# Chimeric CD3 Fusion Receptors Expressed on iPSC-derived Universal TCR-less CAR-T and -NK Cells Synergize with Bispecific Engagers to Enhance Antitumor Activity and Limit Antigen Escape

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## Introduction

Despite success in hematologic malignancies, chimeric antigen receptor (CAR) T cells have been less effective in solid tumors, in part because of the heterogeneous expression of the CAR-specific target antigen (1° Ag) found within the tumor mass. In combination with a CAR, bispecific T cell engagers (BiTEs) can target additional tumor antigens (2° Ag) while engaging CD3 signaling molecules on T cells, providing a unique way to address tumor heterogeneity, mitigate antigen escape and further potentiate durable effector function. However, in generating off-the-shelf universal CAR-T and NK cells, a combination strategy is not feasible as neither modified cell product is compatible to specifically engage with a BiTE. In developing allogeneic CAR-T cells, the T cell receptor (TCR) surface expression must be eliminated to prevent graft versus host disease, but the absence of surface TCR expression leads to loss of surface CD3 expression and BiTE compatibility. Similarly, NK cells naturally lack TCR expression and have no surface CD3 molecules for BiTE engagement.

Here we discuss the development of a novel chimeric CD3ε fusion receptor (3e-28-3e* CFR) with a modified transmembrane and endodomain enabling surface expression in TCR-less T or NK cells, allowing for a novel combinatorial solution between universal CAR-T or NK cells with BiTEs in an allogeneic setting.

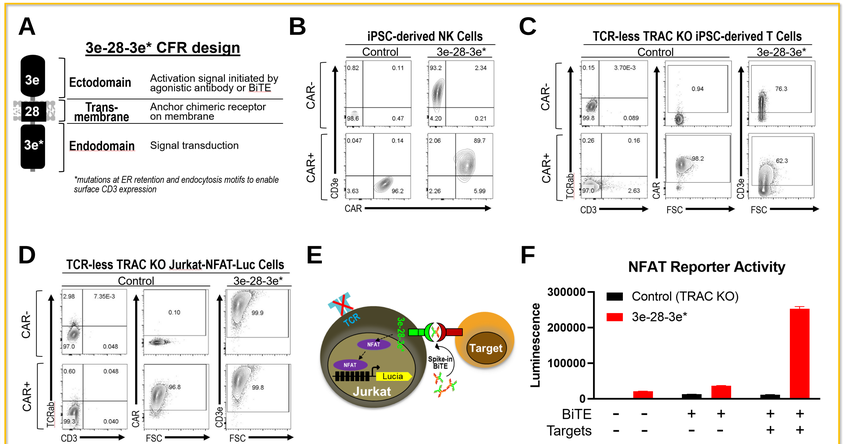


**Figure 1. (A)** Chimeric CD3 fusion receptor (CFR) design allowing surface expression on TCR-less **(B)** iNK cells, **(C)** TRAC knockout iT cells, and **(D)** TRAC KO Jurkat-NFAT-Luc cells. **(E)** Illustration of signal transduction from the CFR via BiTE engagement. **(F)** NFAT reporter activity in CFR+ Jurkats in the presence of BiTE and Targets.

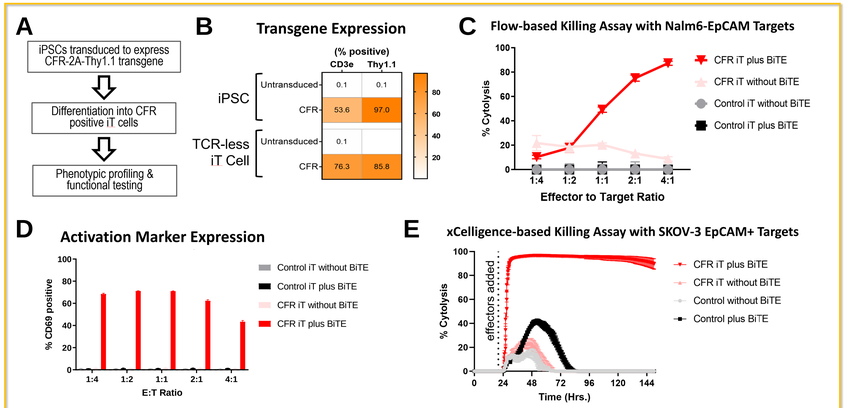


**Figure 2. (A)** Schematic showing the generation of an iPSC population expressing the chimeric CD3 fusion receptor followed by subsequent differentiation of the iPSC population into CFR+ iT cells which can be functionally tested. **(B)** Surface expression of CFR and Thy1.1 marker, indicating transgene expression at the iPSC stage and in fully differentiated TCR-less iT cells. **(C)** Flow-based killing assay with Nalm6 targets overexpressing EpCAM. **(D)** Flow cytometric detection of the activation marker CD69 on iT cells from the co-culture with Nalm6 Targets in Fig. 2C. **(E)** Killing assay with EpCam+ SKOV-3 target cells on the xCELLigence platform. (Note: BiTE: αCD3e x αEpCAM BiTE)

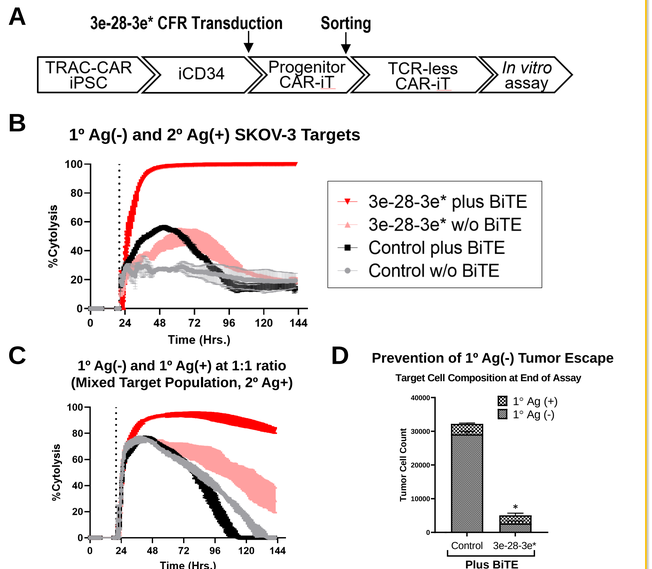


**Figure 3. (A)** Schematic showing the differentiation of iPSCs into TCR-less CAR-iTs and the lentiviral transduction strategy for introducing the 3e-28-3e* CFR at the progenitor CAR-iT stage. **(B)** xCelligence-based killing assay with SKOV-3 target cells expressing only the 2º Ag which can be targeted by engagement of the CFR with a BiTE and the target cell. **(C)** TCR-less CAR-iTs expressing the 3e-28-3e* CFR show robust antitumor function toward a mixed target population with addition of BiTE (αCD3e x αEpCAM). **(D)** Flow cytometric analysis of the target cells at the end of the co-culture from Fig. 3C, demonstrating the ability of 3e-28-3e* CFR+ CAR-iT cells to mitigate escape of 1º Ag negative tumor cells.

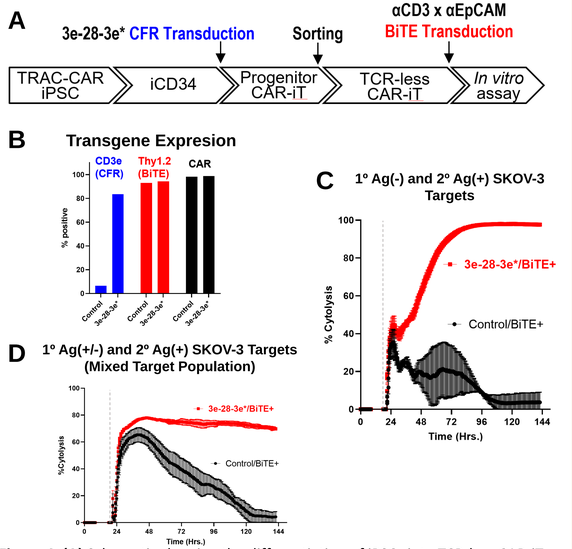


**Figure 4. (A)** Schematic showing the differentiation of iPSCs into TCR-less CAR-iTs and the lentiviral transduction strategy for introducing the 3e-28-3e* CFR construct at the progenitor CAR-iT stage and the BiTE construct (αCD3e x αEpCAM) in fully differentiated TCR-less CFR+ CAR+ iT cells. **(B)** Expression of the CFR, BiTE, and CAR as determined by flow cytometric detection of surface expression for CD3e, Thy1.2, and CAR in the TCR-less effector cells. **(C)** xCelligence-based killing assay with target cells lacking the CAR target antigen (1º Ag) while expressing the EpCAM, the BiTE target antigen (2º Ag). **(D)** xCelligence-based killing assay with a mixed target cell population (1º Ag+/- and 2º Ag+) showing superior heterogenous tumor killing ability of CFR+ BiTE+ CAR+ effector cells.

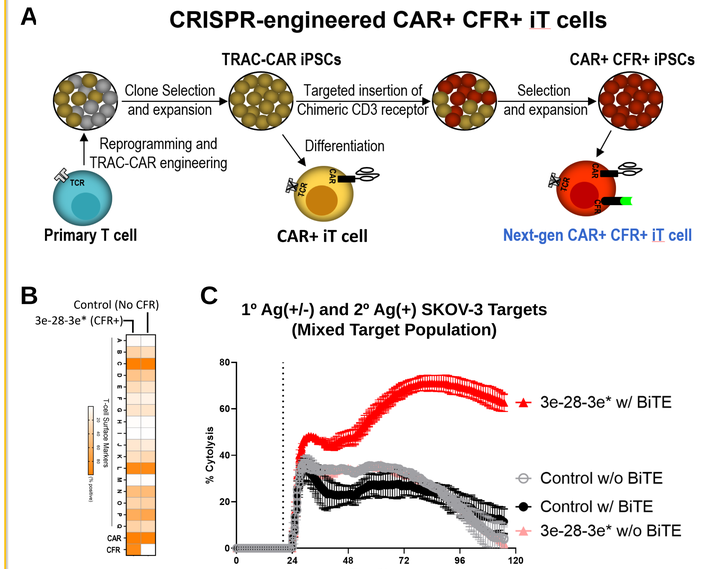


**Figure 5. (A)** Schematic showing the generation of a TRAC-CAR iPSC line followed by CRISPR-targeted insertion of the 3e-28-3e* chimeric CD3 fusion receptor (CFR) into a defined gene locus and subsequent differentiation of engineered iPSC population into CFR+ CAR+ iT cells. **(B)** Phenotypic profiling of Control (no CFR) and CFR+ CAR+ iT cells at the end of the differentiation process showing normal expression of T cell markers and robust expression of the CAR and CFR. **(C)** xCELLigence-based killing assay demonstrating superior cytolytic ability of CFR+ CAR+ iT cells toward a mixed target population (1º Ag+/- and 2º Ag+) in the presence of BiTEs.

## Summary of Results

1. A functional Chimeric CD3 Fusion Receptor (CFR) can be uniquely expressed on the surface of off-the-shelf TCR-less T and NK effector cells **(Figure 1)**.
2. Engineered iPSCs can be differentiated into CFR+ effectors that are compatible with BiTE technology **(Figure 2)**.
3. CFR+ CAR-iT cells were generated from progenitor CAR-iTs engineered with the 3e-28-3e* CFR construct. These effector cells allow targeting of a secondary antigen in heterogenous tumors **(Figure 3)**.
4. CFR+ CAR+ iT cells can be engineered to secrete Bispecific T cell Engagers **(Figure 4)**, resulting in BiTE-dependent activity similar to that seen with spike-in of BiTE **(Figure 3)**.
5. Targeted insertion of the 3e-28-3e* CFR construct at the iPSC stage is feasible, and CAR-iT cells generated from these iPSCs exhibit a normal phenotypic profile while maintaining the added functionality provided by the CFR construct – secondary antigen targeting via BiTEs **(Figure 5)**.

## Conclusion

Chimeric CD3 Fusion Receptors (CFRs) present a unique strategy to enhance the antitumor activity of off-the-shelf cellular therapies that utilize chimeric antigen receptors (CARs) to target a primary antigen on tumor cells. CFR+ CAR+ effector cells can synergize with BiTEs to limit antigen escape in heterogenous tumors and elicit durable responses in challenging tumor settings.