RNAi-Mediated Knockdown of TCR Prevents Surface Expression on T-Cells and Completely Abrogates IL-2 Secretion

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Abstract

Background: CAR T-Cell therapy has been an exciting advancement in the field of oncology by providing the ability to modify a subject's own immune cells to be able to treat their cancer. Although the autologous adoptive cell transfer approach has been successfully employed in the clinic, an allogeneic approach has the potential to significantly streamline the manufacturing process. As a result, this may provide more accessible options for patients as well as enhance safety by reducing the possibility of graft-versus-host disease. Restricting expression of the T-Cell Receptor (TCR) on the modified T-Cells helps eliminate the ability to recognize major and minor histocompatibility antigens in the recipient.

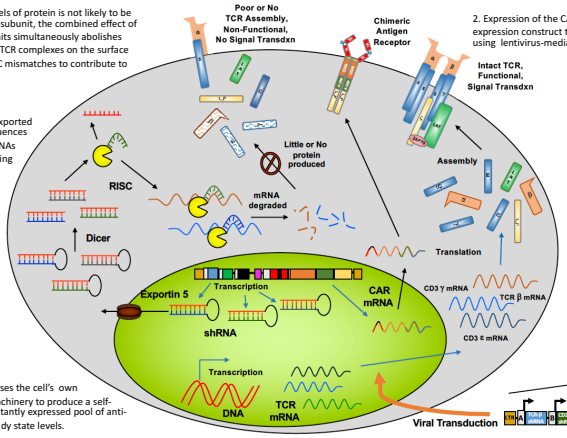
Results: In the approach described, we engineered a recombinant expression construct that produces short hairpin RNAs (shRNAs) against multiple subunits comprising the TCR complex. Individually, each of the highly selective shRNA inhibited protein and mRNA expression by up to 93%, levels still insufficient for therapeutic benefit. However, when multiple shRNAs against the different TCR subunits were concomitantly expressed from the same vector, we observed a nearly complete depletion of the TCR complex from the cell surface (>95%) 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 when measured by ELISA and >98% when measured by qPCR. Importantly, there were no significant changes in the cell cycle of the TCR-less cells and the cells responded robustly to PMA and Ionomycin activation as measured by ELISA for IL2 (~40% vs. control) and IFN-Gamma (100% vs. control). This indicates that the endogenous activation pathways are intact despite TCR knockdown and suggest that these cells may be activated by CAR-T reconstitution. Given their relatively small size, the sequences encoding the shRNAs are intended to be bolted directly into the same lentiviral vectors for co-expression with the CAR protein.

Conclusion: Taken altogether, these data demonstrate that a strategy of knocking out the TCR with multiple shRNAs is viable towards generating allogeneic T-Cells for immunotherapies against certain cancers.

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.

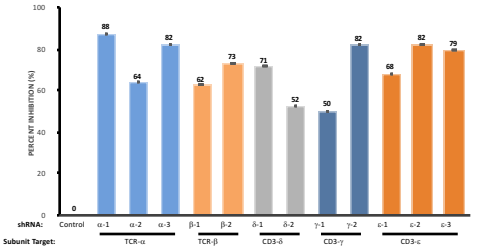


2. Expression of the CAR is driven from the same expression construct that is delivered into T-cells using lentivirus-mediated transduction.

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*.

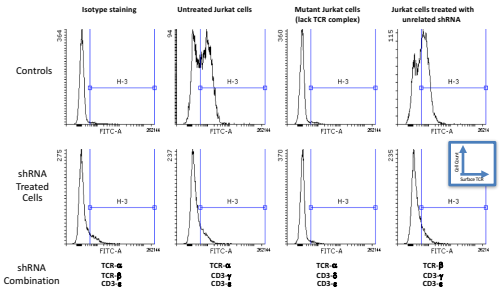
3. The construct uses the cell's own transcriptional machinery to produce a self-replenishing, constantly expressed pool of anti-TCR shRNA at steady state levels.

Knockdown of Individual Subunits of the TCR Complex



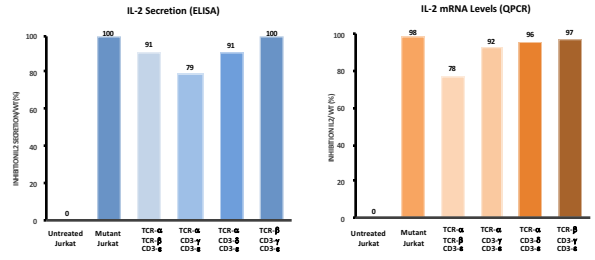
- Jurkat T-Cells were transduced with recombinant constructs that express individual shRNA that have been modeled into miRNA backbones (shmiRs). Each of the shRNA were designed to inhibit one of the subunits of the TCR complex.
- Assessment of knockdown was performed by QPCR analysis and identified the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

Use of Multiple shRNAs Inhibits TCR Surface Expression



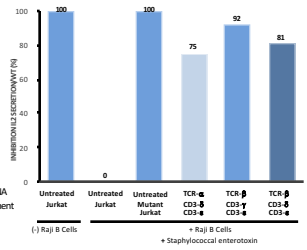
- Jurkat T-cells were transduced with vectors concomitantly expressing multiple shRNAs against the different TCR subunits. A vector that expressed three anti-Hepatitis C virus shRNAs served as a negative control.
- FACS analysis using an anti-TCR-α/β specific antibody showed a nearly complete depletion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



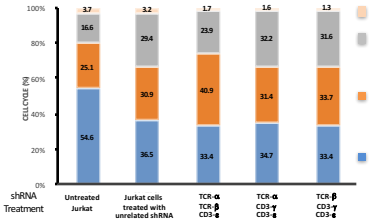
- Jurkat T-cells were transduced with vectors concomitantly expressing multiple shRNAs against the different TCR subunits.
- Transduced cells were subsequently treated with anti-CD3 and anti-CD28 antibodies to measure T-cell functionality upon TCR knockdown.
- T-cell activation was inhibited, as measured 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



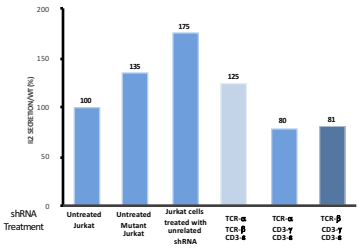
- Jurkat T cells were transduced with vectors concomitantly expressing multiple shRNAs against the different TCR subunits.
- Transduced cells were subsequently co-cultured with Raji B cells loaded with Staphylococcal enterotoxin to measure T-cell functionality upon TCR knock-down.
- T-cell activation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

TCR Knockout Does Not Disrupt Cell Cycle Distribution



- Jurkat T cells were transduced with vectors concomitantly expressing multiple shRNAs against the 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.
- The bar graph demonstrates the percentage of TCR-less cells in each stage of the cell cycle, G0/G1, S, G2/M.
- Importantly, there were no significant changes in the cell cycle distribution in the T-cells lacking the TCR complex.

TCR Knockout Does Not Disrupt TCR-Independent Activation



- Jurkat T cells were transduced with vectors concomitantly expressing multiple shRNAs against the 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 in response to PMA and Ionomycin was largely intact as measured by ELISA for IL2

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.
- This 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 strategy of knocking out the TCR with multiple shRNAs is viable towards generating allogeneic T-Cells for immunotherapies against certain cancers.