APPLICATION NOTE

High-efficiency endogenous TCR knock-out and functional validation of CD8+ CAR T-cells.

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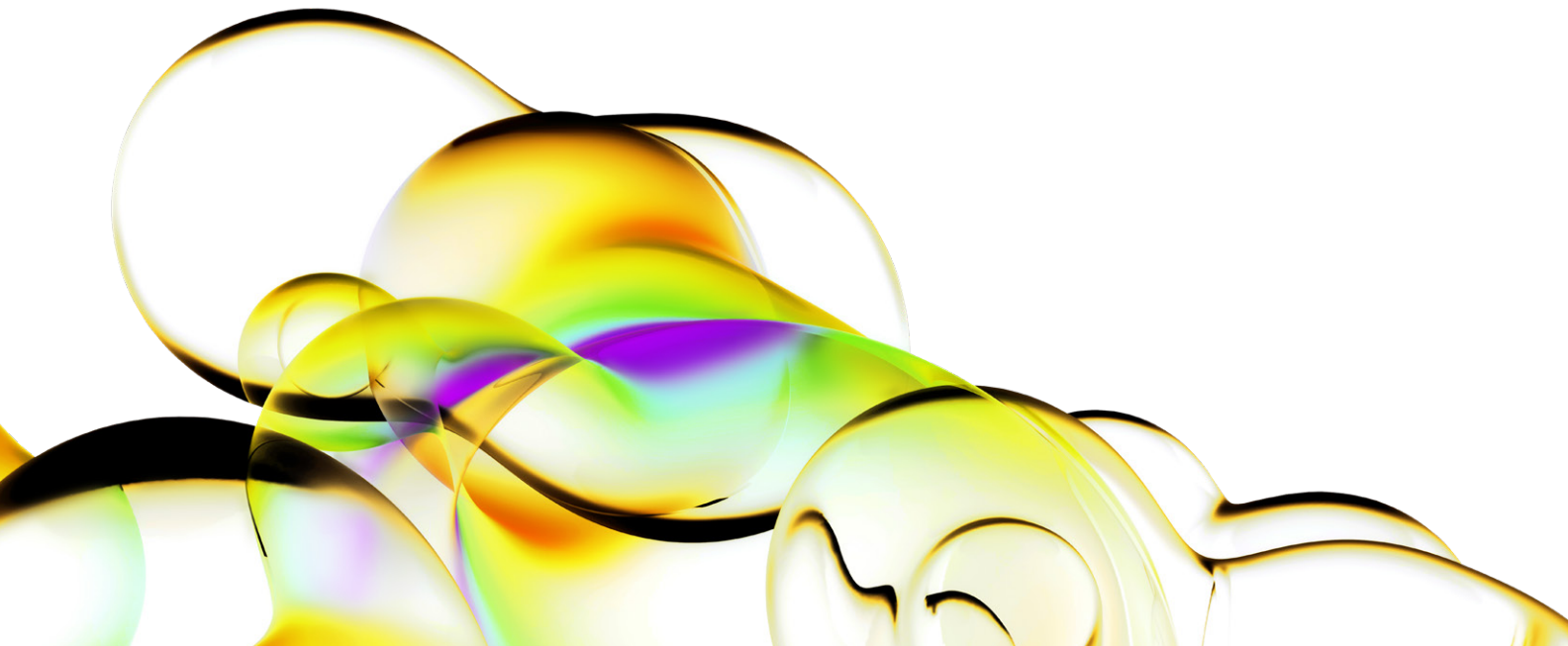
Introduction

In recent clinical advancements, primary immune cells are engineered with chimeric antigen receptors (CARs) to escalate immune responses against specific disease-associated antigens. Immune cells that can be engineered with a CAR can be isolated from many different sources: primary T or Natural Killer T (NKT) cells isolated from donor whole blood from commercial cell repositories, T cells differentiated from induced-pluripotent stem cells (iPSCs), or cancer cell lines that are banked and available for purchase (i.e. Jurkat from ATCC).

However, these cell types (i.e., $CD4^+$, $CD8^+$, NKT cells, etc.), encode and express a native antigen-specific receptor. Each T cell receptor (TCR) is encoded by a unique rearrangement of variable and constant region segments, that undergo multiple genomic shuffling events. TCRs expressed by the T-cells are selected for survival, reassortment, or apoptosis while developing in the thymus to generate a large repertoire of *foreign/aberrant*-antigen recognition for the host. There are two types of TCR: TCR α/β and TCR γ/δ.

The TCR α/β that is present on >90% of T lymphocytes is comprised of an alpha chain that is paired with a beta chain. The alpha chain is formed by the joining of unique variable, and junction (VJ) regions that are spliced at the RNA transcript level to the T cell receptor alpha constant (TRAC) sequence. Similarly, the beta chain is comprised of unique V, diversity (D), and J sequences that are spliced to one of the two T cell receptor beta constant regions (TRBC1 or TRBC2), which are highly homologous.



A very small fraction of T lymphocytes (between 0.5-5%) express a different TCR, the TCR γ/δ. Similar re-arrangements as described above occur to comprise the TCR γ/δ, where the most conserved portion of the delta chain is the delta constant (TRDC) region, and for the paired gamma chain there are two gamma constants (TRGC1 or TRGC2). The natural diversity of these genes to generate a unique TCR inventory in a host coupled with their noncanonical processing into mRNA transcripts, has led to difficulties in efficient automated CRISPR guide designs. However, the most conserved portions of the TCR α/β and TCR γ/δ heterodimers, the constant genomic regions (*TRAC, TRBC, TRDC,* and *TRGC*), have potential to be targeted by highly efficient and specific gRNAs.

To validate high efficiency single guide designs for the *TRAC, TRBC, TRDC,* and *TRGC* genomic regions, PAM sequences were first identified in these genomic loci (Figure 1). All potential spacer sequences for CRISPR-spCas9 were assessed for specificity by the Dharmacon™ Edit-R™ algorithm. An additional requirement for conserved spacer sequences was needed for simultaneous targeting of the *TRBC1* and *TRBC2*, as well as the *TRGC1* and *TRGC2* regions, so that the TRBC or TRGC guide designs would target both constant regions. In total, 45 TCR-targeting guide designs were screened for editing efficiency in primary T-cells from two donors. The best performing guides, 4 TRAC, 5 TRBC and 4 TRDC guides are available as pre-designed validated Edit-R synthetic sgRNAs.

Here, we describe a workflow where isolated primary CD8⁺ T-cells were engineered with an anti-human CD19 CAR lentivirus, after high-efficiency knock-out of the endogenous TCR α/β utilizing the top performing Edit-R TRAC or TRBC synthetic sgRNAs. These TCR-negative (TCR-) CD8⁺ CAR T cells were functionally characterized using flow cytometry to demonstrate specificity of the CAR for CD19⁺ cells, followed by screening for pro-inflammatory cytokine release using the LEGENDplex™ Human CD8/NK panel by BioLegend and the HTRF Human IFN gamma kit by Revvity.

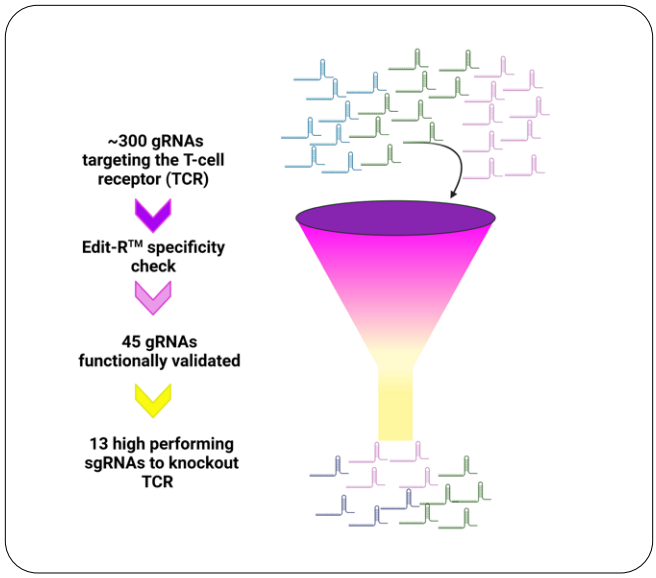
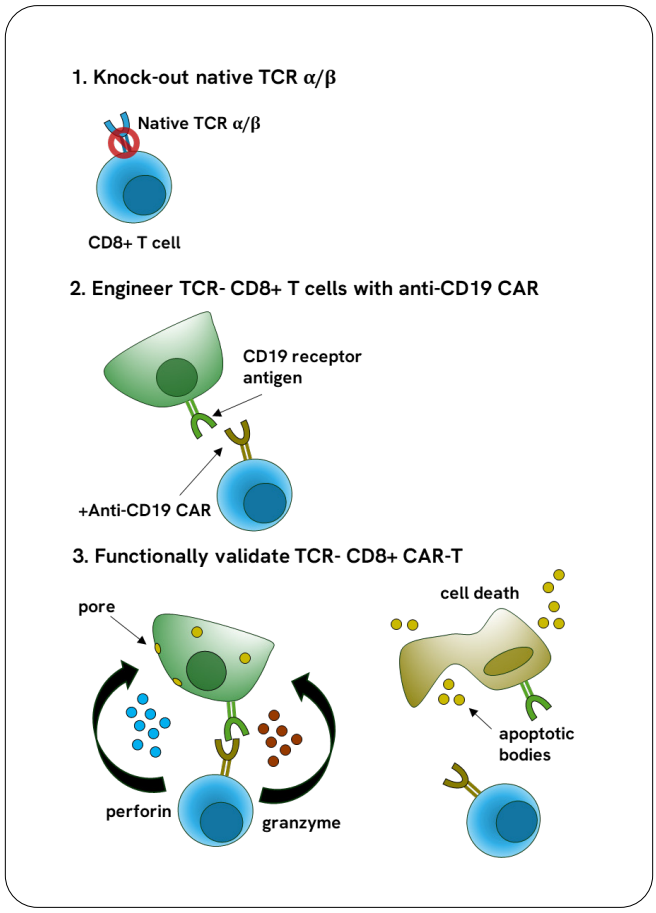


Figure 1: T-cell receptor guide validation pipeline. Created with BioRender.com



Results

Efficient targeted knock-out of endogenous TCR α/β on primary CD8⁺ T-cells

To generate high-quality primary CD8⁺ CAR T-cells, the endogenous TCR α/β was knocked-out with ribonucleoprotein (RNP)-based CRISPR-spCas9 editing, utilizing the best performing TRAC and TRBC targeting pre-designed sgRNAs. To achieve high-efficiency RNP-delivery of Edit-R TCR-targeting sgRNAs, follow the detailed protocol for RNP-based editing in primary cells on our website. Briefly, primary CD8⁺ T-cells were stimulated/activated for 72 hours using Human T-Activator CD3/CD28 Gibco™ Dynabeads + 200 UI/mL IL-2 + 15 ng/mL IL-15 in GM-3 (Lonza, Cat# CC-3211) medium, prior to RNP electroporation. Cells were restimulated every 10 days to maintain activation status. Cells were grown at a concentration between $0.5x10^6$-$2.0x10^6$ cells/mL in LGM3 media with fresh 50 UI/mL IL-2 added with each cell maintenance split (every 2-3 days).

Individually, the top performing Edit-R TRAC sgRNAs and Edit-R TRBC sgRNAs were complexed with Cas9-NLS (CAS1220X) protein at a 2:1 ratio. RNP complexes were electroporated into primary CD8⁺ T-cells in duplicate using the Lonza Bioscience P3 Primary Cell 96-well Nucleofector Kit and program EO-115. 72 hours after RNP-nucleofection, the TRAC-edited and TRBC-edited CD8⁺ T-cells were assessed for endogenous TCR α/β expression by flow cytometry using PE anti-human TCR α/β antibody from BioLegend (Cat# 984702). The two individual TCR-edited CD8⁺ T-cells each achieved >98% TCR α/β knock-out (Figure 2). High-efficiency TCR knock-out is critical for preserving primary cell numbers and minimizing cell handling prior to engineering the CD8⁺ T-cells with a lentiviral CAR.

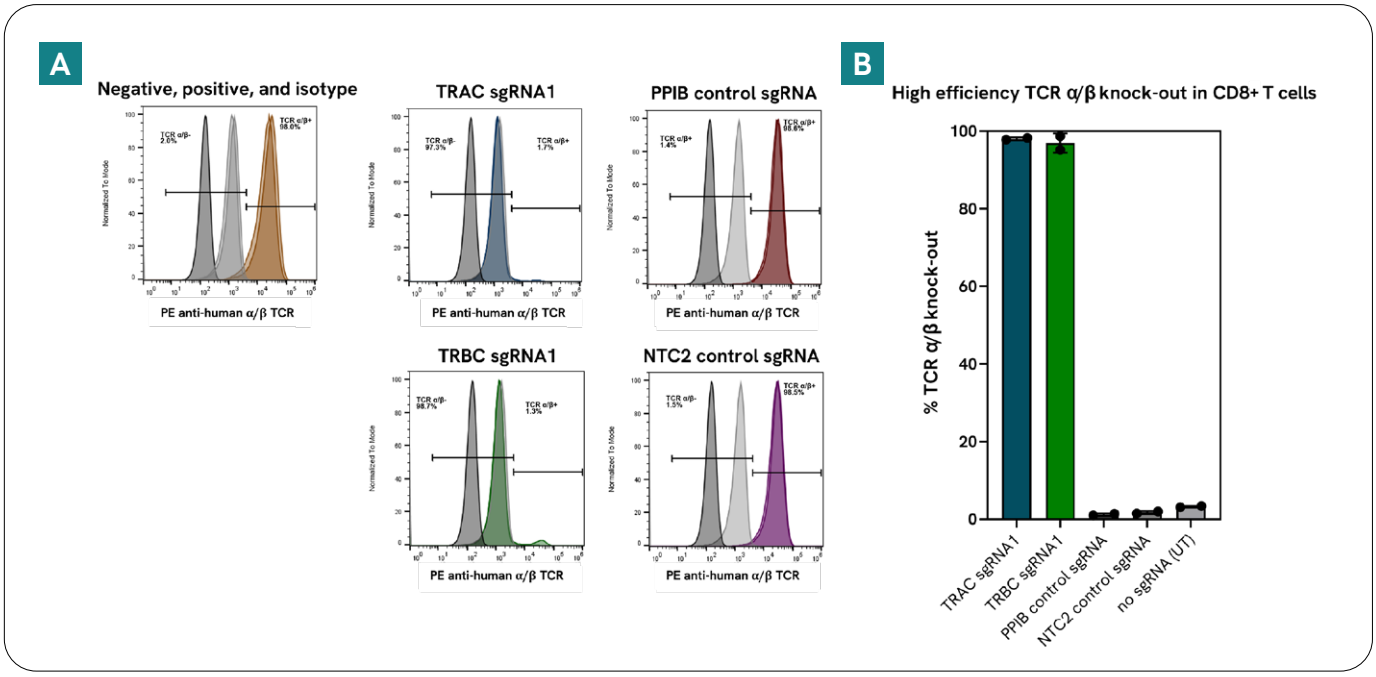


Figure 2: High editing efficiency in primary CD8⁺ T cells with validated guides. (A) Edit-R PPIB positive control, non-targeting control 2 (NTC2), TRAC and TRBC sgRNA-edited primary CD8⁺ T-cells were assessed for TCR α/β knock-out phenotype by flow cytometry at 72 hours. Displayed in overlapping histograms are Zombie Green™ Dye-negative (living) unedited CD8⁺ T-cells unstained (black), isotype control-stained (gray), or PE anti-human TCR α/β-stained cells (orange). The TCR-edited CD8⁺ T-cells were stained with anti-human TCR α/β antibody (blue, green, red, or purple traces in histograms). First, cells were stained 1:400 with the Zombie Green Fixable Viability kit. Then all cells were incubated with a 1:50 dilution of Human TruStain FcX™ (Fc Receptor Blocking Solution) to reduce specific antibody staining background. Finally, cells were stained with a 1:50 concentration of PE anti-human TCR α/β antibody or corresponding PE IgG1κ isotype antibody. (B) Bar graphs quantifying the % TCR α/β phenotypic knock-out on primary CD8⁺ T-cells (n=2, +/- SD) as measured by flow cytometry.

Engineering TCR α/β-negative CD8+ T-cells with anti-CD19 CAR lentivirus

TCR-edited and unedited CD8+ T-cells were subsequently engineered with an anti-CD19 CAR lentivirus. First, the duplicate TRAC or TRBC edited TCR α/β-negative cell populations were pooled and expanded, for a total of 6 days post RNP-delivery. Unedited CD8+ (TCR+) T-cells and TRAC or TRBC edited CD8+ T-cells were split into two groups of 1 million cells each. One group of unedited (TCR+), TRAC and TRBC edited CD8+ T cell lines were transduced with concentrated anti-CD19 CAR lentivirus[1] and the other group of those three lines were cultured without transduction. Briefly, edited (TCR-) and unedited (TCR+) CD8+ T-cells were spinfected in a 48 well plate with anti-CD19 CAR lentivirus in a 32°C table-top centrifuge for 60 minutes at 800xg. 24 hours post spinfection, CAR-transduced unedited (TCR+), TRAC and TRBC edited CD8+ T cell lines were selected with a pulse of 1 ug/mL puromycin, followed by a maintenance dose of 0.5 ug/mL puromycin supplemented LGM3 media supplemented with 50 UI IL-2/mL for an additional 4 days. All CD8+ T cell lines demonstrated >30% observable CAR expression by flow cytometry at this time point (data not shown) and complete resistance to puromycin selection. All CD8+ T cell lines (CAR+ and CAR-) were maintained in selection-free LGM3 media and expanded to cell numbers appropriate for the following killing and characterization assays:

1. Flow cytometry-based assessment of specific-killing in co-culture using BioLegend antibodies.
2. LEGENDplex Human CD8/NK Cytokine Panel from BioLegend
3. HTRF Human IFN gamma kit from Revvity

Co-culture and specific killing of CD19+ peripheral blood mononuclear cells (PBMCs) by TCR-edited CD8+ CAR T-cells

To demonstrate that knocking out the endogenous TCR from CD8+ T-cells, followed by the addition of an engineered CAR lentivirus, did not negatively affect effector function on target cells we performed a co-culture experiment with PBMCs and analyzed the killing of CD19+ PBMCs by flow-cytometry. PBMCs isolated from whole blood are a mixture of 70-90% circulating lymphocytes. Here, the total PBMCs were analyzed by flow cytometry and determined to be comprised of 25% CD19+ cells, 38% CD4+ cells, and 18% CD8+ cells. Both 8:1 and 16:1 ratios of CD8+ CAR T-cells to CD19+ PBMCs demonstrated complete killing after 5 hours of co-culture[2] as measured by flow cytometry. In brief, $1.5x10^5$ total PBMCs were co-cultured in a V-bottom plate with $2.4x10^5$ or $4.8x10^5$ anti-CD19 CAR CD8+ T-cells or unedited CAR-negative CD8+ T-cells as a control.

The co-cultures were mixed gently and incubated at 37°C, 5% CO_2 for 5 hours. Co-culture conditions were set up in duplicate per ratio for unedited or edited CD8+ T-cells engineered with an anti-CD19 CAR or no CAR. After 5 hours of incubation, supernatant was collected to assess cytokine release (Figure 4), and viable cells were stained to quantify CD19+ PBMCs remaining in the co-culture (Figure 3A). Absolute numbers CD19+ PBMCs were calculated per co-culture condition, demonstrating near-complete ablation of the CD19+ PBMC population in the anti-CD19 CAR-T transduced cell lines (Figure 3B). In addition, CD8+ T cell lines without CAR transduction were assessed for TCR α/β expression at this time point. TRAC and TRBC edited CD8+ T cell (no CAR) population maintained a >98% knock-out phenotype (Figure 3C).

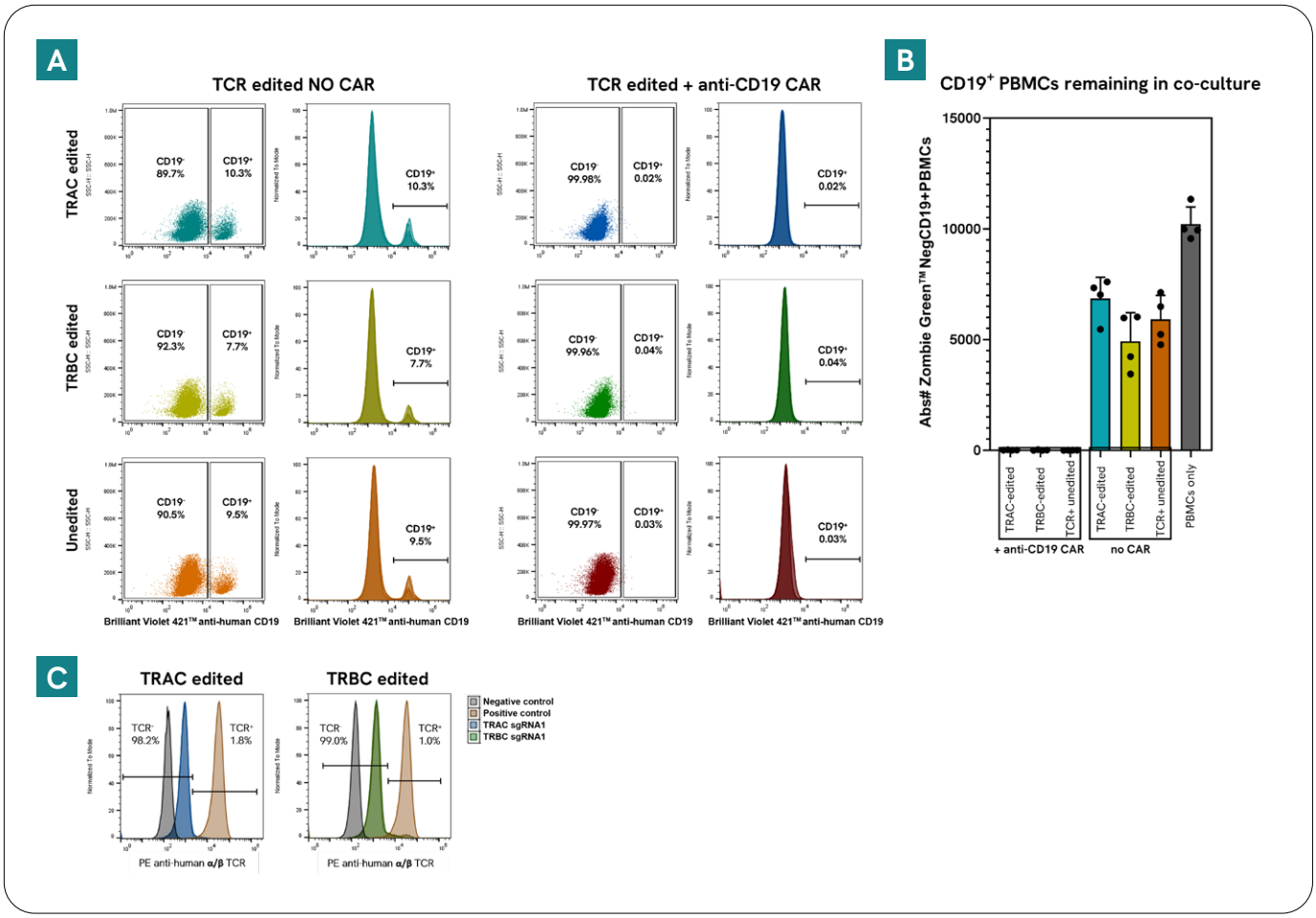


Figure 3: CD8⁺ CAR T-cells specifically kill CD19⁺ PBMCs and endogenous TCR knock-out does not negative effect this function. (A) Edit-R TRAC- and TRBC-edited and unedited primary CD8⁺ T cell lines (no CAR, left columns and +anti-CD19 CAR, right columns) were co-cultured in 8- and 16-fold excess ratios to CD19⁺ PBMCs for 5 hours. Displayed here are the dot plot and histogram representations of living cells from the co-culture (Zombie Green-negative) stained with Brilliant Violet 421™ anti-human CD19 antibody (1:100). As in Figure 1, cells were first stained with 1:400 concentration of Zombie Green Fixable Viability kit. Then all cells were stained 1:50 with Human TruStain FcX (Fc Receptor Blocking Solution) to reduce specific antibody staining background. (B) Bar graph displaying calculated absolute cell numbers of Zombie Green-negative CD19⁺ PBMCs remaining in co-culture (TCR-edited and unedited cells with and without anti-CD19 CAR) after 5 hours of incubation (n=4, +/- SD). (C) Overlaid histograms of TRAC or TRBC-edited CD8⁺ T cells with the same protocol as Figure 2A with 1:50 PE anti-human TCR α/β prior to co-culture killing assay (D8 post RNP-electroporation), unedited CD8⁺ T cells unstained (black), or PE anti-human TCR α/β-stained cells (positive control is orange; TRAC-edited is blue; TRBC-edited is green).

Functional validation of edited CD8⁺ CAR T-cells with LEGENDplex cytokine kit

To assess pro-killing cytokine release from the engineered and edited CD8⁺ CAR T-cells, supernatant collected from the 5-hour 8:1 (CD8⁺ CAR T-cells: CD19⁺ PBMCs) co-culture ratio was evaluated with the LEGENDplex Human CD8/NK Mix and Match Subpanel from BioLegend. Up to 14 cytokines can be quantified per 25 µL supernatant sample, simultaneously, using a flow cytometer and complimentary BioLegend LEGENDplex Data Analysis Software Suite. To quantify supernatant cytokines for this application, 4 cytokines were selected (IL-2, IL-4, IFNγ, and Perforin) for analysis. Lyophilized beads conjugated with antibodies specific to IL-2, IL-4, IFNγ, and Perforin were dissolved in assay buffer and mixed to create a 1x bead solution. Samples were diluted 1:1 and 1:3 in duplicate to measure supernatant cytokines within linear range of the cytokine standards.

Following the LEGENDplex manual, multiplexed beads and supernatant are incubated together, beads are washed, then stained with biotinylated antibodies, followed by binding streptavidin to amplify signal per cytokine. These samples were run on a flow cytometer and exported .fcs files were analyzed using BioLegend LEGENDplex. First, a standard curve was generated using the samples with predefined standard cytokine cocktail (Figure 4A).

Using this standard curve, cytokine concentration in each sample can be calculated. Supernatants of TCR-edited CD8⁺ CAR T-cells when co-cultured with total PBMCs contained a higher concentration of cytokines compared to supernatants from CD8⁺ CAR T-cells cultured alone or total PBMCs alone indicating specific-activation of the engineered CD8⁺ CAR T-cells in co-culture. (Figure 4B). This is an additional piece of evidence that TCR-edited CD8⁺ CAR T-cells are not negatively impaired in killing function by endogenous TCR knock-out. These induced cytokine responses from each TCR-edited and unedited CD8⁺ CAR-T supports the antigen-specific target cell killing observed by flow cytometry.

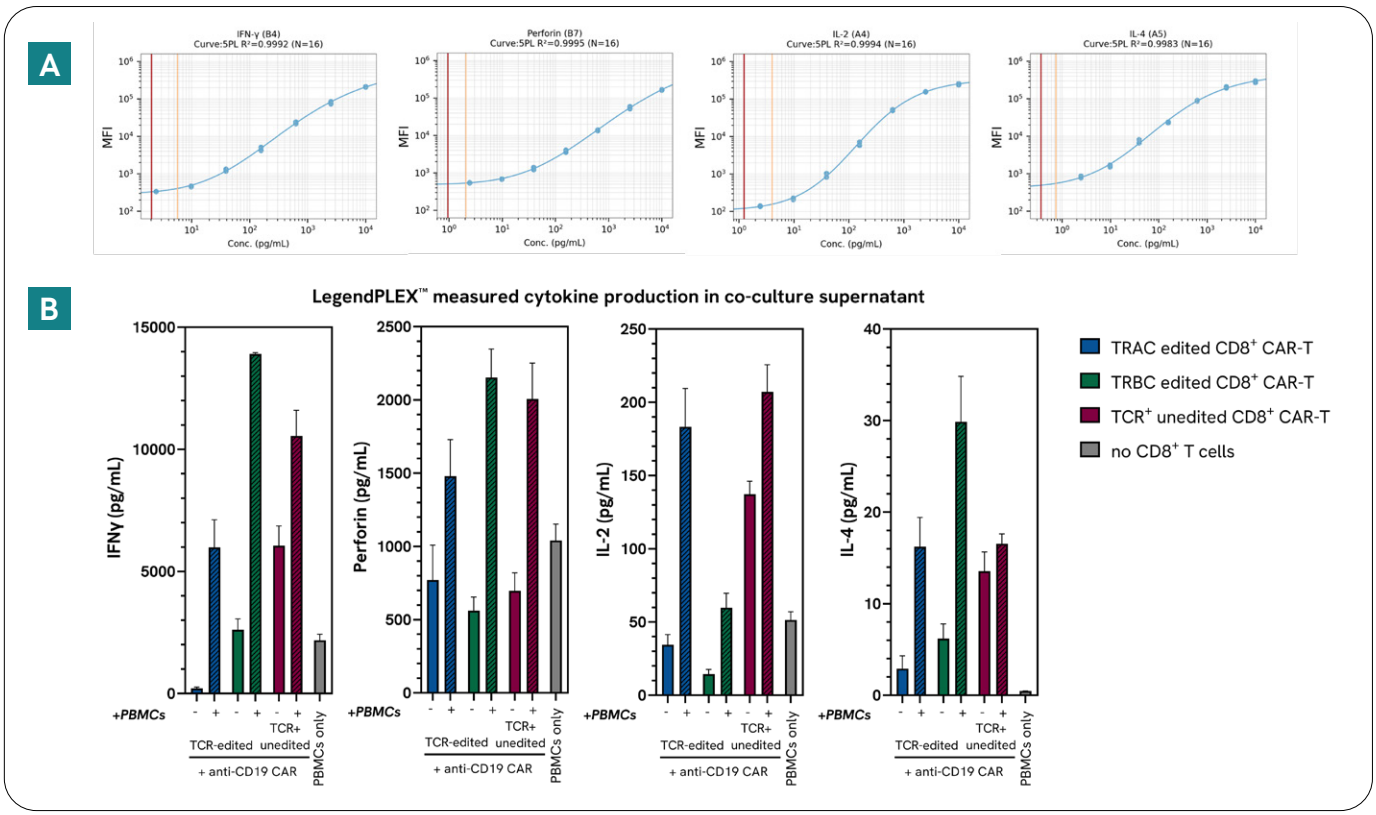


Figure 4: TCR-edited and TCR⁺ CD8⁺ CAR T-cells produce similar levels of pro-killing cytokines. (A) Standard curves generated with LEGENDplex Human CD8/NK Panel Standard. (B) Concentration of IFNγ, Perforin, IL-2, and IL-4 measured in culture supernatant by flow cytometry and analyzed with BioLegend LEGENDplex cloud-based software, arranged from highest induced to production to least (n=2, +/- SD).

HTRF Human IFN-gamma Kit for high-throughput assessment of cytokine production

IFNγ induction from TCR-edited CD8⁺ T-cells occurs when the TCR interacts with MHC molecules presenting antigen on target cells. The HTRF assay was selected to further evaluate IFNγ induction by an orthogonal method. In addition, the HTRF assay supports high-throughput sample collection and analysis for downstream experimentation such as, screening CAR T-cells with small molecules or, editing additional genomic regions to investigate other factors influencing T-cells effector function. Supernatant collected and quantified with the LEGENDplex Human CD8/NK Cytokine Panel was diluted 1:1, 1:5, and 1:10 for an orthogonal measurement of IFNγ using the Human IFN-gamma HTRF Human IFN-gamma kit (catalog 62HIFNGPEG) kit by Revvity. The HTRF assay was performed in 384-well low volume white plates. Antibodies labeled with HTRF donor (620 nm) and acceptor (665 nm) were pre-mixed and added to the diluted supernatant and IFNγ standard in duplicate.

These samples were incubated overnight in sealed 384-well plates at room temperature and measured on the EnVision® plate-reader from Revvity. The 1:10 dilution of supernatant provided an accurate measure (within linear range of the standard) of IFNγ production by TCR-edited and unedited CD8⁺ CAR T-cells when co-cultured with PBMCs. As observed with the LEGENDplex kit measurement for IFNγ induction, supernatant assessed by HTRF calculated a high level of IFNγ induction from TCR-edited CD8⁺ CAR-T when

co-cultured with PBMCs compared to CD8⁺ CAR T-cells cultured alone or PBMCs alone. In further agreement with the LEGENDplex data, the concentration of IFNγ (pg/mL) of the supernatant was comparable (>10000 pg/mL at 5 hours) across all TCR-edited and unedited CD8⁺ CAR T-cells when co-cultured with PBMCs, and greater than unedited CD8⁺ T-cells without an engineered anti-CD19 CAR (Figure 5).

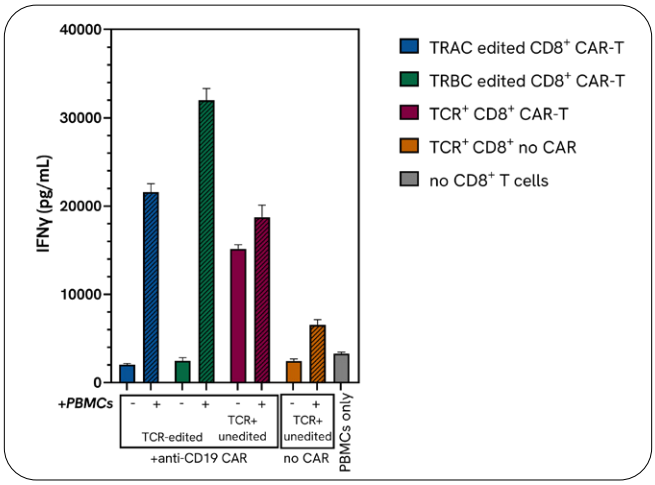


Figure 5: HTRF is an orthogonal method for the measurement of IFN-gamma cytokine release in CRISPR-Cas9 edited CD8⁺ CAR T-cells. Both donor and acceptor measurements acquired by the EnVision® plate-reader to generate a standard curve fitting with the 4 Parameter Logistic (4PL $1/y^2$) model. Plate-reader measurements were analyzed using a Revvity supported online web tool by MyAssays (https://www.myassays.com/Revvity-hifn-gamma-htrf.assay). Bar graphs display the calculated IFN-gamma concentration (pg/mL) measured in the supernatant of each sample (with or without PBMC co-culture; n=2, +/- SD).

Discussion

CRISPR-based editing technologies have revolutionized the field of immunotherapy by enabling engineering of primary immune cells for the development of curative treatments. For the clinical applications of chimeric antigen receptor T cell (CAR-T) therapies, knock-out of immune-specific targets are important for the longevity and efficacy of allogenic T-cells surviving in a patient (i.e., B2M, CD52, TRAC, etc.).

To advance engineered immune cells into clinical trials, well-controlled experiments, screens, and new onco-immune studies must be conducted to better understand and evaluate this groundbreaking technology. Here, we demonstrated the invaluable utility of high-efficiency TCR-knock out using pre-designed TRAC and TRBC targeting Edit-R synthetic guides (sgRNAs) in primary CD8⁺ CAR T cells from Revvity.

Demonstrated here is an easy-to-implement process for your CAR screening workflow. First, the endogenous TCR α/β receptor was edited in primary CD8⁺ T-cells using Edit-R TRAC and TRBC targeting sgRNAs with >98% knock-out phenotype. Next, TCR-edited CD8⁺ T-cells were engineered with an anti-CD19 CAR lentivirus and transiently selected post-transduction. Finally, these TCR-edited CD8⁺ CAR T-cells were tested in co-culture killing assays demonstrating antigen-specific cytotoxic activity. Furthermore, production of effector cytokines was not affected by knocking-out the endogenous TCR α/β.

References

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2. Noto, et al., Cell-based Flow Cytometry Assay to Measure Cytotoxic Activity. J Vis Exp (JoVE). 2013; (82):51105; doi: 10.3791/51105

For more information

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