Abstract #4868

Optimal activation of TCR-T cells in solid tumors through addition of best-in-class single chain CD8 co-receptors, results in CD4 engagement and improved T cell fitness and persistence

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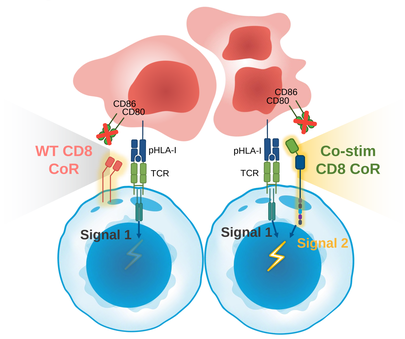
Background

Incorporation of CD8αβ or CD8α co-receptors (CoR) in T cell receptor (TCR) T cell therapy constructs enables CD4 T cell engagement, resulting in broader and/or deeper clinical responses at lower doses in solid tumors. Here, we designed a small, chimeric, single-chain CD8 CoR maintaining all functional elements of the CD8αβ CoR and used it as a scaffold to add intracellular costimulatory signaling motifs (co-stim CD8 CoR). **Incorporation of co-stim CD8 CoR provides optimal co-stimulation simultaneously to TCR engagement in both CD4 and CD8 T cells and prevents TCR-T cell exhaustion caused by the lack of costimulatory molecule (such as CD80/CD86) expression in tumors.**

Methods

Multiple single-chain CD8 CoRs were designed combining building blocks from CD8αβ and CD4 CoRs. *In silico* 3D modeling using the Rosetta software suite was used to model interactions between TCR-CD8CoR with peptide-HLA-I (pHLA-I) complexes. Primary T cells were engineered to express MAGE-A1 or PRAME-specific TCRs and different CD8 CoRs. T cell function was evaluated by measuring cytokine production using multiplex cytokine bead array (LEGENDplex) and T-cell-mediated killing using time-lapse live-cell microscopy in 2D cultures and 3D tumor spheroid models.

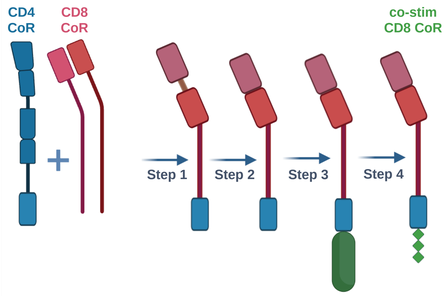
Co-stim CD8 CoR enhances TCR-T therapy by engaging the cytotoxic potential of CD4 T cells and delivering co-stimulation to CD4 and CD8 T cells



Conclusions:

- **Innovative Co-Receptor Design:** Using advanced protein engineering, we have designed a chimeric co-stim CD8 CoR with built-in co-stimulation, enhancing T cell performance beyond the natural wild-type counterpart.
- **Improved Cytotoxic Potential:** Expression of the co-stim CD8 CoR enables HLA-class I restricted cytotoxic activity in CD4 T cells and enhances cytokine secretion, broadening immune functionality.
- **Enhanced T Cell Fitness and Functionality:** The combination of co-stim CD8 CoR with a PRAME TCR leads to improved T cell fitness and higher functional activity compared to alternative PRAME TCR-T approaches, including clinical-stage candidates.
- **Potential for Broader Tumor Responses:** Incorporation of the co-stim CD8 CoR in TCR-T constructs may contribute to deeper and broader responses in hard-to-treat solid tumors.

Development of a single chain CD8 co-receptor with additional co-stimulation



Step 1: A functional **single-chain CD8 CoR scaffold** was generated by integrating building blocks from the CD8α, CD8β and CD4 CoR chains

Step 2: To prevent interference with MHC binding, the **linker peptide** between CD8α and CD8β was designed to **fit into a hydrophobic crevice** in CD8α

Step 3: To compensate for the lack of co-stimulatory molecules on cancer cells and add signal 2 simultaneously with TCR engagement **multiple co-stimulatory domains** were tested

Step 4: Activity enhancement and smaller construct size was achieved by **combination of specific binding motifs** of the construct prioritized in step 3

Step 1: Design of the single-chain scaffold

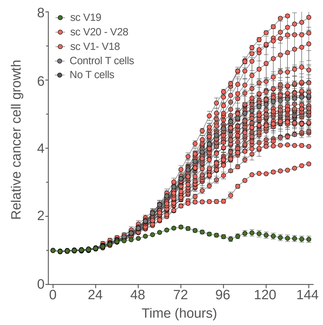


Figure 1. Candidate sc V19 was selected based on superior cytotoxic activity of gene edited CD4 T cells. CD4 T cells expressing a MAGE-A1 TCR and scCoR versions were co-cultured at 3:1 E:T ratio with NCI-H2030 lung cancer cells overexpressing HLA-A*02:01.

Step 2: Design of the optimal linker

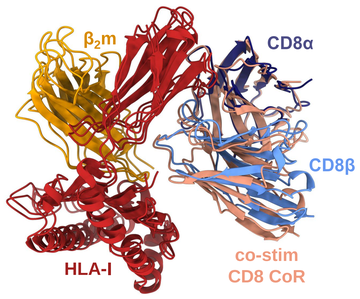


Figure 2. WT CD8 CoR and co-stim CD8 CoR have the same binding mode to the pHLA-I complex. A ribbon diagram depicting superposition of Ig domains of WT CD8 CoR αβ chains, co-stim CD8 CoR, pHLA-I, and β_2m. Computational modeling was performed by **Cube Biotech.**

Step 3: Addition of co-stimulatory signaling

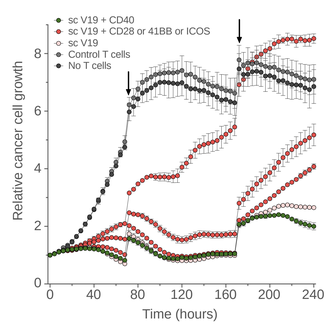


Figure 3. Incorporating CD40 signaling domains enabled sustained cytotoxic activity upon repeated antigen stimulation, superior to commonly used 4-1BB and CD28 domains. CD4 T cells expressing a MAGE-A1 TCR and versions of CD8 CoR were co-cultured with NCI-H2030 lung cancer cell line overexpressing HLA-A*02:01 at 5:1 E:T ratio. Arrows indicate addition of fresh cancer cells.

Step 4: Co-stimulation optimization

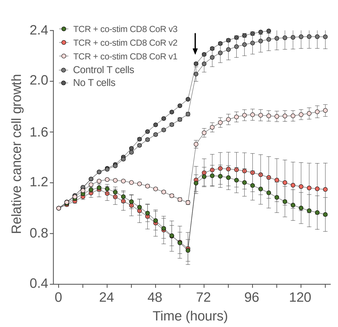


Figure 4. Substitution of WT CD40 cytoplasmic domain with a specific combination of TRAF binding motifs improved cytotoxic efficiency. CD4 and CD8 T cells expressing a PRAME TCR and co-stimulatory single-chain CD8 CoRs were co-cultured with Hs695T melanoma cell line at 1:4 E:T ratio. Arrow indicates addition of fresh cancer cells.

Co-stim CD8 CoR confers superior HLA-Class I restricted effector functions to CD4 T cells compared to commonly used wild-type CD8 CoR approaches

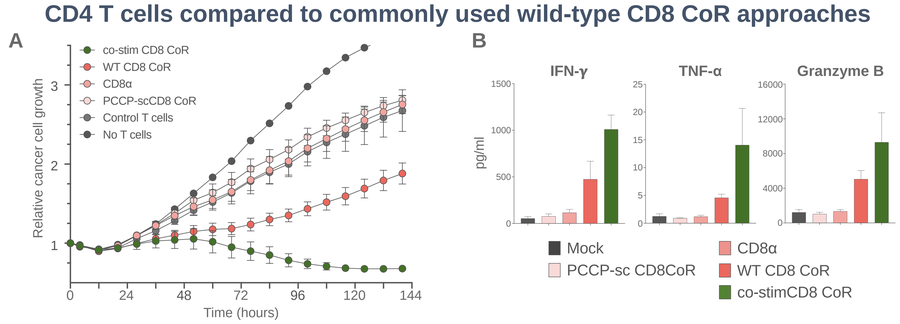


Figure 5. Compared to other commonly used CD8 CoRs, CD4 T cells expressing co-stim CD8 CoR demonstrate superior cytotoxic activity and effector molecule secretion. (**A**) TCR-T CD4 T cells expressing a PRAME TCR and co-stim CD8 CoR or known clinical-stage (CD8α and CD8αβ) or preclinical-stage (PCCP-sc) approaches were evaluated by co-culture with HLA-A*02:01 overexpressing SK-MEL-28 melanoma cells at 4:1 E:T ratio. (**B**) Cytokine secretion was determined 72 hours post start of co-culture. (Data shown as mean ±SEM, n= 3).

Co-stim CD8 CoR expressing CD4 and CD8 T cells display enhanced and durable activity compared to WT CD8 CoR

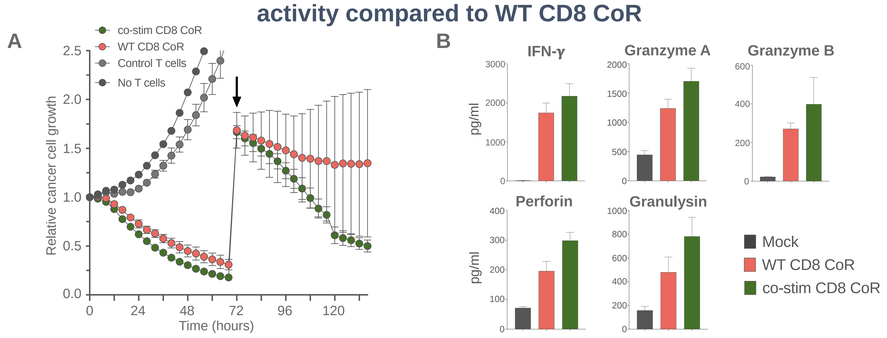


Figure 6. Compared to the most commonly used WT CD8 CoR, co-stim CD8 CoR expressing cells exert more durable anti-tumour responses. (**A**) CD4 and CD8 T cells expressing a PRAME TCR and the indicated CD8 CoRs were co-cultured with NCI-H2030 lung cancer cell line overexpressing HLA-A*02:01 at 4:1 E:T ratio. Arrow indicates addition of fresh tumor cells. (**B**) Cytokine secretion was determined from supernatants collected at 96 hours post start of co-culture with Hs695T melanoma cell line at a 1:6 E:T ratio. (Data shown as mean ±SEM, n= 2-3).

Co-stim CD8 CoR confers enhanced anti-tumor activity in multi-cellular 3D cancer spheroid models

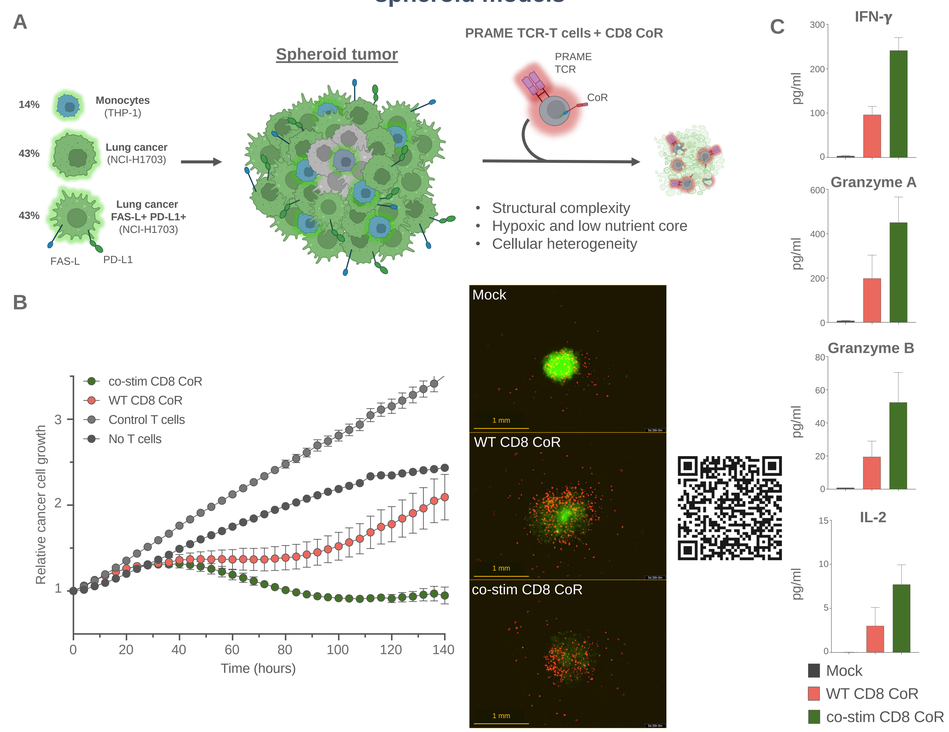


Figure 7. TCR-T cells expressing co-stim CD8 CoR demonstrate superior cytotoxic activity and effector molecule secretion in advanced 3D spheroid model. (**A**) Schematic of 3D spheroid formation and subsequent co-culture assay. GFP+ cancer cell mixtures were grown for 5 days in ultra-low attachment plates. Engineered T cells were stained with Cytolight Red dye prior to co-culture with individual spheroids at a 1:10 E:T ratio. (**B**) Spheroid fluorescence was evaluated by time-lapse live cell microscopy. Representative images taken at the end of culture. (**C**) Cytokine secretion was determined 120 hours post co-culture initiation. (Data shown as mean ±SEM, n= 3).

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