# Generation of Off-the-Shelf TCR-less CAR-targeted Cytotoxic T cells from Renewable Pluripotent Cell for Cancer Immunotherapy

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## ABSTRACT

Genetically engineered T cells are at the vanguard of an emerging wave of scientific breakthrough focused on harnessing the power of the immune system to treat cancer and other immune disorders. Perhaps the most dramatic clinical outcome to date has been demonstrated in clinical trials evaluating autologous chimeric antigen receptor (CAR) therapy for the treatment of refractory B cell ALL where complete responses in the majority of the patients have been reported. While adoptive CAR-T cell treatments hold great promise, pressing challenges remain to ensure multi-parameter genetically engineered T-cell immunotherapies can be successfully derived, cost-effectively and consistently manufactured, and safely and reliably delivered at the scale necessary to support wide patient base commercialization.

Human induced pluripotent stem cell (hiPSC)-derived T cells uniquely represent a practical and renewable supply of well-defined engineered CAR-T cells for various therapeutic applications. We have previously described a novel platform to facilitate multi-gene locus-specific engineering of hiPSCs at the single cell level to establish highly characterized master cell banks which can then be repeatedly applied to our stage-specific lymphocyte directed differentiation process to reproducibly and reliably generate engineered cytotoxic T cells.

Here we present the first set of pre-clinical data for FT819, a first-of-kind off-the-shelf hiPSC-derived CAR-T cell product. To generate FT819, we successfully combined reprogramming of peripheral blood derived T cells with targeted insertion of a CD19 CAR into the T cell receptor α (*TRAC*) locus under the transcriptional control of its endogenous regulatory elements to generate a single cell-derived clonal *TRAC*-targeted CAR expressing master hiPSC line. The clone was characterized to be pluripotent (>95% SSEA4 / TRA181) and consisted of bi-allelic disruption of TRAC locus. During the stage-specific differentiation, the hiPSC line faithfully converted into CD34 positive cells which were then differentiated towards CD8 positive cells with uniform CAR expression (95 +/- 5%) in the absence of TCR expression, eliminating the likelihood of GvHD. In vitro functional studies demonstrated that FT819 elicits an efficient cytotoxic T lymphocyte response to CD19 antigen challenge with production of effector cytokines (IFNγ, TNFα, IL2), degranulation (CD107a/b, Perforin, Granzyme B), proliferation (>85% entry into cell cycle) and upregulation of activation markers CD69 and CD25. Importantly, FT819 targets tumor in an antigen specific manner as demonstrated by lysis of CD19+, but not CD19-, Raji and Nalm6 tumor cell lines. In summary, FT819 holds the promise of a safe and efficacious off-the-shelf cytotoxic CAR-T cell product derived in a renewable and highly reproducible process analogous to biopharmaceutical drug products.

## SUMMARY

1. T cells with a CAR targeted to the *TRAC* locus can be reprogrammed to generate an engineered master pluripotent cell line, TRAC-CAR TiPSC
2. TRAC-CAR TiPSC can effectively and reproducibly differentiate into a potent, antigen specific CAR-T cell product, FT819
3. FT819 TRAC-CAR-iT cells are void of TCR expression and carry no risk of TCR-mediated graft vs host disease (GvHD) or alloreactivity
4. Engineered CAR-expressing iT cells expressing a high-affinity, non-cleavable CD16 Fc receptor represent a second approach to target tumor cells and broadly address antigen escape
5. FT819 manufacturing process is amenable to the scale required for a true off-the-shelf cellular immunotherapeutic

## REFERENCES

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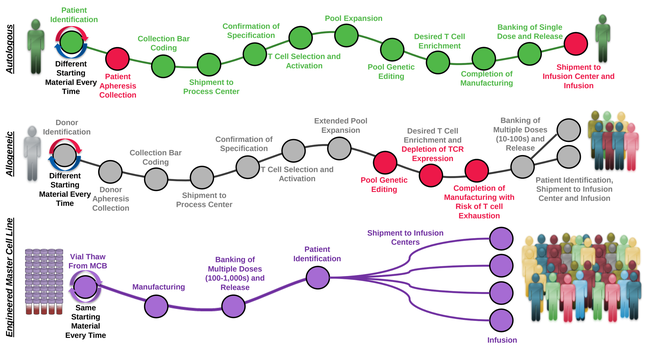
## INTRODUCTION

*Why use human iPSC as a source for off-the-shelf CAR-T cell therapeutics?*

RENEWABLE | REPRODUCIBLE| SCALABLE

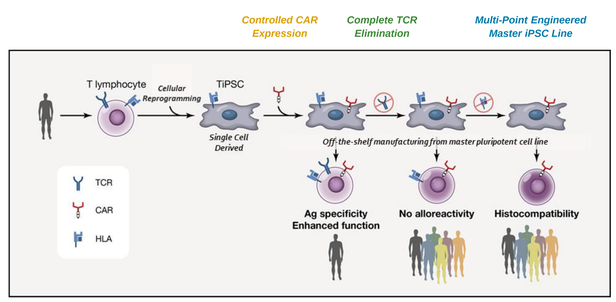



*FT819: first-of-kind off-the-shelf human iPSC-derived CAR-T cells*

OBJECTIVES

- Generation of Single Cell-Derived, Genetically Edited, Pluripotent Master Cell Line
- Derivation of Potent Engineered T cells (αβ and γδ)
- Generation of allogeneic TCR$^{-/-}$ CAR CD16 T cells
- Reproducible Expansion Off-the-Shelf Banking
- Open Clinical Trial

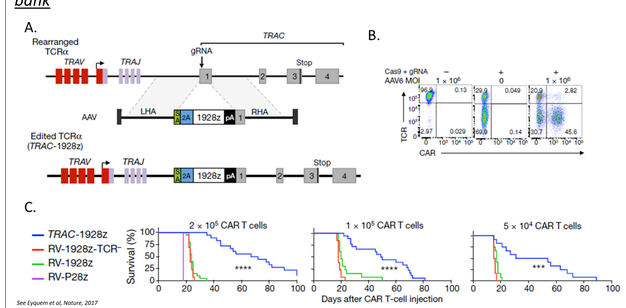
*Genetically engineered iPSC-derived T cells represent a powerful therapy for patients with cancer*




Adapted from: Themeli, Riviere & Sadelain, *Cell Stem Cells*, 2015

## RESULTS

*Concurrent genetic engineering and reprogramming to generate TRAC-CAR TiPSC bank*




*See Eyquem et al, Nature, 2017*

**Figure 1. From Eyquem et al. Nature 2017. Primary *TRAC*-CAR T cells outperform conventional CAR-T cells.** A. CRISPR/Cas9-targeted CAR gene integration into the *TRAC* locus. Top, *TRAC* locus; middle, rAAV6 contain the CAR cassette flanked by homology arms; bottom, edited *TRAC* locus. B. Representative TCR/CAR flow plots 4 days after TRAC targeting. C. Kaplan-Meier analysis of survival of mice treated with NALM-6 tumor.

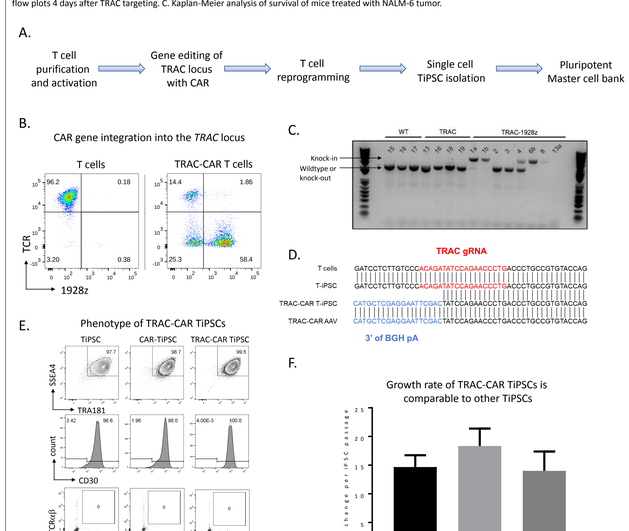



**Figure 2. Concurrent engineering and reprogramming of *TRAC*-targeted T cells.** A. Schematic of the engineering process. B. Flow cytometric analysis of TCR/CAR expression on peripheral blood derived T cells 3 days after TRAC-targeting. C/D. Confirmation of TRAC locus gene editing by PCR (B) and sequencing (C). Top row: TRAC sequence in normal T cells – red indicates sequence targeted by guide RNA. Second row: TRAC sequence in TiPSC (WT control made alongside the TRAC-CAR T-iPSC), showing normal TRAC sequence. Third row: sequence of TRAC-CAR T-iPSC showing disruption of the TRAC locus and insertion of the CAR transgene (sequencing detects the 3' BGH polyA-sequence at the end of the CAR sequence). Bottom row: TRAC-CAR AAV sequence, AAV sequence used to introduce the CAR sequence into the TRAC locus. E. Phenotypic analysis for pluripotency and T cell marker expression in TRAC-CAR TiPSC. F. Gene editing of the *TRAC* locus does not alter the growth rate of TRAC-CAR TiPSC

*Stage-specific T cell differentiation*

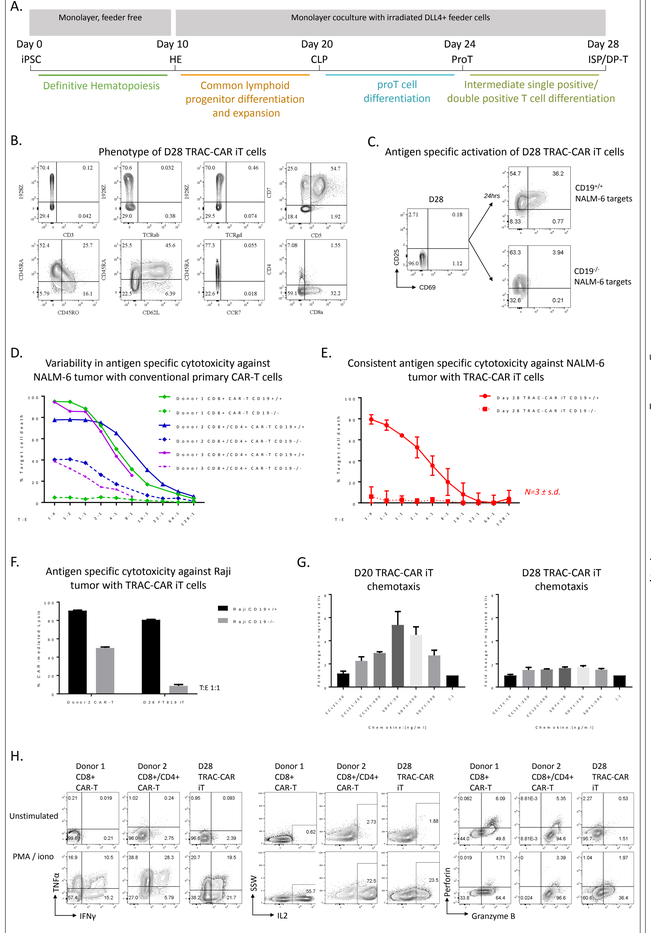



**Figure 3. Generation of functional CAR-T lymphocytes from TRAC-CAR TiPSC.** A. Schematic of iPSC-derived T cell (iT) differentiation protocol. B. D28 TRAC-CAR iT cells were assessed for the expression of T cell maturation markers by flow cytometry. C. D28 TRAC-CAR iT cells were assessed for their functional capacity to respond to CD19 antigen by upregulation of the T cell activation markers CD25 and CD69 after 24hrs of coculture with NALM-6 tumor targets. D/E. In vitro cytotoxicity using a 18hr flow cytometry assay using wildtype (CD19+/+) or knockout (CD19-/-) NALM-6 as target cells. 3 independent experiments on 3 separate primary CAR-T cells. N=3 independent experiments on 3 separate TRAC-CAR iT batches. F. In vitro cytotoxicity using a 18hr flow cytometry assay using wildtype (CD19+/+) or knockout (CD19-/-) Raji as target cells at a 1:1 T:E ratio. G. D28 TRAC-CAR iT cells were assessed for chemotaxis in response to the indicated thymus-derived chemokines in a transwell migration assay. Developing T cells lose migratory capacity to thymus-derived chemokines during maturation. H. D28 TRAC-CAR iT cells were assessed for the generation of the proinflammatory cytokines IFNγ and TNFα, the pro-survival cytokine IL-2 and the cytolytic molecule Granzyme B in response to PMA/ionomycin stimulation for 4 hours.

*T cell maturation and expansion*

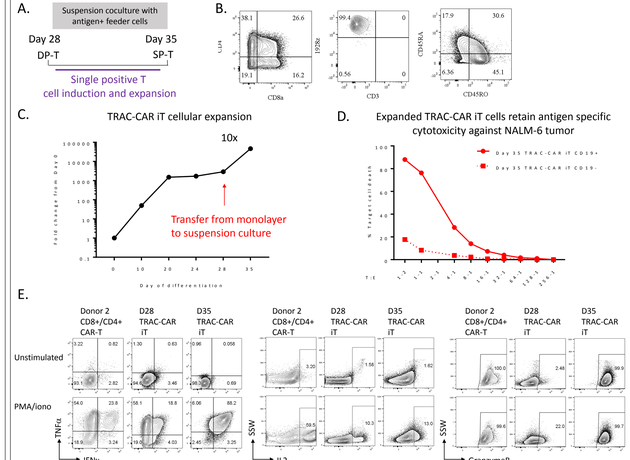



**Figure 4. Expansion and maturation of TRAC-CAR iT cells.** A. Schematic of 7 day expansion protocol using coculture with irradiated CD19+ feeder cells. B. D35 TRAC-CAR iT cells were assessed for T cell maturation markers by flow cytometry. C. Cellular expansion of TRAC-CAR iT cells during the 35 day differentiation process. One production run results in >40, 000 fold increase in cellular yield from starting TRAC-CAR TiPSC. D. In vitro cytotoxicity using a 18hr flow cytometry assay using wildtype (CD19+/+) or knockout (CD19-/-) NALM-6 as target cells. E. D35 TRAC-CAR iT cells were assessed for the generation of the proinflammatory cytokines IFNγ and TNFα, the pro-survival cytokine IL-2 and the cytolytic molecule Granzyme B in response to PMA/ionomycin stimulation for 4 hours.

*Mitigating antigen escape through antibody dependent cell-mediated cellular cytotoxicity (ADCC)*

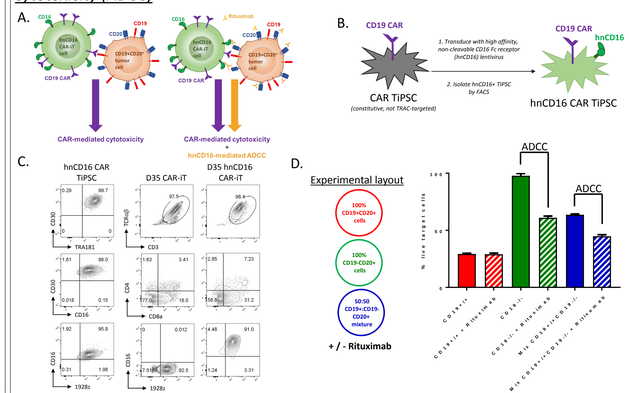



**Figure 5. Engineered CAR-iT cells expressing a high affinity, non-cleavable version of CD16 (hnCD16) represents a secondary approach to target tumor.** A. Schematic of CAR and hnCD16 dual cytotoxic targeting of CD19 and CD20 expressing tumor in the presence of anti-CD20 Rituximab. Engagement of CD16 by the Fc portion of monoclonal antibodies activates T cell cytolytic activity. B. CD16 construct used to make hnCD16 CAR TiPSC utilizes high affinity (F158V) and non-cleavable version of CD16. CAR TiPSC were transduced with hnCD16 and enriched by FACS for CD16. C. hnCD16 CAR TiPSC were assessed for the expression of pluripotency markers, CAR and CD16. hnCD16 CAR TiPSC were differentiated into T cells and assessed for T cell maturation markers. D. CAR and hnCD16 ADCC-mediated cytotoxicity against CD19+/+ and CD19-/- Raji cells. Survival of target cells was quantified by Incucyte Zoom after 36 hours in the presence and absence of anti-CD20 monoclonal antibody Rituximab. hnCD16 CAR iT cells mediated substantial CAR-mediated killing of wildtype CD19+/+ targets and hnCD16 ADCC-mediated killing of CD19-/- CD20+ targets.