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

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Abstract

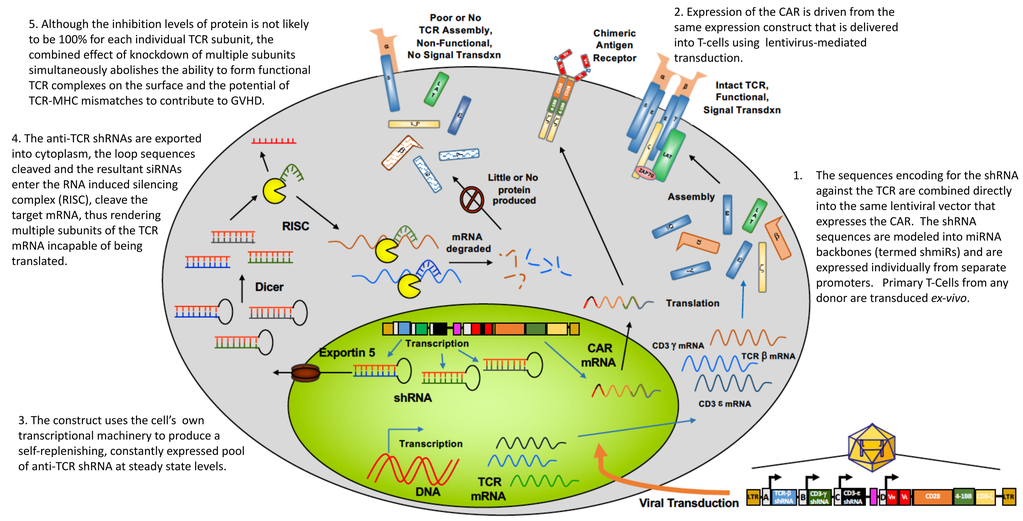
INTRODUCTION: CAR T-Cell therapy is an exciting advancement in the field of oncology that provides the ability to modify a subject's own immune cells to treat their cancer. Although autologous adoptive cell transfer has been successfully employed in the clinic, an allogeneic approach has the potential to significantly streamline the manufacturing process, thus providing more accessible options to patients as well as enhancing safety by reducing the possibility of graft-versus-host-disease from an HLA mismatched donor. The T-Cell Receptor (TCR) is a protein comprised of multiple subunits and functions to activate T-cells by a signal transduction cascade that is initiated upon antigen binding. Thus, restricting or eliminating expression of the endogenous TCR on the modified CAR-T-Cells may help eliminate the ability to recognize major and minor histocompatibility antigens in the recipient. The goal of this study was to assess if the simultaneous expression of multiple short hairpin RNAs that knockdown levels of individual TCR subunits could result in the complete loss of TCR-mediated T-Cell activation.

METHODS: Recombinant DNA expression constructs producing combinations of short hairpin RNA (shRNA) against the various subunits comprising the TCR complex, were transfected into T cells. Cell surface TCR expression was analyzed by FACS. Following CD3 activation or co-culture with B-cells, T-Cell activation was quantified by measuring the levels of IL-2 by ELISA and QPCR.

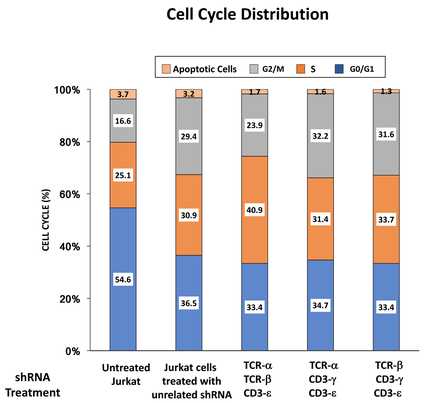
RESULTS: As even modestly low levels of TCR activity might be detrimental in an allogeneic approach, a strategy was employed to completely abrogate TCR activity. When expressed individually, each shRNA inhibited protein and mRNA expression of it's cognate TCR subunit by up to 93% of the endogenous levels. However, upon simultaneous expression of the shRNAs against the different subunits from the same vector, we observed a near complete depletion of the TCR complex from the cell surface (>99%) as measured by FACS analyses. Furthermore, TCR functionality was inhibited when treated cells were stimulated with either CD3 or in B cell co-cultures with Staphylococcal enterotoxins. IL-2 secretion was inhibited to undetectable levels by ELISA by the multi-shRNA treatment and >98% by qPCR.

CONCLUSIONS: Although the level of individual knockdown of any one of the components of TCR never exceeded 93%, simultaneous knockdown of several TCR subunits was sufficient to abrogate surface TCR expression and downstream activation suggesting that disruption of stoichiometric expression levels of the subunits was sufficient to prevent TCR formation. Given the small size of each shRNA expression cassette, the packaging capacity required for three shRNAs (<2 Kb) permits co-expression from the same lentiviral vector as the CAR. Altogether, these data point to a viable strategy towards generating a single vector approach for the production of allogeneic T-Cells for immunotherapies against certain cancers.

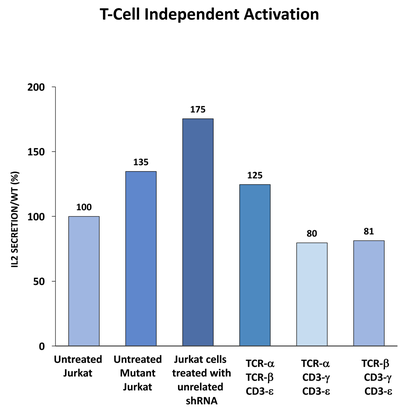
Mechanism of Action: ddRNAi Eliminates TCR Expression and Signal Transduction



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

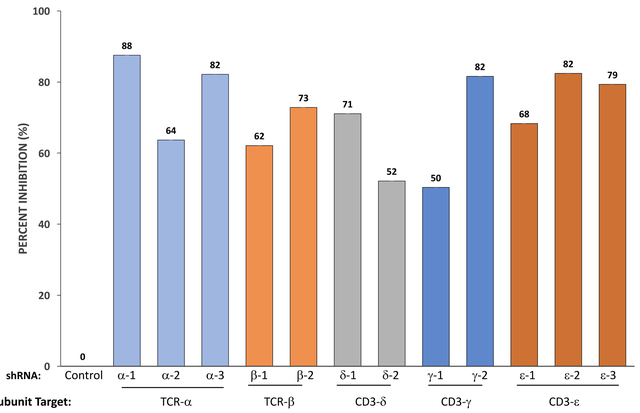


- Jurkat T-cells were transduced with plasmids concomitantly expressing shRNAs against different TCR subunits.
- Cells were pulsed with BrdU. FACs was performed with antibodies against BrdU and 7AAD to determine the percentage of cycling cells. Cells were also co-stained with an anti-TCR-α/β specific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



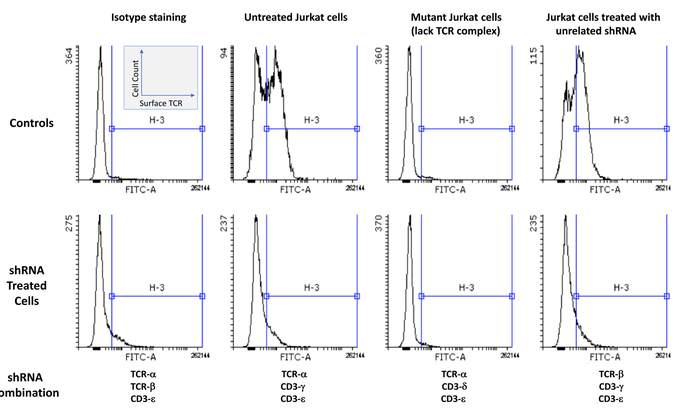
- Jurkat T-cells were transduced with plasmids concomitantly expressing shRNAs against different TCR subunits.
- Cells were treated with PMA and Ionomycin for 4 hours to measure TCR-independent T-cell functionality upon inhibiting TCR complex formation on the cell surface.
- T-cell activation was measured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

Knockdown of Individual Subunits of the TCR Complex



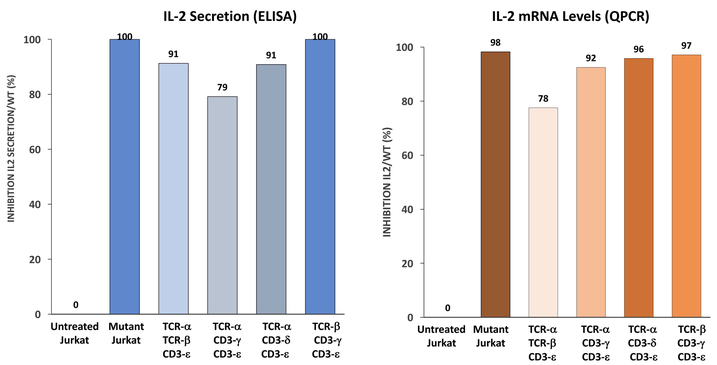
- Jurkat T-Cells were transduced with recombinant plasmids that express individual shRNA that have been modeled into miRNA backbones (shmiRs). Each of the shRNA were designed to inhibit one of the subunits of the TCR complex.
- Assessment of knockdown was performed by QPCR analysis and identified the top candidates capable of inhibiting each subunits by levels up to ~ 88%.

Use of Multiple shRNAs Inhibits TCR Surface Expression



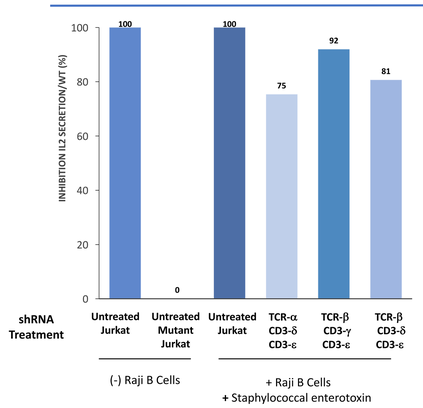
- Jurkat T-cells were transduced with plasmids concomitantly expressing shRNAs against different TCR subunits. A plasmid that expressed three anti-Hepatitis C virus shRNAs served as a negative control.
- FACS analysis using an anti-TCR-α/β specific antibody showed a nearly complete depletion of the TCR complex from the cell surface.

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



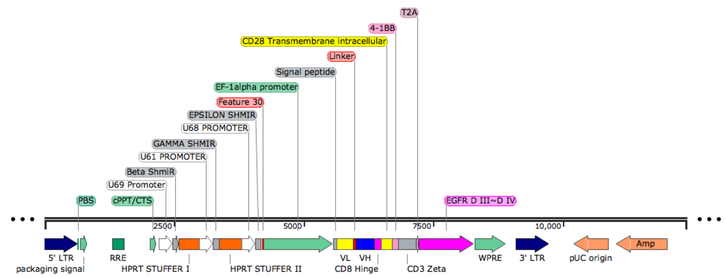
- Jurkat T-cells were transduced with plasmids concomitantly expressing shRNAs against different TCR subunits.
- Transduced cells were subsequently treated with anti-CD3 and anti-CD28 antibodies to measure T-cell functionality upon TCR knockdown.
- T-cell activation was inhibited, as measured by IL-2 secretion, to undetectable levels when measured by ELISA. The levels of IL-2 mRNA were concomitantly knocked down at roughly equivalent levels

Lack of TCR-Mediated Signal Transduction in shRNA Treated Jurkat Cells Activated Through APC Co-Culture

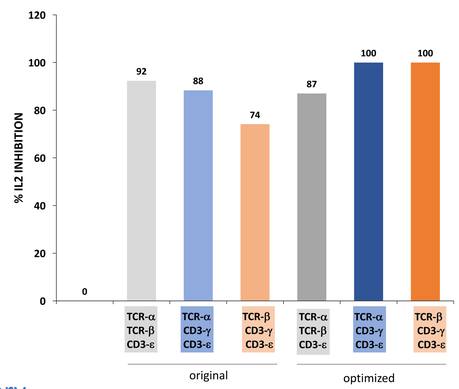


- Jurkat T-cells were transduced with plasmids concomitantly expressing shRNAs against different TCR subunits.
- Transduced cells were subsequently co-cultured with Raji B cells loaded with Staphylococcal enterotoxin to measure T-cell functionality upon TCR knock-down.
- T-cell activation was inhibited by up to ~92% as measured by IL-2 secretion using ELISA.

Single Vector for Simultaneous CAR Expression and TCR Suppression



Optimization of lentiviral vectors for optimal 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 either CD3 or in B cell co-cultures with Staphylococcal enterotoxins.
- 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.