Single cell RNA sequencing reveals functionally validated signatures of cytotoxicity in anti-FRα CoStimulatory Antigen Receptor (CoStAR™) activated CD4+ T cells.

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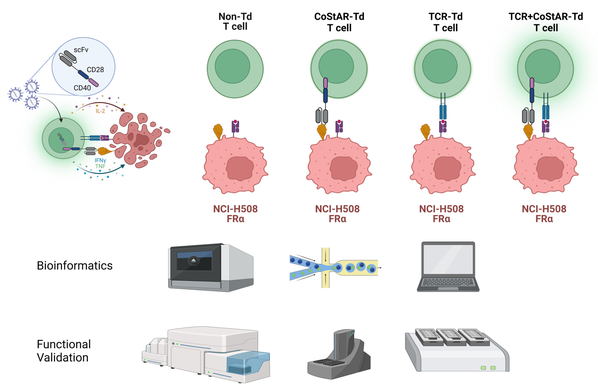
INTRODUCTION

- There are several limiting factors for the development of cell therapy modalities in solid tumors, including tumour cell clonal heterogeneity, high expression of inhibitory receptors and poor endogenous costimulation (1-5).
- Tumor-infiltrating lymphocytes (TILs) contain a hugely diverse T-cell receptor (TCR) repertoire (6), thereby offering the broadest diversity of anti-tumor reactivity.
- TIL therapy has a proven track record of effective clinical efficacy (7-9).
- We have developed the CoStimulatory Antigen Receptor (CoStAR) platform to overcome the suppressive TME.
- The CoStAR described here consists of a FRα-specific single chain antibody fragment (scFv) fused to the signaling domains from CD28 and CD40.
- We have previously shown that engagement of CoStAR amplifies TCR signalling leading to enhanced T cell activity (10,11).
- Here, we use coculture assays and subsequent scRNA-seq analysis with functional in vitro validation techniques to explore the relative contribution of CoStAR to TCR activity in an effort to further understand CoStAR function.

METHODS

- T cells from three healthy donors were engineered with an HLA-A*02/CEA (CEA:691-699 IMIGVLVGV) specific TCR and/or FRα-specific CoStAR and subsequently enriched for expression.
- T cells were cocultured with HLA-A*02+/CEA+ NCI-H508 tumour cells overexpressing human FRα (H508-FRα) before processing through 10X Genomics 5' GEX scRNA-seq workflow.
- Bioinformatic analysis was used to compare TCR and/or CoStAR+ populations, as well as CD4+ and CD8+ populations.
- Functional validation of gene signatures was performed by isolating CD4+ and CD8+ cells and coculturing with H508-FRα, before analysis of cytokine and chemokine by bead arrays and multiplexed analyte analysis (Mesoscale Discovery), flow cytometric staining and xCELLigence cytotoxicity assays.



RESULTS

- A custom gene reference was created and could successfully quantify scRNA-seq reads of anti-FRα-CoStAR, as well as the recombinant TCR introduced into the T cells.
- A cell is CoStAR+ if at least 1 mRNA copy maps to the CoStAR scFv nucleotide sequence included in the custom reference.
- This was compared with flow cytometric analysis of TCR and CoStAR, with GEX and flow cytometry showing good concordance.

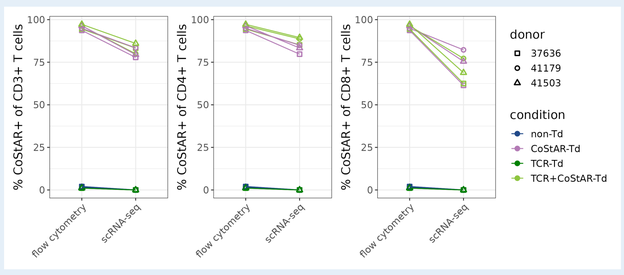


Figure 1: Measurements of CoStAR transduction efficiency are concordant between scRNA-seq and flow cytometry.

RESULTS

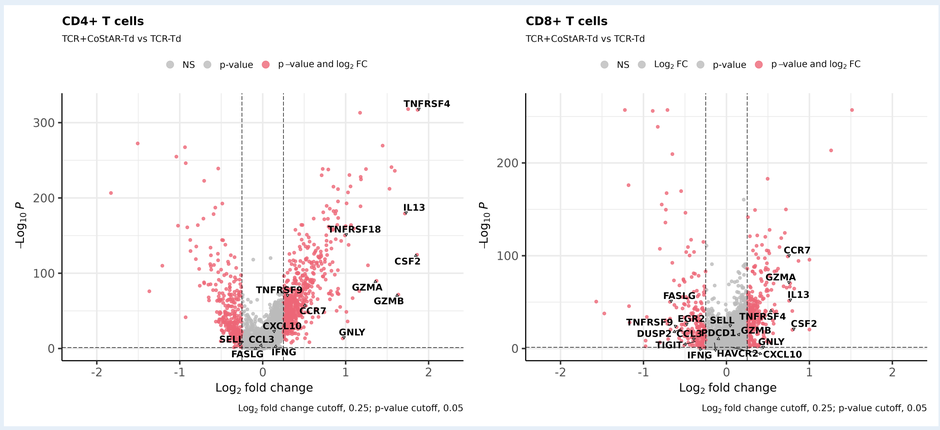


Figure 2: Differential expression analysis reveals CoStAR activity leads to different activation and differentiation states in CD4+ and CD8+ T cells.

- TCR+CoStAR signaling resulted in increased expression of genes associated with activation (e.g. TNFRSF4, TNFRSF9) and a cytotoxic phenotype (e.g. GZMA, GZMB, GNLY) in CD4+ cells compared to TCR alone signaling.
- TCR+CoStAR signaling resulted in increased expression of genes associated with a less differentiated phenotype (e.g. CCR7, SELL) and increased cytotoxicity and decreased expression of genes associated with exhaustion (e.g. HAVCR2, TIGIT, PDCD1) in CD8+ cells compared to TCR alone signaling.

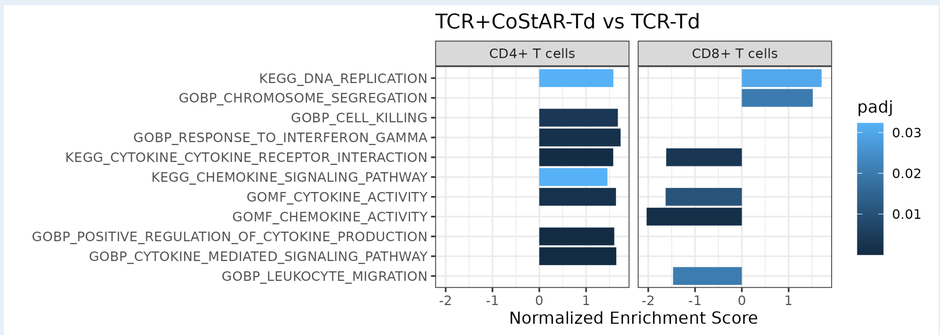


Figure 3: Pathway analysis shows enrichment of pathways associated with cytokine signaling and cell killing in CD4+ T cells and with migration and cell cycle in CD8+ T cells.

- Pathway analysis indicated enhancement of cytotoxic phenotype and cell killing in CD4+ T cells upon CoStAR-mediated costimulation.
- In CD8+ T cells, TCR+CoStAR signaling resulted positive in enrichment of cell cycle related pathways and negative enrichment of leukocyte migration in line with a less differentiated phenotype.

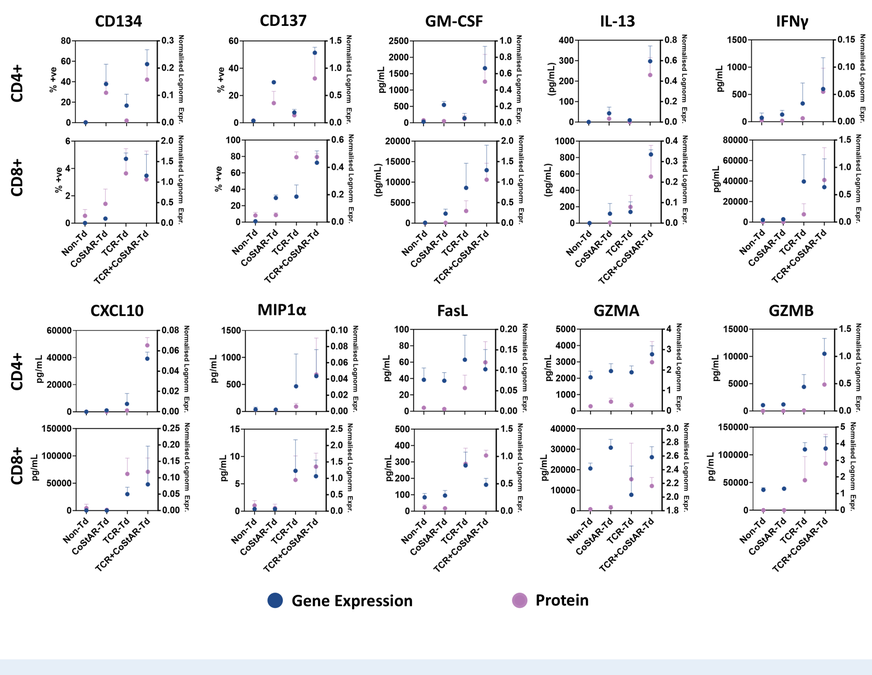


Figure 4: Protein analysis shows good concordance of multiple genes identified through single cell RNA sequencing analysis

- We used flow cytometry and multiplexed immunoassay to measure several cell surface markers and soluble effector molecules produced by negatively enriched CD4+ and CD8+ T cells under conditions of stimulation of TCR and/or CoStAR following coculture with NCI-H508-FRα tumour cells.
- We were able to functionally validate the upregulation of CD134 (TNFRSF4) and CD137 (TNFRSF9) in CD4+ and CD8+ T cells following CoStAR stimulation alone. In fact, in CD4+ T cells both markers were upregulated by CoStAR engagement to a higher degree than following TCR alone stimulation.
- Many other proteins had good concordance with our gene expression data, including the cytokines GM-CSF (CSF2), IL13, and IFNγ; and the chemokines IP10 (CXCL10) and MIP1a (CCL3).
- Our analysis also revealed CoStAR-enhanced upregulation of FasL (FASLG), GZMA and GZMB in CD4+ and CD8+ T cells.

RESULTS

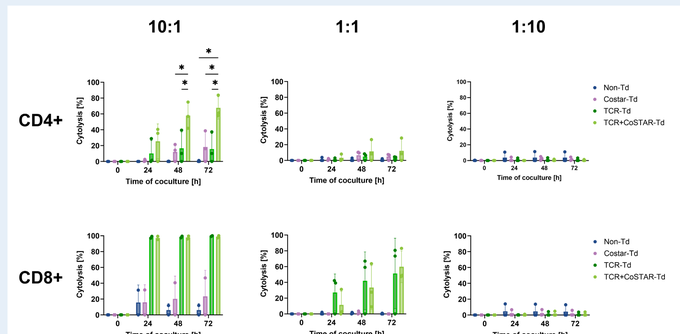


Figure 5: Cytotoxicity of CD4+ and CD8+ T cells expressing TCR and/or CoStAR using xCELLigence.

- The unexpected observation of enhancement of cytotoxicity signatures in CD4+ T cells with CoStAR-costimulation, led us to explore CoStAR-mediated cytotoxicity in vitro.
- TCR and/or CoStAR+ CD4+ and CD8+ T cells were negatively enriched and added to NCI-H508-FRα tumour cells at the indicated effector to target ratios.
- xCELLigence was used to track killing and measurements made at 24, 48 and 72h.
- At a 10:1 E:T we observed efficient killing of target cells by CD4+ TCR+CoStAR-Td T cells vs TCR+ alone at 48 and 72h (P<0.05).

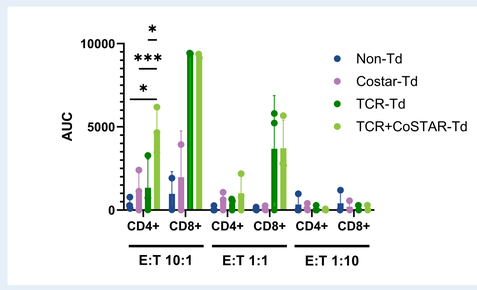


Figure 6: Area under the curve analysis of cytotoxicity by CD4+ and CD8+ T cells engineered with TCR and/or CoStAR

- Area under the curve analysis was performed for CD4+ and CD8+ cells across each E:T ratio over 100h.
- We observed significantly increased cytotoxicity with CD4+ cells expressing CEA TCR + CoStAR vs TCR alone (P<0.05) or CoStAR alone (0.0005).

CONCLUSION

- Anti-FRα CoStAR provide functional benefit to CD4+ and CD8+ T cells in overlapping and distinct ways.
- CoStAR enhancement of TCR signalling enhanced signatures of activation and cytotoxicity in CD4+ cells, and enhancement of genes associated with less differentiation and cytotoxicity in CD8+ T cells.
- Functional validation of these gene changes using flow cytometry and multiplexed immunoassay demonstrated strong concordance between gene and protein expression, including IP-10, GM-CSF and GZMB.
- Furthermore, certain proteins including CD134 could be observed to be upregulated following CoStAR engagement alone, suggesting CoStAR activity can have a broader positive impact on T cell function.
- The cytotoxicity profile seen in CD4+ T cells was functionally validated in isolated CD4+ T cells, demonstrating that CoStAR can imbue CD4+ T cells with cytotoxic activity.
- These results support the clinical exploration of anti-FRα CoStAR in FRα+ tumor indications.

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