinc finger nucleases demonstrated transient control of HIV infection in the course of antiretroviral treatment interruption (Tebas, NEJM, 2014). Our current work hopes to improve on these advances by combining high level of CCR5 gene disruption with preferential selection of gene modified cells. We hypothesize that coupling CCR5 knockout with drug selection will help us achieve therapeutically relevant levels of HIV-protected cells by enabling efficient selection only of CCR5-modified T-cells **(Figure 7)**. We could even possibly use the methotrexate drug- selection system to further enrich for gene-modified cells in vivo.

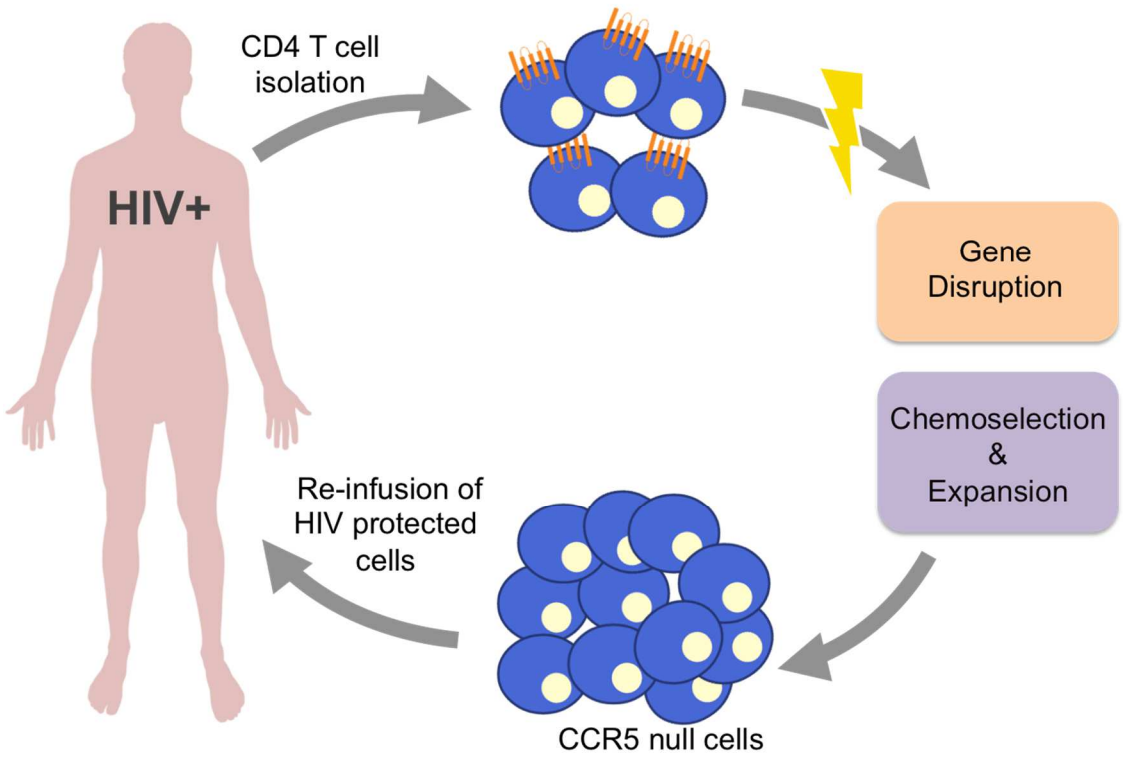


Figure 7: Therapeutic strategy to achieve a functional HIV cure. Cells are extracted from patients, gene-modified, expanded, and reinfused in an autologous transplantation setting. Biallelic mutations in the CCR5 gene are created using the engineered megaTAL nuclease platform, thereby imparting these cells with resistance to HIV infection. A mutant chemoselection marker is inserted at the CCR5 locus, followed by drug selection in methotrexate to enrich for gene-modified CCR5-null cells.

A potential safety concern with traditional gene therapy methods has been insertional mutagenesis and cellular transformation induced by virus integration after the modification of hematopoetic stem cells (HBA 2008). Using targeted insertion of the mDHFR constructs will help overcome such safety concerns. We have demonstrated the utility of mRNA delivery for non-toxic and transient nuclease expression. mRNA delivery methods should be further developed for use in other nuclease platforms and clinical trials also. Advantages of using mRNA: nuclease expression is transient; unlike plasmids or viral-vectors, mRNA cannot accidentally integrate into the genome and cause undesired long-term expression; mRNA electroporation technology has already been established for use in large-scale good manufacturing practice (GMP) conditions; it is easier to move into clinical application in some regulatory environments because it is considered to be a pharmaceutical product as opposed to viral vectors which are considered genetically modified organisms.

Our approach is especially beneficial in the case of gene editing for diseases in which the modified cells do not have a survival advantage by virtue of the correction of their disease phenotype. In such disease models we could exert artificial selection pressure using low-dose MTX to achieve therapeutically relevant levels of gene-modified cells to ameliorate the disease phenotype.

This study has potential for therapeutic application since we use a transiently expressed, highly specific nuclease to efficiently insert a donor transgene into primary cells. This protocol could be

adapted for use in future studies using adoptive T cell transfer as a treatment modality. MTX administration has the ability to induce lymphopenia and reinforce transgene expression simultaneously making it useful to drive selective engraftment of gene-modified cells following a transplant.

Ultimately this methodology could be used to enhance the *in vitro* and *in vivo* selection of T cell therapeutics that are being designed to target a broad spectrum of cancers. We envision a possible clinical methodology that can combine chemo-and immunotherapy; one such instance has been previously described in the treatment of brain cancer (Lamb 2013). Additionally, MTX is active against a variety of CD19+ hematologic malignancies, hence a combination therapy of drug-resistant gene-modified cells could have additive anti-tumor effects. It is tempting to speculate that the combinatorial effects of chemotherapy and the preferential enrichment of modified T cells may improve the anticancer response of the patient beyond that of either therapeutic used alone.

The targeted insertion followed by low-dose drug enrichment can be especially beneficial when incorporated into CAR T cell manufacturing processes. The AAV genome can accommodate constructs less than 4.4kb in size. The most well characterized CARs, currently used in clinical trials, could easily be linked to a mutant DHFR using a T2A sequence. These CAR-DHFR constructs measure around ~2kb, leaving room for CCR5 homology arms which have been shown to be long enough to facilitate robust HR (Sather 2015). These CARs can then be enriched ex vivo during clinical grade manufacturing, or in vivo by putting patients on low-dose MTX immunosuppression.

CELLULAR ENGINEERING AND CHEMOSELECTION IN CD4 T CELLS: MATERIALS AND METHODS

megaTAL Nuclease

The CCR5-megaTAL nuclease was generously shared by bluebirdbio Inc (formerly Pregenen, Inc). The HE platform was engineered as previously described (Romano 2016). It is bound to a 10.5-repeat variable di-residue TAL array that recognizes and cleaves at 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 procured form American Type Culture Collection (ATCC) and cultured in RPMI-1640 Medium with added 10% FBS and 1% penicillin- streptomycin by volume. Cells were maintained at a cell concentration between 1 x 10^5 and 1 X 10^6 viable cells/mL in Corning® T-75 flasks or 12 well plates. Media was renewed every 3-4 days by pelleting cells and adding fresh medium depending on cell density.

PBMC were isolated from blood of healthy human donors in accordance with institutional guidelines. Untouched CD4 T cells were enriched from this population using either CD4+ T cell Isolation kit (Miltenyi Biotec) or EasySep™ Human CD4+ T Cell Enrichment Kit (StemCell Technologies). Frozen stocks of CD4 cells were thawed, activated *ex vivo* for 48 hours using CTS Dynabeads CD3/CD28 (Life Technologies) at a bead to T cell ratio of 1:1 and cultured in T cell media. Media consisted of the following: RPMI-1640 Medium, 20% FBS, 50ng/mL IL-2, 5ng/mL IL-7, 5ng/mL IL-15, and no antibiotics. T cells were seeded 1-2 × 10^6 T cell ml^{-1} in 48 well plates with a half media change every 2-3 days.

Gene-modified T cells were expanded long-term according to a modified rapid expansion protocol using pooled irradiated feeder cells, growth factor IL-2 and anti-CD3 antibody. Between

3×10^5 – 1x 10^6 T cells were seeded in T25 flasks, along with 25x10^6 irradiated allogeneic PBMCs and 5x10^6 irradiated TM LCL. Cells were grown without antibiotics in 50U/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. Gene-modified T cells were also expanded long-term using G-Rex 10 flasks (Wilson Wolf Manufacturing). Activated T cells were seeded at between 5-10x 10^6 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.

Production of Viral vectors

Lentivirus was produced as previously described at the Fred Hutch Viral Core(Becker 2010). Briefly, virus was produced by co-transfection of third generation lentiviral production plasmids into HEK-293 cells, concentrated and titered using flow cytometry. The EFDIG lentiviral vector was produced using a self-inactivating, HIV-1 based backbone and pseudotyped with vesicular stomatitis virus G protein envelope (Gori 2007). The construct contains an hEF-1alpha promoter which drives the expression of the Tyr22-DHFR variant, and a GFP reporter linked via a picornavirus internal ribosomal entry site (IRES).

AAV stocks were produced as previously described at the SCRI Viral Core (Khan 2011). Briefly, HEK293T cells were co-transfected with plasmids for AAV vector, serotype helper, and an adenoviral helper. These cells were harvested after 48h, 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. Stocks used in this study were around 1x10^{11} viral copies per mL.

Genetic modification of cells

Activated T cells were transduced with lentiviral vectors at a multiplicity of infection of 1. Excess virus was washed off after 24 hours and efficiency of transduction was assessed at day 2 by flow cytometry.

Targeted integration at the CCR5 locus was performed as previously described (Romano 2016). Briefly, activated T cells were transfected with 1ug of CCR5 megaTAL mRNA in the Neon Transfection system using the settings 1400V, 10ms, 3 pulses. After 2-3 hours they were transduced with AAV at 20% of the culture volume regardless of viral titer. Excess virus was diluted by adding fresh media after 24 hours and efficiency of transduction was assessed at day 2 by flow cytometry. After a day-long 30 degree incubation, T cells were cultured for two weeks in T cell media at 37 degrees and fresh media added every 2-4 days.

In vitro selection of gene modified cells

Chemoselection in the presence of the drug methotrexate (GeneraMedix) was achieved by carefully titrating the amount of drug and timing of introduction into culture. Methotrexate was diluted in phosphate buffered saline and aliquots were stored at -20 degrees. Cells were cultured over the course of 14 days with 100nM of drug being added once on day 4 of the REP stimulation. Drug-induced selection was quantified by total cell counts, and flow cytometric analysis of transgene selection and viability.

Flow cytometry

Healthy T cells were stained with antibodies against surface markers CD4, CD8, CD125, CD62L, and CD45RA according to manufacturers recommendations. Live/dead discrimination was performed using Propidium Iodide staining. HIV-infected cells were fixed and permeabilized using the CytoFix/CytoPerm kit (BD Biosciences) according to manufacturers recommendations. Cells were intracellularly stained using anti-p24 KC57 antibody (Beckman

Coulter). Stained cells were processed on a FACSCanto II (BD Biosciences), and analyzed using FlowJo software (Tree Star).

TCR Spectratyping

The Immune Monitoring Core (Fred Hutch) performed spectratyping analysis of TCR VB CDR3 subfamilies 1-25 using multiplex transcription PCR (Akatsuka 1999).

Statistics

Statistical analyses were conducted using Prism6 (GraphPad Software). Differences were considered to be statistically significant where $P<0.05$.

Cloning of DHFR mutant constructs

Dual-expression GFP/Methotrexate resistant dihydrofolate reductase (DHFR) gene editing templates were created by modification of the previously described CCR5 gene editing template (Romano and Sather) which introduced a GFP transgene under an MND promoter, resulting in CCR5 knockout. Briefly two DHFR gBlocks were synthesized (Integrated DNA Technologies) containing mutations – L22F/F31S or F31R/Q35E - previously described to confer methotrexate resistance (Jonnalagadda 2013 & Volpato JP 2009 respectively). These cassettes were introduced to the CCR5 editing template using Gibson assembly (InFusion HD EcoDry), downstream of the MND promoter and upstream of the GFP, bi-cistronically linked via a t2A peptide.

SUMMARY

Engineered nucleases can be used to induce double stranded breaks at specific sites in the genome to facilitate therapeutic gene disruption or gene insertion. Gene insertion strategies may have a significant advantage over traditional lentiviral gene therapy since we have much greater

control over transgene integration site, copy number, and expression. However, a major limitation in the clinical application of gene insertion technologies is the low efficiency of homology driven repair (HDR) achieved in hematopoietic cells. In this chapter, I have reported the knock-in of a selection marker, which allows us to enrich for modified CD4 T cells. To overcome the decline of HIV-resistant cells, observed in clinical trials using CCR5-disruption, we coupled gene-disruption with gene insertion of mutant human dihydrofolate reductase (mDHFR). The mDHFR chemoselection system has been used to render cells resistant to lymphotoxic concentrations of the drug methotrexate (MTX). We first tested our experimental approach by transducing cells with a lentiviral vector encoding a mDHFR cassette followed by chemoselection in MTX at 0.1uM. This approach resulted in a six-fold enrichment of gene modified primary CD4+ T cells *ex vivo*. For gene insertion, combining megaTAL treatment with adeno-associated virus (AAV) transduction produces very high rates of homology-driven repair (HDR) in primary human T cells. Hence, we merged the megaTAL nuclease and AAV gene-delivery treatments to integrate mDHFR into the CCR5 locus, thus producing a population of MTX-resistant CD4+ cells that also lack CCR5. Primary human CD4+ T cells were transfected with CCR5-megaTAL mRNA and transduced with AAV6 containing a mutant DHFR donor template flanked by 0.8kb CCR5 homology arms. They demonstrated a greater than five-fold enrichment in 0.1uM MTX compared to untreated controls *ex vivo*. To our knowledge we are the first group to report MTX-mediated chemoselection and expansion of CD4+ T cells following targeted integration at the CCR5 locus. Eventually this system could be used to select for modified cells *in vivo* in patients using previously established protocols for MTX administration in the clinic.

Chapter 5. THE FUTURE OF GENOME EDITING THERAPEUTICS

Targeted anti-HIV gene editing

Targeted gene editing could modify the alleles of different human genes, which are associated with protection from HIV. HIV has adapted such that human restriction factors – genes with antiviral properties – no longer suppress the virus. However, the nonhuman primate orthologs of genes such as TRIM5a, tetherin, and APOBEC3G retain their capacity for virologic control (Xu 2004, Yap 2005, Gupta 2009). The human alleles could be modified to resemble the nonhuman primate alleles, thus providing a mechanism for viral control. Alternatively, there are certain alleles that are associated with greater viral control that have been found in the human population (Chinn 2010). These naturally occurring human allelic variants could be introduced into cells to inhibit HIV-1.

mDHFR for selection in CART cell immunotherapies

The relatively small size of the mDHFR constructs make them an attractive selection marker that can be used in conjunction with CAR T cell constructs in AAV delivery systems. The human DHFR mutants are roughly 600bp and can easily be packaged along with a CAR T cell construct (~2kb) into an AAV with a packaging limit of ~4.4kb. mDHFR has already been used in tandem with CAR T cell constructs in a lentiviral system (Jonnalagadda 2013). Targeted insertion of the CAR construct using AAV and CCR5-megaTAL, followed by MTX selection can be a promising new therapeutic approach exhibiting more precision in gene delivery.

Utilizing CCR5 as a safe harbor locus

An important step in enhancing safety of gene editing therapies is identifying sites in the genome that are at minimal risk of unintentional insertional mutagenesis when exogenous DNA is added at the site. These sites, called genomic safe harbors, allow for more exquisite control over transgene expression and its effects on neighboring genes. CCR5 has been utilized as a safe harbor, even though it exists in a dense genomic region. Integration at CCR5 was tolerated well without any adverse outcomes in several pre-clinical mouse studies. Our studies show the utility of targeted insertion at CCR5 in T cells. Long term follow up data from the clinical trials will help demonstrate definitively that CCR5 can indeed be used as a safe harbor in the future.

Combining Gene insertion with Gene disruption in T cells

Chemoselection could be combined with targeted gene disruption at various other T cell loci to create novel treatment modalities. We have already demonstrated the safety and efficacy of this approach with respect to the CCR5 locus. Examples where such a combination would be useful are: disruption of endogenous TCR or MHC to generate universal donor cells (Torikai 2012, 2013); disruption of programmed cell death 1 (PD1) gene to prevent tumor evasion (Hamid 2013); disruption of cytotoxic T-lymphocyte antigen 4 (CTLA-4) gene to help T cells destroy tumors (Snyder 2014).

Increasing the frequency of Homology directed repair

In future studies we could use a host of small molecules and other reagents, that are currently under investigation, to modify the intracellular environment to promote the DNA-repair events we desire. One such promising area of research uses viral proteins that modify the nuclear landscape post viral-entry. Different viruses have evolved proteins to help the viral genetic material escape degradation and replicate within the cell. Transient expression of adenoviral proteins has been shown to increase the levels of gene editing in primary human T cells (Gwiazda 2016). Two mutants proteins have been characterized that enhance gene editing in the context of the CRISPR/Cas9 system: E4orf6/E1b55k-H354 and E4orf6/E1b55k-H373A. In particular, the E4orf6/E1b55k-H373A mutant adenoviral helper protein has been shown to significantly increase HR by the CCR5-megaTAL. While the exact mechanism of how the co-transfection of these proteins are enhancing gene editing has not been ascertained, the researchers hypothesize that these proteins probably promote DSB repair during S phase.

Editing in CD34 hematopoietic stem cells

Recently, two groups have successfully shown HR-mediated editing in CD34 cells at levels >15% (Sather 2015, Wang 2015). These cells are especially relevant for the treatment of many hematologic conditions (such as hemoglobinopathies, chronic granulomatous disease, XSCID). Modification of HSCs is also important for diseases that require long term modification of self-renewing cells that give rise to various hematopoietic cell types. Hence, transient expression of a nuclease in a primitive HSC can give rise to permanently modified progeny. Also, HSCs have

been successfully used in several autologous transplant settings and the protocols for their isolation, culture, and transplantation are well established. Furthermore, a clinical trial using CD34 HSCs that were modified using ZFN targeting CCR5 (trial #NCT02500849). Hence, HSCs are an important treatment modality that is being explored. However, we need to keep in mind the limitations of working with HSCs. In contrast to T cells, HSCs are less easy to expand, manipulate, and gene edit. For instance, we have consistently achieved 2-5 fold higher rates of HR-mediated editing in T cells when compared to HSCs.

In vivo gene editing

Even though my work has focused on manipulating cells ex vivo before transplanting them into patient, gene editing can also be carried out by in vivo injection. In vivo editing consists of delivering nucleases, and if necessary, repair templates directly to the disease affected cells in the native tissues. This kind of therapy is especially useful to (a) modify cell types that are not amenable to manipulation ex vivo, and (b) to treat diseases that affect multiple different tissues types in the body. Several different AAV serotypes have, and continue to be developed, with different tissue tropism such that we can target delivery of our nuclease and repair templates after systemic injection. For example, co-delivery of ZFN and a donor cassette using intravenous injection of AAV helped restore production of factor IX and treat hemophilia B in mice (Anguela 2013). In other studies, successful AAV delivery of Cas9 and guide RNAs was used to delete mutant exons in the mouse model of Duchenne muscular dystrophy helped restore dystrophin expression and improve muscle function (Long 2016, Tabebordbar 2016, Nelson 2016).

Chapter 6. ETHICAL AND REGULATORY CONSIDERATIONS OF GENE EDITING

Genome editing of the germline: the need for a global discussion

Recent advances in genome editing, a process to modify the heritable genetic material inside a person's cell, have gradually encouraged scientists, lawmakers, and the public to rejoice over the promise of significant breakthroughs in research. However, two recent studies by investigators from Chine reported using genome editing in human embryos which raised a flurry of ethical and regulatory concerns across the globe (Liang 2015, Kang 2016). Human embryos are now at the center of an international debate over gene editing: Is this ethical? Can we regulate it? What safety thresholds need to be met before proceeding? These are questions that scientists, lawmakers, governments, non-profit groups, and the public need to address immediately.

Genome editing: changing the "spelling" of your genes

Genome editing is defined as the process of precisely modifying the DNA sequence of the genome at the nucleotide level. Even though genome editing or the modification of specific genes in human cells has been around for a decade, significant advances made in the last two years have made it more accessible. Special DNA-cutting enzymes called zinc finger nucleases (ZFN), first described in 2006, have since been systematically tested and have recently entered human clinical trials. They cost around $5000 and are complicated to manufacture. This has limited the adoption of ZFNs. The discovery of DNA sequences called clustered regularly interspaced short palindromic repeats (CRISPRs), a bacterial defense system against viral infection, in 2012 has revolutionized the field (Jinek 2012). The total cost of this genome editing technique can be as little as $30. The CRISPR system is easy to manufacture and users can specify which target cellular DNA sequences they want cut. Subsequently, users can insert desired sequences into the cut site. By changing the arrangement of the four letter of the genetic

code – A, T, C and G – we are able to change the "spelling" of our gene-of-choice. CRISPR has changed the landscape of genome editing by making this cheap, easily usable technology available to startup laboratories anywhere in the world.

Current limitations of Genome editing technologies

There are several concerns around the use of genome editing in the human germline. First, the technique is not very precise. According to Keith Joung, a gene editing expert in Boston, the CRISPR system cuts at non-specific sites with frequencies ranging from 0.1% to more than 60% (Fu 2013). It can be argued that several of the drugs used currently cause side effects due to working off target. However, their side effects have been extensively studied and undergone rigorous testing. The CRISPR/Cas9 system on the other hand, has still not reached a place of development where it can be used safely in clinical settings. Second, the technique cannot modify all the genes in a population of cells, or even all copies of the gene in a single cell. When carried out in embryos, CRISPR-based editing would produce a genetic mosaic of cells – some of which would be edited and some that wouldn't – resulting in incomplete protection or elimination of a disease. Finally, negative results from prematurely using this technique in embryos could set the field of gene editing back by several years. Controversy and outcry by interest groups and policy watchdogs can sway public opinion and eventually harm investment in, and support for, useful science. It is imperative that we do not allow ethical pushback against germline editing to impede progress in the development of groundbreaking technologies and treatments to edit somatic cells.

Challenges in regulating Gene editing in human embryos

Tensions about genome editing in embryos came to a head in April 2015 when Junjiu Huang's team used CRISPRs to cut and replace DNA in non-viable human embryos designed to not

survive longer than 14 days (Liang 2015). This unregulated study highlights how close we are to replicating this editing process in embryos that could be carried to term. In response to this study, the US National Institutes of Health (NIH) reiterated its blanket ban on research that involves gene editing of human embryos based on ethical and legal grounds (Collins 2015). On the other hand, authorities in the United Kingdom ruled that such research could use germ cells – including human embryos up to 14 days old (Ariana 2015). However, these measures do not necessarily solve the problem. While the NIH ban might limit federally funded research in the US, researchers can always use private funding sources. Likewise, while the UK ban addresses research within established institutes in countries with proper channels of oversight, it does not address the issues of culpability in countries where research operates outside the purview of regulatory bodies.

For the last several decades, various groups have been concerned about the advent of technologies that could alter the characteristics of the human germline, namely the sperm, eggs and embryos. These changes once introduced would then be passed on to all offspring and future generations. The advent of the accessible, affordable and uncomplicated CRISPR genome editing system has brought the possibility of *designer babies* much closer to reality. There is a real threat of this technology being used in experimental therapies in regions of the world with little or no regulation over embryonic research. For example, we have seen a large rise in medical tourism over the last decade. People from western countries have flocked to fertility clinics in countries with lax regulations like China, India, and Russia -- and collectively spent millions of dollars on unproven and illegal stem cell therapies (Einsiedel 2012). It seems only a matter of time before illegal genome editing therapies would also be added to the roster of "medical" services offered at such clinics.

While no one disputes that gene editing will be instrumental in finding cures to thousands of currently incurable disease states, scientists and ethicists alike are raising concern over what will inevitably become a demand for the 'enhancement' of normal human traits. The private sector will then have a strong motivation to develop enhancement therapies as marketable products in response to consumer demand. This is cause for concern since it is tougher to regulate private markets. Furthermore, with rapid globalization and transfer of information worldwide, it is foreseeable that people will locate the technology and travel to whichever country provides the procedures they seek.

Continuing multi-stakeholder dialogue on Gene editing

We need to carefully evaluate how we want to use gene editing as a society. The history of eugenics in the first half of the 20th century serves as a chilling example of how an ideology can capture the imagination and hearts of entire communities, which result in human rights abuses. Scientific research was quoted as reason to remove *feeble-mindedness*, *criminality*, and *pauperism* from the human gene pool. This idea enjoyed support from educated thought-leaders and scientific professionals which led to widespread immigration restrictions on, and sterilizations in, people perceived to be genetically inferior. It took Nazi atrocities and a world war for the theory to be re-examined and ultimately discredited.

We might eventually find out that the long-term harms caused by gene editing outweighs the short-term benefits. Hence, it is prudent to engage in multi-stakeholder dialogue over a prolonged period of time before we start to edit the genetic code of humanity. Any risk-benefit analysis should look beyond just the medical and scientific risks. Ethical questions such as what it will mean to be human if our genomes can be modified at will, should be included in the debate. To jumpstart such conversations across nations the International Summit on Gene

Editing was convened in December 2015 in Washington DC. The summit issued a statement with their recommendations on the proper use of gene editing at this time (Intl Sum 2015). They said that while every nation has the right to regulate activities under its own jurisdiction, the human genome is shared among nations. Hence they urged nations to convene ongoing forums to discuss issues and formulate recommendations and guidelines that include a wide range of perspectives and expertise. They posited that, ultimately, governance on gene editing "can be exerted through laws, regulations, guidelines, standards, professional norms, and public expectations."

Interim moratorium on Gene editing in human embryos

At this point, a worldwide moratorium on the use of CRISPR and other gene editing technologies in germline cells is the best way forward. Under the moratorium scientists at institutions across the globe would agree to not pursue any attempts to modify the genomes of human reproductive cells. There is a precedent that shows that such a moratorium, though not legally enforceable, does work. In 1975, scientists, led by Nobel laureate David Baltimore, issued a moratorium on using recombinant DNA technologies to manipulate genes, until ethical and safety guidelines had been established (Berg 2008). This gathering, now referred to as the Asilomar Conference, is an example of applying the precautionary principle when it comes to implementation of new technologies. While a moratorium on germline-editing would be temporary, it would give us time to thoroughly investigate the ramifications of using this technology. We could open up the field for democratic public debate to inform decision-making. Key stakeholders in such debates would be disease advocacy groups and families whose lives have been impacted over multiple generations by the transmission of devastating genetic diseases through their family tree. A moratorium on gene editing in embryos, until we understand the technique completely, does not

necessarily leave concerned at-risk parents stranded. The existing technique of preimplantation genetic diagnosis—where cells are removed from an early embryo produced by in vitro fertilization and tested for genetic disorders—can be used to decide which embryos to implant and actually carry to term. Genetic counseling, donor eggs or sperm, and adoption are other existing options for parents with heritable disorders who wish to save their children from inheriting a disease. While making gene-editing decisions the ethical issue that parents also need to keep in mind is that they are treating the embryo as property or commodity, and not its own entity.

Beyond Asilomar: lessons on improving engagement and oversight

While Asilomar is a good example of a group of like-minded scientists getting together and effecting change in science and policy, it is not without flaws. There are several key takeaways from the Asilomar example of what could be improved upon. One of the main problems of the conference was it lack of inclusion. While it included several scientists and experts, it left out several key stakeholders. An unanticipated and problematic result of the moratorium proposed by the conference was that it led with the assumption that recombinant DNA-modified organisms were a high-risk category. This led to overly stringent NIH regulations and oversight committees that slowed down the progress of research. Another unintended consequence of treating the new technology as threatening was that it sent a message of danger to the general public. This message was reinforced by the increased regulatory scrutiny by NIH (and later other federal agencies), and to this day the general public view genetically modified organisms (GMOs) with suspicion. Some scientists argue that the seeds of public mistrust were sown due to overly conservative and poorly understood recommendations that came out of Asilomar (Miller 2015).

The issue to address moving forward is whether the drawbacks of Asilomar were a problem of process or a problem of substance. Was the problem caused by the flawed process of inviting a select few scientists and experts who did not represent a wide-variety of concerns? Or was the problem that what came out of that gathering was not substantive enough since there just wasn't enough information at the time to make decisions? The recommendations from the conference were later codified into regulatory frameworks and processes, which further stilted the gathering of new information. What took place at Asilomar did not have a well-defined process. A couple of experts decided to come together and call for self-regulation of the use of emerging technologies and self-regulatory entities do not inherently have a process of multiple checks and balances. Moving forward we need to remember that true inclusion would bring in opinions from all sides of the issues being discussed, especially since we are now discussing issues that have the capacity to influence the human genome globally. It is also helpful to move into such multi-stakeholder discussions assuming the principle of mutual beneficence – we need to assume that we are working towards a common goal instead of imagining ourselves as being on different sides. Eventually when these discussions start getting codified into recommendations we need to remember that rule-making needs to have participation at all levels. At every step along the way there needs to be a process to check whether the substance of the rules that are being put forth is actually accurately reflecting what stakeholders discussed.

Halt all current efforts towards heritable Gene editing in humans

So should genome editing in human egg, sperm and embryos be allowed? At this point, the answer is no (Lanphier 2015, Baltimore 2015). At present there aren't any set standards that need to be met for this work to be considered safe. It is also not known who will set the threshold of what constitutes successful therapeutic modification, and how these thresholds would be

enforced. In the distant future, we as a worldwide community will also need to determine what applications, if any, using genome editing could be considered ethical for reproductive selection. Until then it is safest to test the waters using somatic gene editing in non-germline cells since this technique is easier to regulate. These therapies are limited to and affect only the patient being treated and hence can be evaluated within already existing regulatory framework for such clinical trials. At present there are no set regulatory framework for evaluating the clinical efficacy of gene editing conducted in germline cells. There is also no broad societal consensus on whether such modifications are ethical and morally permissible. Thus, rushing headlong into irreversible germline modification is irresponsible, and is akin to "playing god".

The most important point to reiterate here is that the moratorium is not a ban. It merely asks for a halt in germline modification until the risk-benefit analyses and safety concerns are addressed. Germline editing should be revisited as scientific knowledge advances and societal views evolve. In summary, genome editing has revolutionized biomedical research and its applications. However, the CRISPR technology is not yet ready to move into modifying germline cells for clinical applications. A worldwide moratorium on genome editing research in human embryos and reproductive cells is necessary until ethical, safety, and regulatory concerns are addressed.

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