Defining An Allogeneic CAR-T Approach by shRNA-Mediated Knockdown of the T-Cell Receptor

Patty Garcia[1], Vanessa Strings-Ufombah[1], Natalie Suhy[1], Peter Roelvink[1] and David Suhy[1]

[1]*Benitec Biopharma, Hayward, CA and Sydney, AU*

BENITEC BIOPHARMA
silencing genes for life™

Abstract

Background: An allogeneic CAR T-Cell therapy can streamline manufacturing, provide greater accessibility to patients and enhance safety by reducing graft-versus-host-disease. The T-Cell Receptor (TCR) is comprised of multiple subunits and functions to activate T-cells by a signal transduction cascade initiated upon antigen binding. Eliminating the endogenous TCR on modified CAR-T-Cells may eliminate the ability to recognize major and minor histocompatibility antigens in the recipient. This study assessed if simultaneous expression of multiple short hairpin RNAs (shRNA) that knockdown levels of individual TCR subunits results in loss of TCR expression and TCR-mediated T-Cell activation.

Methods: Recombinant DNA producing shRNA against the TCR was transfected into T-cells. Cell surface TCR was analyzed by FACS. Following CD3 activation, T-Cell activation was quantified by assessing levels of IL-2.

Results: Expressed individually, each shRNA inhibited it's cognate TCR subunit up to 93%. Simultaneous expression of shRNAs against different subunits resulted in near complete depletion of the TCR from the cell surface as measured by FACS. IL-2 secretion was inhibited to undetectable levels by ELISA by the multi-shRNA treatment and >98% by qPCR.

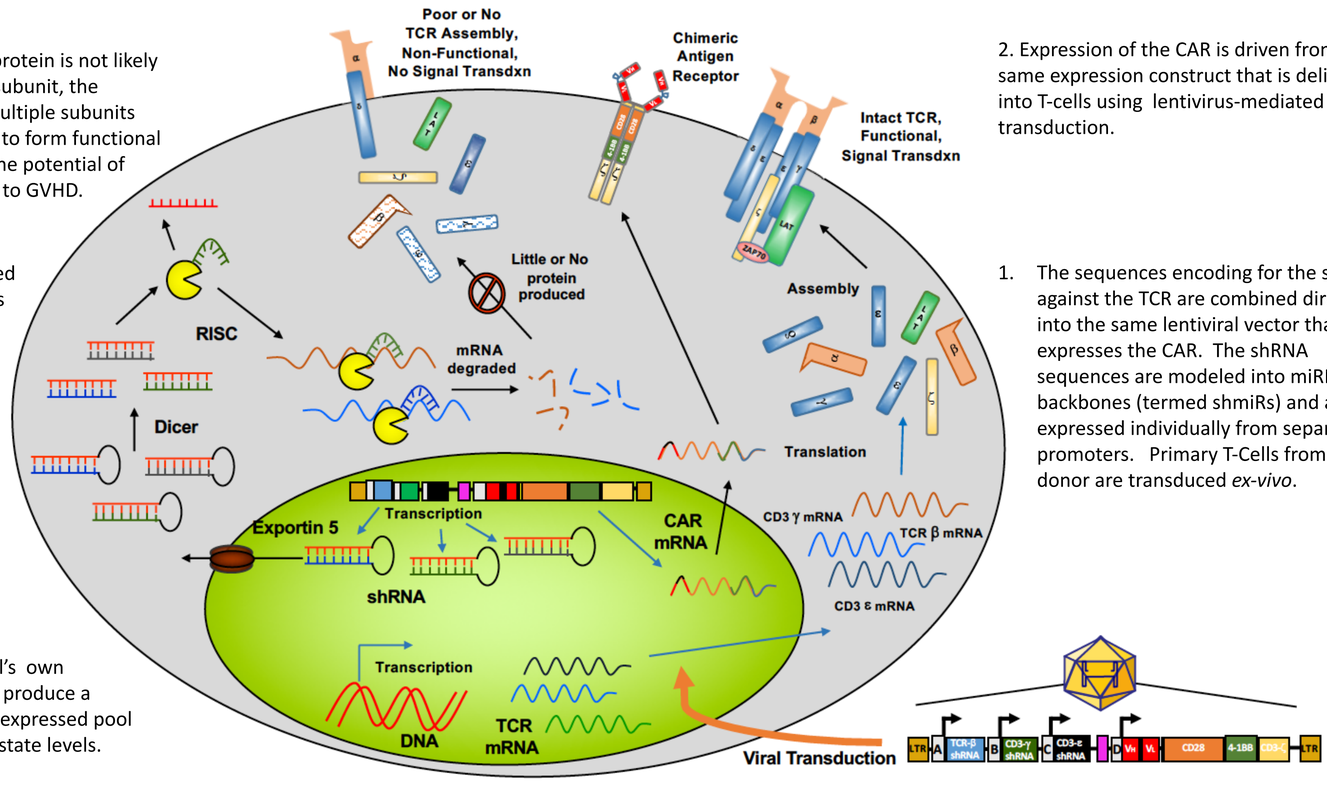
Conclusions: Though knockdown of any single component of TCR never exceeded 93%, simultaneous knockdown of several TCR subunits abrogated surface TCR and downstream activation suggesting that disruption of stoichiometric expression the subunits prevented TCR formation. The small size of shRNA expression cassette (<2 Kb) permits co-expression from the same lentiviral vector as the CAR. Altogether, these data point to a strategy towards generating a single vector approach for producing allogeneic T-Cells for immunotherapies.

Mechanism of Action: ddRNAi Eliminates TCR Expression and Signal Transduction

5. Although the inhibition levels of protein is not likely to be 100% for each individual TCR subunit, the combined effect of knockdown of multiple subunits simultaneously abolishes the ability to form functional TCR complexes on the surface and the potential of TCR-MHC mismatches to contribute to GVHD.

4. The anti-TCR shRNAs are exported into cytoplasm, the loop sequences cleaved and the resultant siRNAs enter the RNA induced silencing complex (RISC), cleave the target mRNA, thus rendering multiple subunits of the TCR mRNA incapable of being translated.

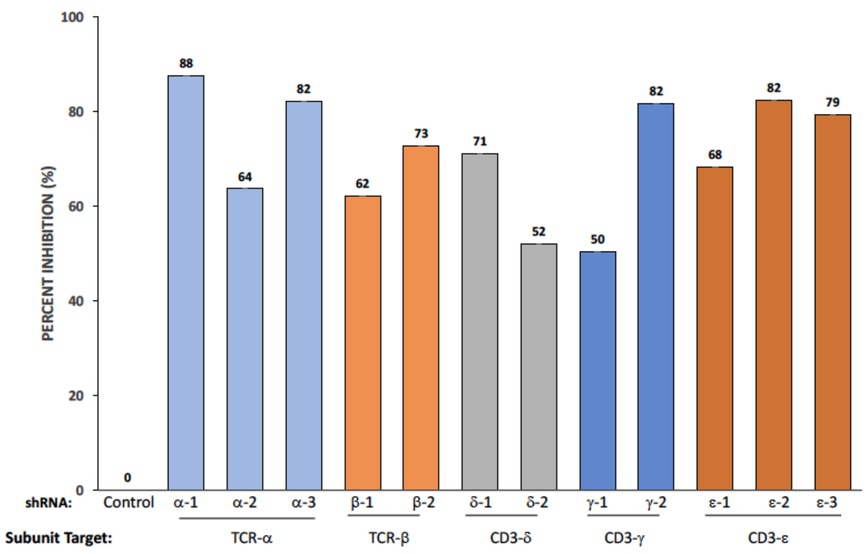
3. The construct uses the cell's own transcriptional machinery to produce a self-replenishing, constantly expressed pool of anti-TCR shRNA at steady state levels.



2. Expression of the CAR is driven from the same expression construct that is delivered into T-cells using lentivirus-mediated transduction.

1. The sequences encoding for the shRNA against the TCR are combined directly into the same lentiviral vector that expresses the CAR. The shRNA sequences are modeled into miRNA backbones (termed shmiRs) and are expressed individually from separate promoters. Primary T-Cells from any donor are transduced *ex-vivo*.

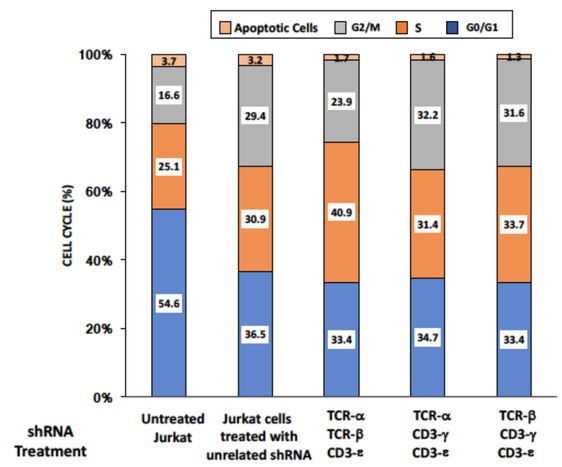
Knockdown of Individual Subunits of the TCR Complex



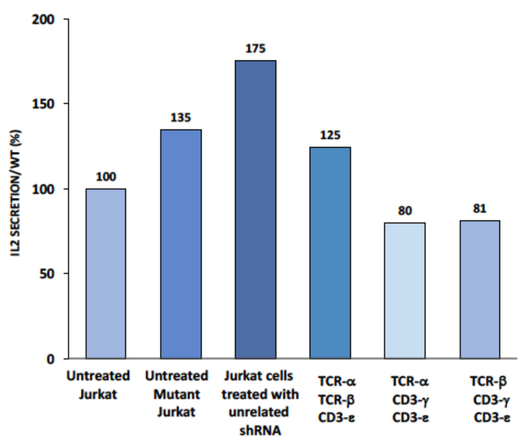
- Jurkat T-Cells were transduced with recombinant plasmids that express individual shRNA that have been modeled into miRNA backbones (shmiRs). Each shRNA was designed to inhibit one of the subunits of the TCR complex.
- Assessment of knockdown was performed by QPCR analysis

Abrogation of TCR Expression Does Not Disrupt Cell Cycle Distribution or TCR-Independent Activation

Cell Cycle Distribution



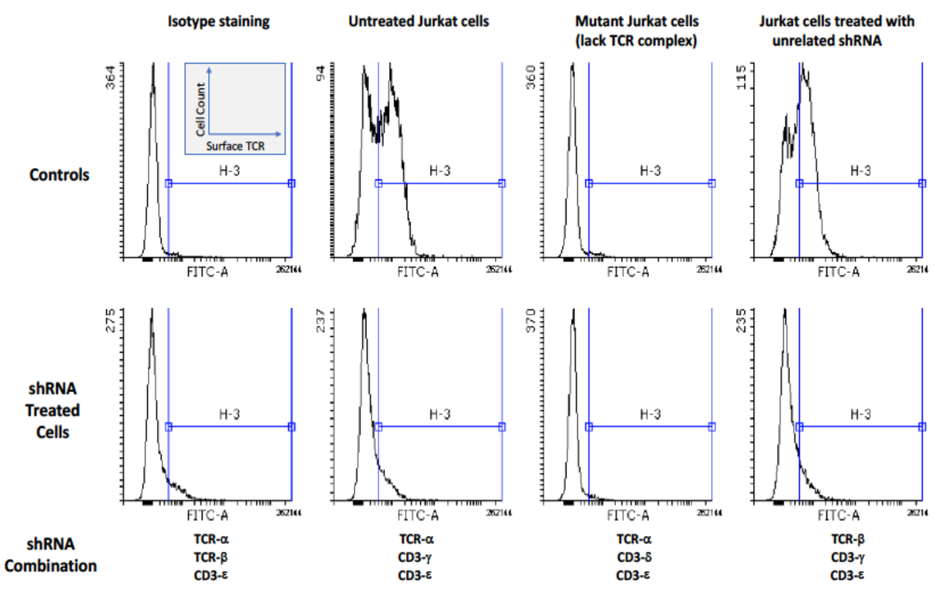
T-Cell Independent Activation



- Jurkat T-cells were transduced with plasmids concomitantly expressing shRNAs against different TCR subunits.
- Cells were pulsed with BrdU. FACs was performed with antibodies against BrdU and 7AAD to determine the percentage of cycling cells. Cells were also co-stained with an anti-TCR-α/β specific antibody to identify T-cells lacking the TCR complex.

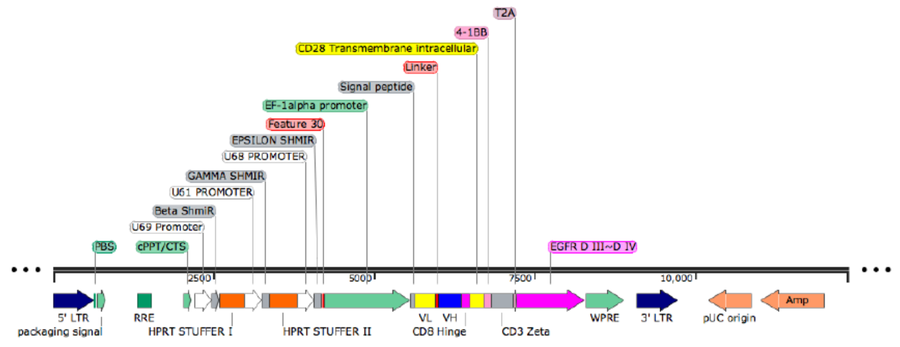
- Jurkat T-cells were transduced with plasmids concomitantly expressing shRNAs against different TCR subunits.
- Cells were treated with PMA and Ionomycin for 4 hours to measure TCR-independent T-cell functionality upon inhibiting TCR complex formation on the cell surface.
- T-cell activation was measured by IL2 ELISA

Use of Multiple shRNAs Inhibits TCR Surface Expression

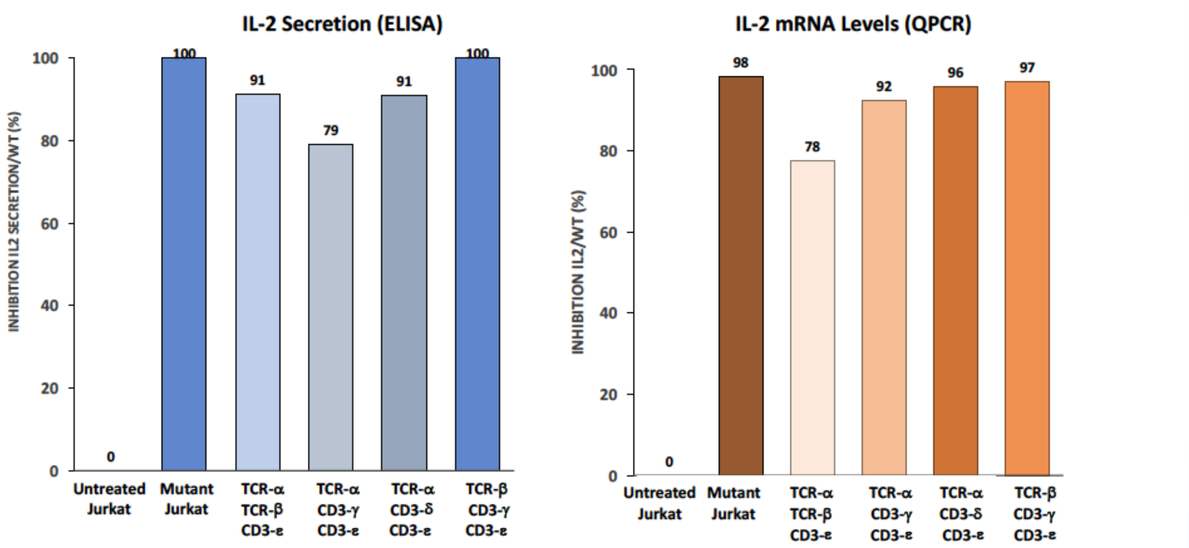


- Jurkat T-cells were transduced with plasmids concomitantly expressing shRNAs against different TCR subunits. A plasmid that expressed three anti-Hepatitis C virus shRNAs served as a negative control.
- FACS analysis for TCR expression was performed using an anti-TCR-α/β specific antibody.

Single Vector for Simultaneous CAR Expression & TCR Suppression



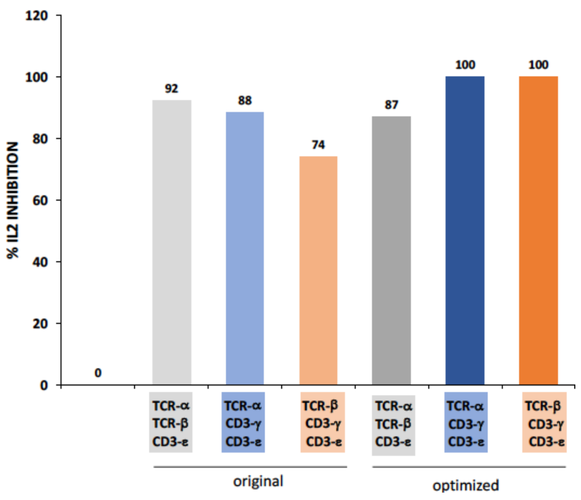
Lack of TCR-Mediated Signal Transduction in shRNA Treated Jurkat Cells Activated with CD3 and CD28 Antibodies



- Jurkat T-cells were transduced with plasmids concomitantly expressing shRNAs against different TCR subunits. Transduced cells were subsequently treated with anti-CD3 and anti-CD28 antibodies to assess T-cell functionality after TCR knockdown.
- T-cell activation was inhibited, as measured by a reduction of IL-2 secretion (left) or loss of IL-2 mRNA (right)

Optimization of Lentiviral Vectors for Potent TCR Suppression

- All shRNAs were cloned into a single lentiviral expression vector for simultaneous expression. Because the efficiency of IL-2 inhibition was less from each of the triple vectors as compared to combinations with individual plasmids, regulatory elements within the triple shRNA expression lentivirus vectors were optimized for maximal inhibition of TCR activation.
- Combinations of CR-α/CD3-γ/CD3-ε and TCR-β/CD3-γ/CD3-ε inhibited levels of IL-2 below the assay LLOD of 15.6 pg/ml.



Summary

- Using multiple shRNAs, we observed a nearly complete depletion of the TCR complex from the cell surface as measured by FACS.
- TCR transduction was robustly inhibited when treated cells were stimulated with CD3.
- There were no significant changes in the cell cycle of the TCR-less cells
- TCR-less cells responded robustly to TCR-independent activation with PMA and Ionomycin and indicates that the endogenous activation pathways are intact despite TCR knockdown and suggest that these cells may be activated by CAR-T reconstitution.
- Collectively, these data demonstrate that a single vector strategy of knocking out the TCR with multiple shRNAs is viable towards generating allogeneic T-Cells for immunotherapies against certain cancers.