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Homology-Independent Targeted Insertion (HITI) for Therapeutic T-Cell Engineering

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Commentary

In this commentary we discuss our recent work on delivering an anti-GD2 CAR (chimeric antigen receptor) via homology independent targeted insertion (HITI) using the CRISPR/Cas9 technology [1]. HITI relies on Non-Homologous End Joining (NHEJ) that is predominantly exploited by both dividing and non-dividing cells to repair double stranded DNA breaks (DSBs). We explore considerations when using HITI based strategies. Furthermore, we discuss a method for post-HITI **C**RISPR **E**nrich**MENT** (CEMENT) within the context of large-scale clinical manufacturing of non-viral CAR-T cells [2].

Transgene Insertion Strategies and Genome Editing Biology

NHEJ is the primary mechanism for DNA repair of DSBs throughout the course of the cell cycle program. Unlike homology directed repair (HDR), which is active only during the G2 and S phase of the cell cycle, NHEJ is independent from the cell cycle. Therefore, it presents a unique strategy for therapeutic editing of non-activated T-cells. Many groups have described the use of HDR for performing targeted genomic insertion of a CAR into a variety of loci including *TRAC, AAVS1, B2M,* or *PDCD1*, where the activation state of the T-cell is imperative for a successful insertion [3-5]. In contrast, HITI, which utilizes NHEJ for target gene insertion, can be explored in non-activated T-cells, thereby facilitating rapid manufacturing of CAR-T cells. Outside T-cell editing, HITI has been explored for applications of knock-in of large reporter genes in ESCs (Embryonic Stem Cells) and post-mitotic cells [6]. Most importantly, HITI has also been explored *in vivo* in rat models [7]. Recently, in addition to the NHEJ and HDR pathways, HMEJ (Homology Mediated End Joining) has also been explored for insertion of therapeutic CARs into T-cells. This approach relies on short homology arms (~48 bp), which has been shown to have more efficient integration of larger genetic cargo compared to HDR-based approaches [8].

Repair Template Designs

In the last decade various vectors for delivering transgenes using nuclease-based genome editors have been established (Adeno associated viruses, mRNA, transposons, single stranded and double stranded DNA and plasmid donor DNA vectors) [1]. Unlike viral vectors, manufacturing plasmid DNA from bacteria is relatively easy and low-cost. Therefore, plasmid DNA has recently been explored more for T cell therapy. We employed the Nanoplasmid backbone which is only 450bp. The use of Nanoplasmid DNA technology is desirable for T cell therapy applications as it has been reported to have higher expression levels and reduces the cell transfection related toxic effects in comparison to dsDNA templates [9]. Furthermore, the timeline for production of clinical grade Nanoplasmid vectors is anticipated to be shorter and less cost intense as compared to viral vector based on our experience with non-GMP grade nanoplasmid [8].

For HITI, we and others have demonstrated 1 cut site (cs) to yield in higher KI efficiencies as compared to 0cs and 2cs [2,7]. In turn, for HDR some groups have been able to insert at high efficiencies without employing any cut sites [5,10]. Alternatively, Chavez and colleagues reported simultaneous editing of the insertion site locus and cutting within their HDR template delivered via an integrase deficient lentiviral vector

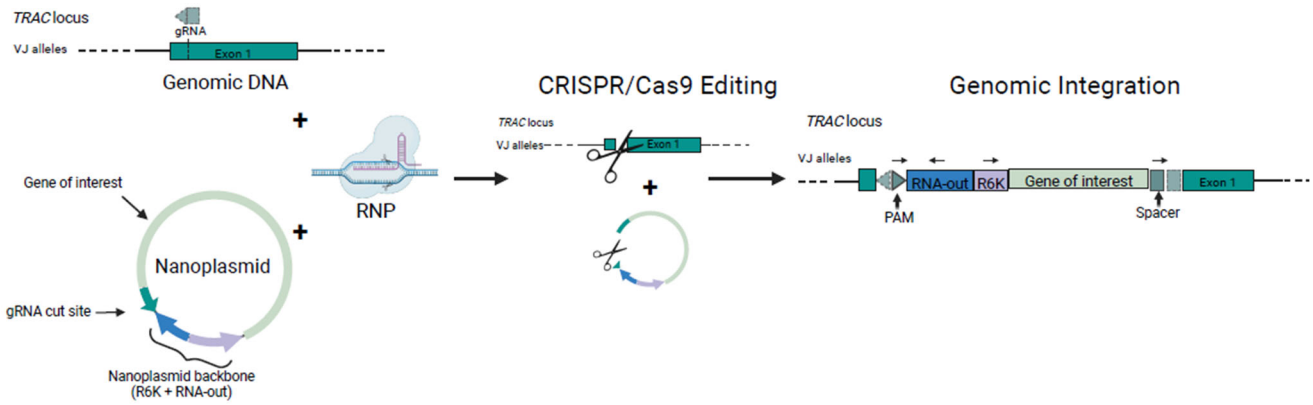

Figure 1. Schematic representation of homology independent targeted insertion via CRISPR/Cas9 editing. The guide RNA is designed to target a specific site within the genomic sequence. The nanoplasmid contains the gene of interest, with an arrow pointing to the site, which is an internal cut site where the Cas9 will make a cut. The process shown in the schema shows the simultaneous cutting of the TRAC locus and the Nanoplasmid DNA by the RNP complex, which results in the insertion of the transgene into the TRAC locus via a homology independent targeted insertion.

to enable targeted insertion of large transgenes. Here, they compared donor DNA with regular and truncated cut sites and determined that processing and linearization of donor DNA caused by the Cas9 RNP complex results in an enhanced knock-in efficiency [11].

CAR-T cell Enrichment Strategies

To achieve higher purity and CAR+ cell yield, enrichment methods to increase the target population needs to be considered. Early approaches in non-viral gene editing used feeder cells to aid the growth of the positively edited cell populations [12]. However, these methods are inconsistent and complex. Depending on the manufacturing feasibility and selection strategies, it can include metabolic selection through integration of a methothrexate (MTX) resistant version of the Dihydrofolate Reductase (DHFR-FS) or more complex methods through surface markers including, but not limited to tNGFR (truncated Low-affinity Nerve Growth Factor Receptor) or tEGFR (truncated Epidermal Growth Factor Receptor). We evaluated the DHFR-FS system versus separation column based tEGFR (truncated Epidermal Growth Factor Receptor) and tNGFR (truncated Low-affinity Nerve Growth Factor Receptor) based enrichment to compare the different approaches to selection of edited cells [13,14]. Importantly, we optimized the MTX treatment schedule and shortened the duration of MTX exposure. Our work demonstrates that by using the DHFR-FS system we can enable an efficient MTX-based enrichment of transgene positive cells and generate anti-GD2 CAR-T cells with up to ~80% purity. The DHFR-FS system offers a more controlled, consistent and scalable alternative and allows for an easier implementation into large-scale manufacturing process, whereas the use of cell surface selection markers requires additional cell processing and purification steps, which can significantly decrease the cell yield and increase manufacturing complexity and cost. Alternatively, transgene knock-in into essential gene loci has also been explored for enrichment of transgene positive populations [15]. Recently, others have explored the use of HDR repair templates containing a splice acceptor and splice donor targeting the intronic sequence of endogenous surface receptor loci, thereby enabling the magnetic depletion of unedited cell populations [16]. However, both technologies are limited to targeted insertion into essential genes and surface receptors respectively.

Strategies for Building Closed System Scale Up Systems

Using a semi-closed system electroporation protocol, we show that the co-delivery of the genome editing components can be performed in a clinically relevant context. We establish a modular manufacturing process using the Maxcyte GTx electroporation unit that generated 5.5 x 10^8 – 3.6 x 10^9 GD-2 CAR-T cells from a starting population of 5 x 10^8 T-cells in a G-REX 100M cell culture system, across 3 independent donors. The Maxcyte GTx, a clinically relevant electroporation platform, enables semi-closed or closed system flow-through electroporation. It can be aseptically connected to the G-REX (Wilson Wolf) platform, offering a highly efficient method for closed system scale-up. To edit T cells on the Maxcyte GTx, we used the preset Expanded T-Cell 4 protocol, which has been optimized for electroporation of T cells. However, one should consider optimization of parameters such as electroporation voltage, pulse width, cell concentration, DNA vector concentration, electroporation buffer parameters, Cas9:gRNA ratio, time of electroporation, temperature, and pre-electroporation and post-electroporation handling of the T-cells to allow for the most efficient editing process.

Investigating and Addressing Genotoxicity Events

As CRISPR/Cas9 therapies are more commonly being applied clinically, it is important to ensure their safe and effective use. CRISPR/Cas9 can cause unintended double-stranded breaks at off-target sites, leading to insertions, deletions, or translocations [17]. Careful selection of gRNA should involve an empirical design process. It's crucial to consider challenges like the presence of a PAM sequence near the target site, the gRNA's ability to form secondary structures, mismatch tolerance, delivery efficiency, and accessibility to chromatin. Therefore, the gRNA design process must incorporate *in silico*, and *in vitro* methods to screen gRNAs with efficient on-target and minimal to no off-target activity. The recent FDA approval of exagamglogene autotemcel (exa-cel) provides us with more insights into the standards that need to be established in the process [18]. We outline a roadmap for assessing and mitigating CRISPR/Cas9 genotoxicity events. When designing CRISPR/Cas9 gene editing therapies, it is important to consider early on mitigation strategies of off-target editing by carefully selecting and screening guides. We utilize *in silico* tools like COSMID (CRISPR Off-target Sites with Mismatches, Insertions, and Deletions) and CCTop (CRISPR/Cas9 Target Online Predictor) to exclude gRNAs with predicted high off-target effects [19,20]. We selected a gRNA targeting TRAC, encoding a mismatch base for optimal on-target performance as confirmed via GUIDE-Seq [21]. Sequencing approaches like GUIDE-seq, CIRCLE-seq, Discover-seq, Digenome-seq, and SITE-seq combine sequencing data and *in silico* analysis to identify off-target effects and should be considered for off-target assessment [22-26]. It is important to note that these tools rely on the human reference genome, and for a more comprehensive off-target nomination, tools like CRISPRme, which account for human genetic diversity and perform variant-aware off-target assessment, are essential [27]. Post editing, next-generation sequencing (e.g. rhAMP Seq) should be used as it allows for quantification and precise identification of off-target mutations induced by CRISPR/Cas9 by providing high-resolution sequencing data [28]. On-target editing outcomes could include Indels, long deletions/truncations, inversions, insertions, copy-neutral LOH (loss of heterozygosity), and chromothripsis [29]. While Indels are easier to detect, standard sequencing methods do not capture the full range of other outcomes. More complex methods, like long-read sequencing and single primer amplification, are required for comprehensive detection [29-31]. Additionally, on and off-target insertions can be assessed via Targeted Locus Amplification (TLA), which provides an unbiased way to assess insertion sites [32].

Previous reports have provided evidence of low-level chromosome 14 aneuploidy due to the editing of the TRAC locus by CRISPR/Cas9 using a clinically relevant gRNA [33]. Therefore, monitoring chromosomal translocations utilizing ddPCR (droplet digital PCR) over time should be considered for long-term patient follow up assessments. Tsuchida et al. conducted a systematic analysis of Cas9-induced chromosome loss events and recommended protocol adjustments to reduce the occurrence of chromosome loss [34]. These improvements include activating/stimulating T-cells after delivering genome editing components into non-activated T-cells, which helps mitigate aneuploidy linked to elevated TP53 expression. This effect may be reduced utilizing the HITI method and provides further rationale for exploring HITI as a T-cell engineering platform.

To conclude, HITI in non-activated T-cells has its advantages, as it can streamline and accelerate the manufacturing process. Importantly, editing non-activated T-cells using HITI may also enhance the safety profile of the engineered T-cells.

References

1. Balke-Want H, Keerthi V, Cadinanos-Garai A, Fowler C, Gkitsas N, Brown AK, et al. Non-viral chimeric antigen receptor (CAR) T cells going viral. Immuno-Oncology and Technology. 2023 Jun 1;18:100375.

2. Balke-Want H, Keerthi V, Gkitsas N, Mancini AG, Kurgan GL, Fowler C, et al. Homology-independent targeted insertion (HITI) enables guided CAR knock-in and efficient clinical scale CAR-T cell manufacturing. Molecular Cancer. 2023 Jun 26;22(1):100.

3. Eyquem J, Mansilla-Soto J, Giavridis T, van der Stegen SJ, Hamieh M, Cunanan KM, et al. Targeting a CAR to the TRAC locus with CRISPR/Cas9 enhances tumour rejection. Nature. 2017 Mar 2;543(7643):113-7.

4. Zhang J, Hu Y, Yang J, Li W, Zhang M, Wang Q, et al. Non-viral, specifically targeted CAR-T cells achieve high safety and efficacy in B-NHL. Nature. 2022 Sep 8;609(7926):369-74.

5. Oh SA, Senger K, Madireddi S, Akhmetzyanova I, Ishizuka IE, Tarighat S, et al. High-efficiency nonviral CRISPR/Cas9-mediated gene editing of human T cells using plasmid donor DNA. Journal of Experimental Medicine. 2022 May 2;219(5).

6. Kelly JJ, Saee-Marand M, Nyström NN, Evans MM, Chen Y, Martinez FM, et al. Safe harbor-targeted CRISPR-Cas9 homology-independent targeted integration for multimodality reporter gene-based cell tracking. Science Advances. 2021 Jan 20;7(4):eabc3791.

7. Suzuki K, Tsunekawa Y, Hernandez-Benitez R, Wu J, Zhu J, Kim EJ, et al. In vivo genome editing via CRISPR/Cas9 mediated homology-independent targeted integration. Nature. 2016 Dec 1;540(7631):144-9.

8. Webber BR, Johnson MJ, Skeate JG, Slipek NJ, Lahr WS, DeFeo AP, et al. Cas9-induced targeted integration of large DNA payloads in primary human T cells via homology-mediated end-joining DNA repair. Nature Biomedical Engineering. 2023 Dec 13:1-8.

9. Williams JA, Paez PA. Improving Cell and Gene Therapy Safety and Performance using Next-Generation NanoplasmidTM Vectors. Molecular Therapy-Nucleic Acids. 2023 Apr 7;32:494-503.

10. Foy SP, Jacoby K, Bota DA, Hunter T, Pan Z, Stawiski E, et al. Non-viral precision T cell receptor replacement for personalized cell therapy. Nature. 2023 Mar 23;615(7953):687-96.

11. Chavez M, Rane DA, Chen X, Qi LS. Stable expression of large transgenes via the knock-in of an integrase-deficient lentivirus. Nature Biomedical Engineering. 2023 May;7(5):661-71.

12. Kebriaei P, Singh H, Huls MH, Figliola MJ, Bassett R, Olivares S, et al. Phase I trials using Sleeping Beauty to generate CD19-specific CAR T cells. The Journal of clinical Investigation. 2016 Sep 1;126(9):3363-76.

13. Bonini C, Grez M, Traversari C, Ciceri F, Marktel S, Ferrari G, et al. Safety of retroviral gene marking with a truncated NGF receptor. Nature Medicine. 2003 Apr 1;9(4):367-9.

14. Paul B, Ibarra GS, Hubbard N, Einhaus T, Astrakhan A, Rawlings DJ, et al. Efficient enrichment of gene-modified primary T cells via CCR5-targeted integration of mutant dihydrofolate reductase. Molecular Therapy-Methods & Clinical Development. 2018 Jun 15;9:347-57.

15. Allen AG, Khan SQ, Margulies CM, Viswanathan R, Lele S, Blaha L, et al. A highly efficient transgene knock-in technology in clinically relevant cell types. Nature Biotechnology. 2024 Mar;42(3):458-69.

16. Chang CR, Vykunta VS, Goodman DB, Muldoon JJ, Nyberg WA, Liu C, et al. Ultra-high efficiency T cell reprogramming at multiple loci with SEED-Selection. bioRxiv. 2024:2024-02.

17. Kosicki M, Tomberg K, Bradley A. Repair of double-strand breaks induced by CRISPR–Cas9 leads to large deletions and complex rearrangements. Nature Biotechnology. 2018 Sep;36(8):765-71.

18. US FDA. Cellular, Tissue, and Gene Therapies Advisory Committee October 31, 2023 Meeting Announcement. https://www.fda.gov/advisory-committees/advisory-committee-calendar/cellular-tissue-and-gene-therapies-advisory-committee-october-31-2023-meeting-announcement-10312023

19. Stemmer M, Thumberger T, del Sol Keyer M, Wittbrodt J, Mateo JL. CCTop: an intuitive, flexible and reliable CRISPR/Cas9 target prediction tool. PloS One. 2015 Apr 24;10(4):e0124633.

20. Cradick TJ, Qiu P, Lee CM, Fine EJ, Bao G. COSMID: a web-based tool for identifying and validating CRISPR/Cas off-target sites. Molecular Therapy-Nucleic Acids. 2014 Dec 2;3(12):e214.

21. Kath J, Du W, Pruene A, Braun T, Thommandru B, Turk R, et al. Pharmacological interventions enhance virus-free generation of TRAC-replaced CAR T cells. Molecular Therapy-Methods & Clinical Development. 2022 Jun 9;25:311-30.

22. Tsai SQ, Zheng Z, Nguyen NT, Liebers M, Topkar VV, Thapar V, et al. GUIDE-seq enables genome-wide profiling of off-target cleavage by CRISPR-Cas nucleases. Nature Biotechnology. 2015 Feb;33(2):187-97.

23. Tsai SQ, Nguyen NT, Malagon-Lopez J, Topkar VV, Aryee MJ, Joung JK. CIRCLE-seq: a highly sensitive in vitro screen for genome-wide CRISPR–Cas9 nuclease off-targets. Nature Methods. 2017 Jun;14(6):607-14.

24. Wienert B, Wyman SK, Richardson CD, Yeh CD, Akcakaya P, Porritt MJ, et al. Unbiased detection of CRISPR off-targets in vivo using DISCOVER-Seq. Science. 2019 Apr 19;364(6437):286-9.

25. Kim D, Bae S, Park J, Kim E, Kim S, Yu HR, et al. Digenome-seq: genome-wide profiling of CRISPR-Cas9 off-target effects in human cells. Nature Methods. 2015 Mar;12(3):237-43.

26. Cameron P, Fuller CK, Donohoue PD, Jones BN, Thompson MS, Carter MM, et al. Mapping the genomic landscape of CRISPR–Cas9 cleavage. Nature Methods. 2017 Jun;14(6):600-6.

27. Cancellieri S, Zeng J, Lin LY, Tognon M, Nguyen MA, Lin J, et al. Human genetic diversity alters off-target outcomes of therapeutic gene editing. Nature Genetics. 2023 Jan;55(1):34-43.

28. Kurgan G, Turk R, Li H, Roberts N, Rettig GR, Jacobi AM, et al. CRISPAltRations: a validated cloud-based approach for interrogation of double-strand break repair mediated by CRISPR genome editing. Molecular Therapy-Methods & Clinical Development. 2021 Jun 11;21:478-91.

29. Hunt JM, Samson CA, Rand AD, Sheppard HM. Unintended CRISPR-Cas9 editing outcomes: A review of the detection and prevalence of structural variants generated by gene-editing in human cells. Human Genetics. 2023 Jun;142(6):705-20.

30. Kosicki M, Tomberg K, Bradley A. Repair of double-strand breaks induced by CRISPR–Cas9 leads to large deletions and complex rearrangements. Nature biotechnology. 2018 Sep;36(8):765-71.

31. Park SH, Cao M, Pan Y, Davis TH, Saxena L, Deshmukh H, et al. Comprehensive analysis and accurate quantification of unintended large gene modifications induced by CRISPR-Cas9 gene editing. Science Advances. 2022 Oct 21;8(42):eabo7676.

32. De Vree PJ, De Wit E, Yilmaz M, Van De Heijning M, Klous P, Verstegen MJ, et al. Targeted sequencing by proximity ligation for comprehensive variant detection and local haplotyping. Nature biotechnology. 2014 Oct;32(10):1019-25.

33. Nahmad AD, Reuveni E, Goldschmidt E, Tenne T, Liberman M, Horovitz-Fried M, et al. Frequent aneuploidy in primary human T cells after CRISPR–Cas9 cleavage. Nature Biotechnology. 2022 Dec;40(12):1807-13.

34. Tsuchida CA, Brandes N, Bueno R, Trinidad M, Mazumder T, Yu B, et al. Mitigation of chromosome loss in clinical CRISPR-Cas9-engineered T cells. Cell. 2023 Oct 12;186(21):4567-82.