CRISPR-Cas9 CAR knock-in in primary T cells

Abstract

The ever-evolving cancer research landscape has led to the use of T cells, and more specifically chimeric antigen receptor (CAR)-T cells, to target cancer cells, determine their phenotype along with the avidity of tumor specific cell receptors, and ultimately find ways to optimize their immune response [1]. This is a promising new technology that when coupled with CRISPR gene editing technology has rising potential. These CAR-T cells are engineered to contain the CAR sequence in the genome and express the CAR protein on the cell surface. CRISPR is used here to place the CAR gene in a specific location of the T cell genome and can be used to edit the existing T cell receptor gene via insertion of the tumor-targeting CAR gene, resulting in cells with enhanced anti-tumor activity. The result is increased CAR expression along with improved anti-tumor activity [2].

In a laboratory setting, scientists need workflows for integrating large transgenes into the genome with a targeted non-random method like CRISPR-mediated homology directed repair (HDR) [3]. As CAR-T cell therapy is growing, there is a critical need for improved workflows for high-efficiency HDR, particularly in primary T lymphocytes. IDT has optimized methods for high-efficiency CRISPR-Cas9-mediated HDR for engineering CAR-expressing primary T cells. This workflow features reagents and methods for primary T cell handling, activation, and transfection timepoints optimized for electroporation of CRISPR reagents. Since HDR is so tightly tied to cell cycle, proper timing of cell handling is essential to achieving high knock-in rates. Alt-R™ CRISPR-Cas9 sgRNA from IDT, Cas9 mRNA and Nanoplasmid HDR templates from Aldevron®, and products from other commercial sources can be used together to achieve up to approximately 20% CAR knock-in.

Introduction

Cancer research has come a long way in providing insight into the best way to approach the disease, however current options available are constrained by non-specific targeting as well as extensive side effects including the destruction of healthy cells. The search has been on for a more localized approach that would allow for more precise cancer cell recognition and more directed cellular focus. This is where the use of T cells has come in.

T cells play a major role in cellular immunity and immune-mediated responses. Immune regulation and T cell biology, including cytokine regulation, are all important factors to consider when developing the best methods for triggering a T cell attack on a cancer cell and determining how to promote a focused immune response [4]. T cell receptors must go through a checkpoint screening before they are released from the thymus upon maturing. At this point, thymocytes that could attack healthy cells are removed and those that could help fight off foreign cells mature and move to the spleen or lymph nodes. In addition, T cells recognize an antigen and activate a cascade of cellular events that recruit cells like natural killer (NK) cells, neutrophils, and macrophages that can take part in coming together to destroy unwanted cells in the body. All these factors are important when using CAR-T cells as a potential way to target cancer cells.

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The T cell receptors are protein complexes located on the surface of a T cell and their main role is to recognize a distinctive antigen, such as a foreign cancer antigen. When this antigen is detected, a signal transduction pathway is initiated, resulting in T lymphocyte activation triggering a specific immune response. This T cell receptor protein complex is often composed of an alpha (α) and a beta (β) chain, so the strategy most often used for CRISPR editing in T cells is to utilize the alpha region of the receptor or the T cell receptor (TCR) alpha constant chain (*TRAC*) gene locus. A chimeric antigen receptor (CAR) is introduced into the cells at the *TRAC* gene locus using CRISPR technology. This approach has been effective in preventing excessive expression, allows for control of site integration, and permits monitoring of off-target effects [2]. The *TRAC* region contains the endogenous T cell receptor gene and CRISPR editing can be used to modify the existing T cell receptor gene via insertion of a tumor-targeting CAR gene. This has been shown to result in not only improved CAR expression but also enhanced anti-tumor activity [2,8].

To target a specific antigen, CAR-T cells are engineered to express a recombinant gene for CAR by combining both an antigen-binding and a T cell activating function into one single receptor and creating an artificial T cell receptor. This receptor recognizes a specific antigen and allows it to target cancer cells more exclusively. These recombinant CARs are comprised of four domains: (1) an extracellular domain with the antigen recognition/binding region; (2) a hinge or spacer region; (3) a transmembrane domain; and (3) the intracellular domain that has three immunoreceptor tyrosine-based activation motifs (ITAMs), costimulatory molecules (CM1), and an interleukin-12 (IL-12) domain that stimulates the innate immune system (Figure 1) [2,7]. This structure allows CAR-T cells to recognize antigens on the cancer cells which trigger the cascade of cellular events mentioned above and allows for the destruction of these cells using the host's own immune system.

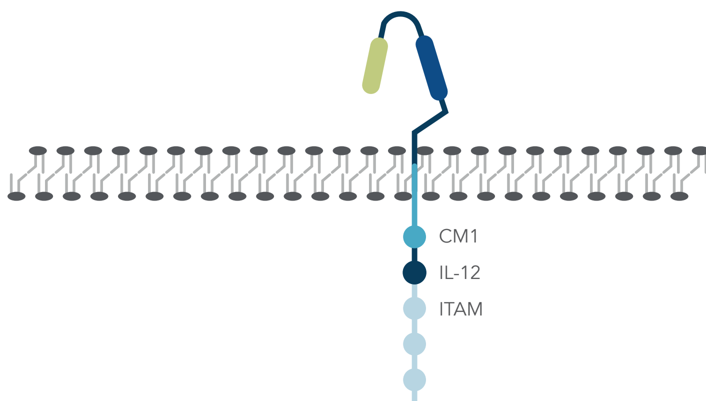

Figure 1. Recombinant CARs contain four domains. A CAR has an extracellular antigen recognition site, hinge, transmembrane domain, and an intracellular domain. The extracellular domain has a single-chain variable domain that recognizes the tumor cell. Although the intracellular domains have evolved, the fourth generation of receptors contains a costimulatory molecule (CM1) domain that improves proliferation and sustained response. The interleukin-12 (IL-12) domain attracts and activates innate immune cells. Finally, the three immunoreceptor tyrosine-based activation motifs (ITAMs) are phosphorylated by a tyrosine kinase upon antigen binding, thus activating the intracellular signaling pathways associated with T cell attack on the cancer cell.

The potential for using CAR-T cells to target specific cells is there, but the knock-in of CAR constructs into primary T cells is often inefficient. This is where CRISPR technology can be used to precisely edit the genome of T cells to enhance their effectiveness in targeting cancer cells. This can be done by modifying specific genes involved in T cell function by removing inhibitory receptors such as PD-1 or introducing specific mutations to enhance T cell activation and persistence. In doing so, researchers can potentially improve the potency and durability of CAR-T cell targeting. CRISPR technology can also be employed to minimize off-target effects and enhance the safety of CAR-T cell methods. By using CRISPR-based methods to screen and validate potential off-target sites, researchers can reduce the risk of unintended genetic alterations and improve the specificity of CAR-T cell targeting.

Advantages of this approach include:

1. Enhanced targeting—CAR-T cells can more precisely target cancer cells only, reducing side effects caused by collateral damage to healthy cells. In addition, CRISPR has been used to modify CAR-T cells to recognize more than one antigen at once so that it can target them simultaneously. This has been shown to be beneficial in avoiding antigen escape or immune evasion, certain types of toxicity, and on-target tissue damage [5]. In addition, this can allow CAR-T cells to be designed to recognize heterogeneous tumors [5-7].
2. Potency—CAR-T cells are highly effective at recognizing and eliminating cancer cells, and they can persist in the body for an extended period of time to prolong their effects. By taking advantage of editing genes involved in T cell exhaustion or apoptosis CRISPR is being used to attempt to extend the survival and activity of CAR-T cells within the tumor microenvironment [6,7].
3. Reduced adverse results—Off-target effects, adverse immune reactions, and uncontrolled proliferation can occur with CAR-T cells due to their role in the immune system. By utilizing CRISPR-mediated genome editing these effects can be reduced and/or eliminated with properly designed and optimized CAR-T modifications [7].

Top-of-the-line reagents and workflows for generating CAR-T cells are vital to the success of this new field. IDT is a leading provider of products, protocols, and scientific support for genome editing. Further, Alt-R CRISPR-Cas9 gRNAs, nucleases, and HDR Enhancers are demonstrably efficient for genome editing in primary T cells as discussed in this webinar Optimized methods for CRISPR editing in primary T cells. The scientists at IDT worked to optimize the use of CRISPR-mediated editing in primary T cells looking at activation methods (soluble and bound), duration of activation, electroporation parameters, use of electroporation enhancer, optimal ribonucleoprotein (RNP) dose, use of a HDR enhancer for HDR experiments, as well as the length of the homology arm for HDR. They discovered that many activation methods can result in successful non-homologous end-joining (NHEJ) editing, the optimal dose of RNP for CRISPR editing in primary cells is between 2 and 4 µM, and that the editing efficiency is increased with the Alt-R Electroporation Enhancer. For HDR, it is important to optimize Cas9 and the electroporation parameters for each experiment. There are several important parameters to take into consideration when planning these experiments. Alt-R HDR donor blocks improve HDR efficiency and using blocking mutations in the donor sequence prevents Cas9 re-cleavage. Small molecules that modulate the DSB pathway also increase HDR efficiency, so using the Alt-R HDR enhancer V2 improves performance and allows for efficient, low toxicity knock-ins up to 2 kb when design is optimized. The WT and HiFi Cas9 nucleases offered by IDT have both been shown to be readily taken up by human primary T-cells while also resulting in high editing efficiency at several different genomic loci (Figure 2).

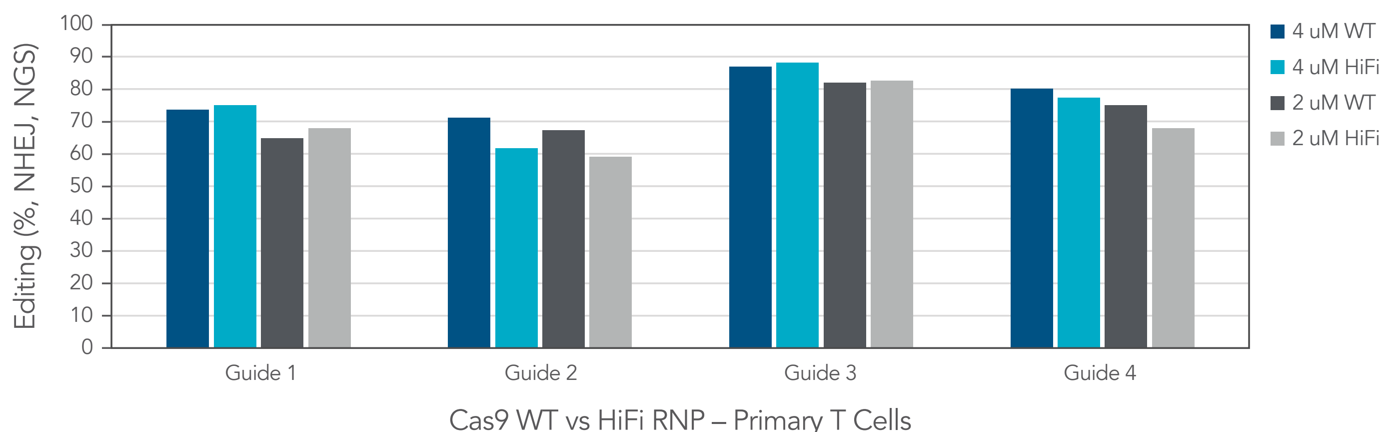

Figure 2. In primary T cells, wild type (Alt-R S.p. Cas9 Nuclease V3) and high-fidelity (Alt-R S.p. HiFi Cas9 Nuclease V3) Cas9 nucleases deliver high editing efficiency. Four highly modified Alt-R CRISPR-Cas9 sgRNAs targeting individual genomic loci were complexed at a 1.2:1 sgRNA:Protein ratio with either Alt-R S.p. Cas9 Nuclease V3 *or* Alt-R S.p. HiFi Cas9 Nuclease V3. Following activation and a 48-hour incubation, single donor primary T cells (Stemcell Technologies) were transfected with either 2 or 4 µM RNP alongside 4 µM Electroporation Enhancer using the Lonza 4D-Nucleofector® system. Following another 48-hour incubation period, genomic DNA was harvested and targeted amplicon sequencing was performed using the rhAmpSeq CRISPR Libraries Kit. Libraries were sequenced using the Illumina® MiSeq platform, and editing rates were analyzed via an internal version of the rhAmpSeq CRISPR Analysis system. *n* = 1 transfection per condition.

Having shown that CRISPR editing had been optimized for both NHEJ and HDR using primary T cells in the lab at IDT, efforts then moved to development of a protocol for CAR knock-in in primary T cells. This optimized protocol is as follows.

Methods

Frozen human primary T cells (Stemcell™ Technologies) were thawed into Immunocult™-XF T cell expansion medium containing 300 IU IL-2 (Cytiva) and 10 µL/mL TransAct™ T cell activator (Miltenyi Biotec) into a 6 well G-rex® gas-permeable plate (WilsonWolf) at a density of 1e6 cells/mL. Two biological replicates were used and carried through to the end of the experiment. Cells were incubated at 37 °C for 48 hours. For electroporation, a 96-well Lonza Nucleofector® Shuttle was used following the appropriate protocol. Briefly, cells were counted, pelleted, washed in PBS, and pelleted again. Pellets were resuspended in P3 buffer with 5e5 cells in 20 µL buffer per nucleofection. Cas9 mRNA (Aldevron) was added at 4 µg per nucleofection, along with 3.3 µg Alt-R CRISPR-Cas9 sgRNA and 1, 2, or 3 µg anti-CD19 CAR Nanoplasmid for inserting CAR at the *TRAC* locus. For comparison purposes, 50 µM of an HDR Enhancer Protein (new product coming soon) was also added to half of the reactions while no enhancer protein was used in the comparative reaction. Each reaction was nucleofected using the Lonza program FI-115. Immediately following nucleofection, cells were allowed to recover in the nucleocuvette plate at 37 °C for 20 minutes before being transferred to a 96-well tissue culture plate containing warmed IL-2 media with 10 µL/mL TransAct™ T cell activator for reactivation. Cells were allowed to grow for 72 hours before lysis with QuickExtract™ (Lucigen). Crude lysates were used for amplification of the *TRAC* region with KOD Xtreme™ polymerase (Millepore), and HDR was quantified as a ratio of the CAR insert-containing amplicon to the WT amplicon size.

Results and discussion

CD19 is a common antigen on the surface of cells associated with lymphomas and leukemias [5]. CAR-T cells targeting CD19 represent many of the methods that currently utilize CAR-Technology. High efficiency knock-in of CD19-targeting CAR into the *TRAC* locus is necessary to generate these cells and for this method to be useful.

In this experiment, CRISPR reagents were delivered into primary T cells to insert an anti-CD19 CAR construct into the *TRAC* locus. Across both biological donors, the optimal concentration of the Nanoplasmid HDR template was 2 µg which reached 22% integration. The other tested doses also reached high levels of knock-in at 20 and 21% for 1 and 3 µg, respectively. Including an HDR Enhancer Protein further increased the level of knock-in for the 2 µg and 3 µg doses tested.

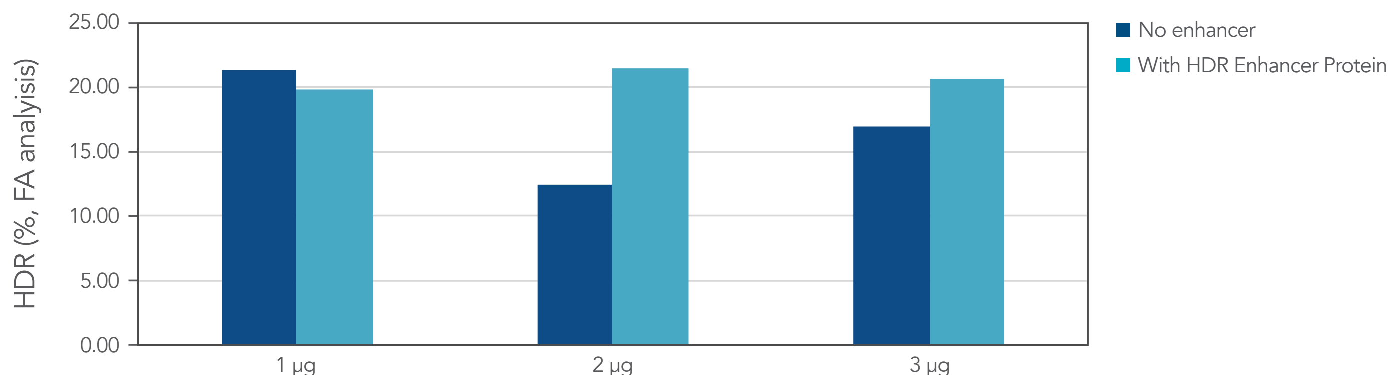

Figure 3. Knock-in of anti-CD19 CAR at the TRAC *locus* in primary T cells. Cas9 mRNA (Aldevron) was added at 4 µg per nucleofection, along with 3.3 µg Alt-R CRISPR-Cas9 sgRNA and 1, 2, or 3 µg anti-CD19 CAR Nanoplasmid for inserting CAR at the *TRAC* locus. Following activation and a 48-hour incubation, primary T-cells (Stemcell Technologies) were transfected with this RNP alongside 50 µM HDR Enhancer Protein using the Lonza 4D-Nucleofector® system. Following a 72-hour incubation period, genomic DNA was harvested, and crude lysate was used for amplification of the TRAC region. n = 1 transfection per condition. HDR was quantified as a ratio of the CAR insert-containing amplicon to the WT amplicon size.

Conclusions

Primary T cells are a challenging environment for genome editing, and precise optimization of protocols is required for success. Similarly, targeted knock-in of large inserts is a difficult bottleneck to overcome for many scientists in laboratory settings. The workflow described here is designed to increase the efficiency of targeted knock-in of a 2 kb anti-CD19 CAR construct into primary T cells to support scientists working in the field of CAR-T cell therapy.

The following is the optimized protocol:

- Thaw and activate cells in ImmunoCult media + 350 IU IL-2 in G-Rex 6 well plates
- Keep cells at 1e6/mL density
- Activate 48hrs with Miltenyi TransAct activator (10uL/mL)
- 4 ug ALD Cas9 mRNA, 4 uM sgRNA, 2 µg Nanoplasmid, 50 µM HDR Enhancer Protein
- Lonza program 96-FI-115
- Let cells recover in cuvette plate immediately post-nucleofection for 10 minutes
- Plate cells at 0.8e6/mL density
- Incubate in IL2 media + 10 µL/mL TransAct activator for 72 hrs

Many DSB repair modulators have been described in recent literature, though few have been shown to be effective at increasing HDR rates. The HDR Enhancer Protein used here is demonstrably effective in immortalized cell lines and is shown here to improve the rate of CAR knock-in in primary T cells.

These methods and reagents used together create a complete workflow for using CRISPR to mediate CAR knock-in. With improved protocols for efficiency of cell engineering, CAR-T cell methods can continue to improve and provide important advancements in cancer research.

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