40. Nuclease-Targeted Gene-Editing of *FOXP3* in Primary T Cells Creates a Stable and Functional T_{reg} Phenotype

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Reprogramming T cells to adopt regulatory T cell (T_{reg}) functions represents a promising strategy for treating autoimmune disorders and graft-versus-host disease. The development and maintenance of T_{reg} cells is dependent on FOXP3 expression, which in turn is tightly controlled through epigenetic modification of intronic conserved non-coding sequences (CNS) surrounding the first coding exon. Two major challenges associated with FOXP3 gene-transfer approaches for T_{reg} reprogramming are: achieving adequately high FOXP3 expression, and avoiding eventual gene silencing. We devised two gene editing strategies with the goal of driving stable expression of FOXP3 in primary human T cells. Using a combination of TALEN-mediated gene disruption and adeno-associated-virus (AAV) delivered donor repair templates, we introduced either an MND promoter upstream of the first coding exon, or we deleted intronic CNS implicated in transcriptional silencing. The donor templates were also designed to co-express FOXP3 with either GFP or EGFRt (truncated Epidermal Growth Factor receptor), to allow tracking and purification of edited cells. Both strategies resulted in T cells stably expressing FOXP3 at high levels (~60 %). Introduction of the MND promoter resulted in the greatest levels of cellular FOXP3 expression (MFI), and these cells showed phenotypic and functional changes consistent with Treg cells, including: surface marker expression ($CD25^{high}$, $CD127^{low}$, $CTLA4^{high}$, $LAG3^{high}$), suppression of cytokine production (IL-2, IL-17 and IFN-γ), and resistance to Rapamycin. Edited cells also suppressed the proliferation of stimulated T cells *in vitro*, demonstrating their effective 'reprogramming' towards a T_{reg} lineage. Thus, our gene modification strategy allowed us to over-ride (using the MND promoter) or modulate (by deleting CNS elements) endogenous *FOXP3* regulatory mechanisms to enforce stable, long-term FOXP3 expression in T cells that were not previously committed to the Treg lineage. This approach, used alone or in combination with selection for disease-relevant TCR specificity or with delivery of a chimeric antigen receptor, is likely to be broadly applicable for producing stable, functionally active Tregs for a range of future clinical applications.

41. Therapeutic Level CRISPR-Oligomer-Mediated Correction of X-CGD Patient Hematopoietic Stem Cells Using Non-Viral, cGMP Compliant, Scalable, and Closed System

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Gene therapy using integrating viral vectors in hematopoietic stem cells (HSC) has shown clinical benefit in genetic diseases. However, there remain safety concerns associated with random integration and the lack of regulation of gene expression. Efficient and site-specific correction of mutation(s) in HSC using non-viral methods may improve safety and regulation of gene expression. Chronic granulomatous disease (CGD) due to defective phagocyte NADPH oxidase complex and lack of bactericidal superoxide and other reactive oxidative species (ROS) is characterized by severe infections and hyperinflammation. Although the X-linked form of CGD with gp91phox deficiency results from mutations that span the CYBB gene, we identified a 'hotspot' mutation at Exon 7 c.676C>T, causing a premature stop codon in 17 out of 285 patients with X-linked CGD at the NIH. Here we report the result of the efficient correction of the hotspot CYBB mutation using highly efficient CRISPR (Cas9 and sgRNA) system with an oligomer as donor repair template, using MaxCyte's commercially/clinically validated cGMP/regulatory compliant and closed platform technology. Plasmids encoding Cas9 and gRNA were purchased from the Genomic Engineering Center at Washington University (St. Louis, MO). The mRNA encoding Cas9 and gRNA were in vitro transcribed at MaxCyte using mMESSAGE mMACHINE® T7 Ultra kit, (Ambion, Austin, TX). We screened and selected best gRNA from four gRNA candidates for correction and then optimized transfection conditions with EBV-transformed B cell line (B-LCL) derived from an adult patient (P1) with the hotspot CYBB mutation. Transfected B-LCL exhibit 80±6% viability, minimal detectable toxicity as determined by cell proliferation rate referenced to control cells, and efficient site-specific gene correction with 20-50% WT gp91 expression. These developed protocols were used to treat G-CSF and pleraxifor mobilized CD34+ HSC from P1. Following optimization, *in vitro* treated HSC from P1 achieved 20-30% WT gp91 expression, with >50% viability, and minimal loss of cell proliferation capacity compared with control cells. CD34+ HSCs undergo myeloid differentiation in DMEM supplemented with G-CSF prior to functional evaluation using flow cytometric dihydrorhodamine (DHR) assay, demonstrating ~20% ROS+ cells in treated samples compared to ~80% in normal controls. P1's HSC treated the same way were transplanted into immunodeficient mice, and analyzed 8 weeks later. Bone marrow from mice transplanted with P1 treated cells showed CD45+ human cell engraftment rates at 50-80%, and of the forward/side scatter-gated granulocytes, 11-26% express gp91phox relative to 68% in normal control. Peripheral blood from mice demonstrated 11-23% human CD45+ cells, of which 9-21% expressed gp91phox, compared to 79% in normal controls. Deep sequencing of human CD45+ cells sorted from mouse bone marrow confirmed high rates (up to 21%) of genetic correction from the 'T' mutation to the wildtype 'C'. Since female carriers of X-CGD with ~10-15% normal functioning neutrophils appear to have normal resistance to infections, this level of correction at 10-20% in human CD45+ cells from transplanted mice suggest CRISPR/oligo approach a feasible therapeutic option for treatment of CGD patients with the Ex7, c.676C>T mutation.

42. Correction of SCID-X1 by Targeted Genome Editing of Hematopoietic Stem/Progenitor Cells (HSPC) in the Mouse Model

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Targeted genome editing by engineered nucleases has brought the goal of gene correction within the reach of gene therapy. A candidate disease for HSPC gene correction is SCID-X1, because gene therapy trials with integrating vectors showed robust clinical efficacy even from few corrected cells but also the occurrence of leukemias due to insertional mutagenesis and unregulated transgene expression. To model SCID-X1 gene correction in preclinical studies, we developed a mouse model carrying the *IL2RG* human gene harboring a common disease-causing mutation in place of the murine *Il2rg*, allowing to use of the same reagents developed for gene correction of human cells. These mice have impaired lymphoid development which phenocopies

that reported for *Il2rg-/-* mice. To assess the minimal level of corrected HSPC required to achieve immune reconstitution we performed competitive transplants with wild-type (WT) and *Il2rg-/-* HSPC and found that 1% of WT cells are sufficient to reconstitute in part the T and B cell compartments. We then tested gene correction of the murine Lin- HSPC by the delivery of donor DNA template by IDLVs followed by transfection of ZFN mRNAs. This protocol yielded high on-target nuclease activity (40%) and a mean of 6% transgene integration by HDR but also high cytotoxicity (65% cell loss) under the conditions we used. The surviving cells remained capable of expansion in culture and maintained their clonogenic potential. Importantly, upon transplant into lethally irradiated mice, only the gene corrected cells were able to generate lymphoid lineages (B and T cells), showing a clear selective advantage over the un-corrected SCID cells. These data indicate functional correction of the defective *IL2RG* gene by targeted editing. Furthermore, upon challenging the mice with a murine pathogen we observed viral-specific γIFN production by CD8+ gene corrected cells, proving their *in vivo* functionality. Yet, measuring the percentage of edited cells (either by NHEJ or HDR) within the HSC compartment long-term, we found that it was nearly undetectable. Despite the lack of HSC marking, gene corrected lymphoid cells persisted in the mice up to 7 months post transplantation within all the hematopoietic organs, indicating successful editing of at least 1% progenitors able to sustain long-term lymphopoiesis and partially correct the disease phenotype. We then developed a new protocol exploiting CRISPR/Cas9 technology that enabled to achieve substantial levels of targeted DNA repair by NHEJ (up to 70%) and HDR (up to 25%) with minimal cytotoxicity and provided stable engraftment of the edited cells in transplanted mice. By this strategy we are now assessing the impact of HSC vs. progenitor targeted editing and conditioning regimen for the extent and stability of disease correction. These studies will help establish the key factors underlying safe and effective rescue of the disease by HSPC gene editing and assist in the design of the protocol for its first clinical testing.

43. CRISPR/Cas9 and rAAV6-Mediated Targeted Integration at the CCR5 Locus in Hematopoietic Stem and Progenitor Cells

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CRISPR/Cas9-mediated genome editing relies on guide RNAs to direct site-specific DNA cleavage mediated by the Cas9 endonuclease. In this study, we identified a highly potent single guide RNA (sgRNA) targeting exon 3 of *CCR5*. This sgRNA was chemically synthesized with three modified nucleotides at each terminus with 2´O-Methyl and phosphorothioate modifications, and electroporated into cells either with Cas9 mRNA or complexed with Cas9 protein (RNP). Using Tracking of Indels by DEcomposition (TIDE) to quantify insertions and deletions (INDELs), we observe up to 80% INDELs in CD34+ hematopoietic stem and progenitor cells (HSPCs).

To achieve targeted integration by homology-directed repair (HDR), we produced rAAV6 vectors carrying a GFP expression cassette flanked by *CCR5* homology arms. Electroporation with Cas9 RNP followed by rAAV6 transduction led to targeted integration in up to 30% of the cells. Interestingly, we observed that cells with targeted integration expressed GFP at fluorescence intensities more than 10-fold higher than from episomal AAV vectors. This allowed us to sort targeted cells as early as four days after nucleofection and transduction. Upon fluorescence-activated cell sorting and culture of this population, >99% of cells remained GFP^+ 20 days post sort. In a methylcellulose-based colony-forming unit (CFU) assay, we identified multipotent and lineage-committed progenitor cells in this population, and PCR of gDNA extracted from colonies confirmed targeted integration at *CCR5* in at least 98% of cells. Phenotypic characterization of this targeted population confirmed the presence of $CD34^+$ $CD38^-$ $CD90^+$ $CD45RA^-$ cells, indicating genome editing of hematopoietic stem cells. We transplanted edited cells into immunodeficient NSG mice and analyzed the bone marrow 8 weeks post-transplant. In mice transplanted with cells that were not enriched for targeted integration, we found 0.1-1.9% GFP^+ cells among the engrafted human cells. This was a significant decline compared to the 12-13% GFP^+ cells in the input cell population following culture, which is a phenomenon consistent with findings reported by other groups using different nuclease platforms. In contrast, when transplanting cells enriched for targeted integration, we found that 75% of the engrafted human cells were GFP^+, confirming the presence of cells with long-term engraftment potential in the enriched population.

In conclusion, we have found that the combination of CRISPR/Cas9 and rAAV6 is an effective platform for HDR-mediated targeted integration of a transgene into the *CCR5* locus. Furthermore, the GFP MFI shift observed when episomal rAAV6 vectors are integrated into the chromosome following HDR enables early isolation of a population highly enriched for targeted integration at this locus. Since *CCR5* is considered a 'safe harbor' for targeted insertion of a gene, this approach might find general use in therapeutic genome editing. Additionally, since CCR5 is an important co-receptor during HIV infection, our findings may be used to generate immune cells resistant to HIV infection.

44. Novel Combination of megaTAL Nuclease-Driven Genome Engineering with a Drug Selection Cassette Increases Efficiency of HIV Gene Therapy

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Human Immunodeficiency Virus (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 represent an alternative therapeutic target. 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 improves on these advances by combining high level of CCR5 gene disruption with preferential selection of gene modified cells. The CCR5-targeting megaTAL combines a LAGLIDADG homing endonuclease scaffold with an eleven repeat transcription activator-like (TAL) effector array to achieve site specific DNA cleavage. This nuclease produces highly efficient CCR5 targeting in primary human CD4+ T cells *in vitro* (70-90% disruption). To test the protective effects of megaTAL treatment, primary human CD4+ T cells treated with CCR5-megaTAL were transplanted into NOD/SCID/γc-null (NSG) 'humanized' mice and 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. Based on the decline of CCR5 modified cells in the clinical trials to date, we hypothesized that we could improve maintenance of HIV resistant cells by expanding them either *ex vivo* or *in vivo*. We propose that coupling megaTAL nuclease treatment with drug selection will help us achieve therapeutically relevant levels of HIV-