

## Journal Pre-proof

Chimeric STAR receptors enable high-sensitivity TCR-negative CD8 $\alpha\beta$ - CD3-positive T-cell development from human-induced pluripotent stem cells

Chaoqi Zhang , Michael Rassner , Yohei Kawai , Qingyi Guo , Akihiro Ishikawa , Bo Wang , Shin Kaneko

PII: S1465-3249(25)00896-5  
DOI: <https://doi.org/10.1016/j.jcyt.2025.102001>  
Reference: JCYT 102001



To appear in: *Cytotherapy*

Received date: 18 August 2025  
Accepted date: 8 November 2025

Please cite this article as: Chaoqi Zhang , Michael Rassner , Yohei Kawai , Qingyi Guo , Akihiro Ishikawa , Bo Wang , Shin Kaneko , Chimeric STAR receptors enable high-sensitivity TCR-negative CD8 $\alpha\beta$ - CD3-positive T-cell development from human-induced pluripotent stem cells, *Cytotherapy* (2025), doi: <https://doi.org/10.1016/j.jcyt.2025.102001>

This is a PDF of an article that has undergone enhancements after acceptance, such as the addition of a cover page and metadata, and formatting for readability. This version will undergo additional copyediting, typesetting and review before it is published in its final form. As such, this version is no longer the Accepted Manuscript, but it is not yet the definitive Version of Record; we are providing this early version to give early visibility of the article. Please note that Elsevier's sharing policy for the Published Journal Article applies to this version, see: <https://www.elsevier.com/about/policies-and-standards/sharing#4-published-journal-article>. Please also note that, during the production process, errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

© 2025 Published by Elsevier Inc. on behalf of International Society for Cell & Gene Therapy.

## Highlight

- Chimeric STAR receptor restored CD3 $\epsilon$  expression
- Chimeric STAR receptor supported T cell differentiation from iPSC
- STAR-expressing iPSC-derived T cells demonstrated enhanced sensitivity and anti-tumor efficacy than CAR-expressing iPSC-derived T cells

**Chimeric STAR receptors enable high-sensitivity TCR-negative  
CD8 $\alpha\beta$ - CD3-positive T-cell development from human-induced pluripotent stem  
cells**

Chaoqi Zhang<sup>†,1</sup>, Michael Rassner<sup>†,1,2</sup>, Yohei Kawai<sup>†,1</sup>, Qingyi Guo<sup>1</sup>, Akihiro Ishikawa<sup>1</sup>, Bo Wang<sup>1</sup>, Shin Kaneko<sup>1,\*</sup>

<sup>1</sup>Shin Kaneko Laboratory, Department of Cell Growth and Differentiation, Center for iPS Cell Research and Application (CiRA), Kyoto University, Kyoto, Japan

<sup>2</sup>Department of Medicine I, Medical Center – University of Freiburg, Faculty of Medicine, University of Freiburg, Freiburg, Germany

<sup>†</sup>Contributed equally

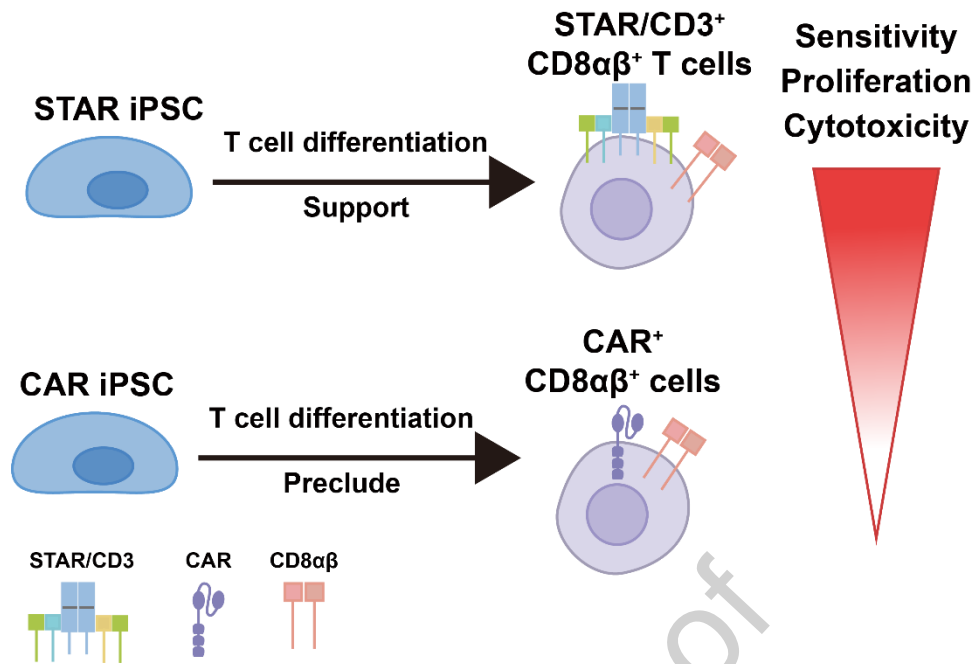
**\*Correspondence**

Prof. Dr. Shin Kaneko

Email: [kaneko.shin@cira.kyoto-u.ac.jp](mailto:kaneko.shin@cira.kyoto-u.ac.jp)

**Key words:** induced pluripotent stem cell, T-cell receptor, cytotoxic T lymphocyte, chimeric antigen receptor, synthetic T cell receptor and antigen receptor, tumor-associated antigen

**Graphic Summary**



## Abstract

Induced pluripotent stem cell (iPSC)-derived T cells hold great promise for scalable and standardized allogeneic immunotherapies but face critical challenges in differentiation, antigen sensitivity, and inaccessibility to intracellular tumor-associated antigens (TAAs), particularly when engineered with chimeric antigen receptors (CARs). Here, we introduce iPSC with Synthetic TCR and Antigen Receptor (STAR) structure, which fuses a single-chain variable fragment (scFv) to the constant regions of TCRαβ. Using a Wilms tumor 1 (WT1)-specific STAR construct, we demonstrate that STAR-iPSC-derived T cells (STAR-iT cells) restore CD3 expression to levels similar to TCR, thereby supporting efficient CD8αβ<sup>+</sup> CD3<sup>+</sup> iPSC-T cell development in the absence of TCR. STAR-iT cells exhibit superior antigen sensitivity compared to CAR-based counterparts. STAR-iT cells display robust cytotoxicity against WT1-expressing tumor cells, enhanced cytokine production, and efficient in vitro expansion. Our findings establish STAR as a scFv-based, next-generation chimeric receptor format and advance the development of universal, off-the-shelf iT cell

immunotherapies.

## Introduction

Adoptive T cell therapy has revolutionized cancer treatment by engineering T cells with tumor-specific T cell receptors (TCRs) or chimeric antigen receptors (CARs) to redirect T cells against tumors(1, 2). However, current T cell therapies rely primarily on autologous T cells, posing challenges in batch variability, long manufacturing time, and limited scalability(3, 4).

Induced pluripotent stem cells (iPSCs) offer a promising alternative. Advances in feeder-free differentiation protocols have facilitated the generation of iPSC-derived T cells (iT cells) with clinical applicability(5, 6), enabling the generation of genetically modified, off-the-shelf T cells with potent anti-tumor activity(7-10).

Despite these advances, developing effective strategies to target solid tumors by CAR-iT cells remain a major challenge. First, solid tumors often lack specific surface antigens, as most tumor-associated antigens (TAAs) are expressed intracellularly and presented via HLA molecules at low levels(11-14). This limitation prevents CAR-T cells from effectively targeting such tumors, as they only recognize surface antigens. Second, although CAR typically has higher affinity than TCR(11, 12), it requires higher antigen density to effectively deliver activation signals. Distinct from CAR, TCR signaling can be triggered by a single HLA/peptide(13, 14), indicating that CAR possesses lower antigen sensitivity than TCR. Third, CAR cannot replace TCR in supporting iPSC-T cell differentiation. iT cells differentiation requires TCR/CD3 signaling but CAR cannot integrate into the CD3 signaling machinery(15). Instead, the second generation CAR containing

CD28 domain disrupt differentiation by inducing tonic signaling(8, 16).

Although TCR offers many advantages, selecting one with both appropriate affinity and minimal cross-reactivity is technically challenging. Because TAAs are weakly expressed in normal tissues, only low-affinity TCRs escape thymic deletion(11, 12), while artificially enhancing TCR affinity risks off-target reactivity(13, 14). By contrast, CAR generally exhibits suitable affinity and rare cross-reactivity issues.

To combine the advantages of CAR and TCR while compensating for their limitations, we propose a novel approach using a Synthetic TCR and Antigen Receptor (STAR) structure(17, 18) to support iPSC-T cell differentiation. The STAR receptor fuses the scFv variable regions to the constant regions of TCR $\beta$  and TCR $\alpha$  chains. These TCR constant regions interact with CD3(17, 19) and support iPSC-T cell differentiation. Previous studies have shown that STAR-expressing primary CD8 T cells have higher sensitivity against low antigen-expressing target cells than CAR-T cells using the same scFv(17). A similar design, HLA-independent TCR, also demonstrated higher antigen sensitivity compared to CAR in primary CD8 T cells(18). Therefore, we propose that incorporating a validated high-affinity, low-cross reactivity scFv into the STAR structure and expressing it in iPSC-derived T cells could confer improved antigen sensitivity and bypassing TCR affinity optimization.

To target intracellular antigens, we selected HLA-restricted scFvs. Previous research reported an scFv specific to WT1<sub>235–243</sub>/HLA-A\*24:02 complex, which shows strong, HLA-dependent cytotoxicity and no cross-reactivity across various HLA alleles—including HLA-B57:01(20), previously linked to cross-reactive WT1-specific TCR(21).

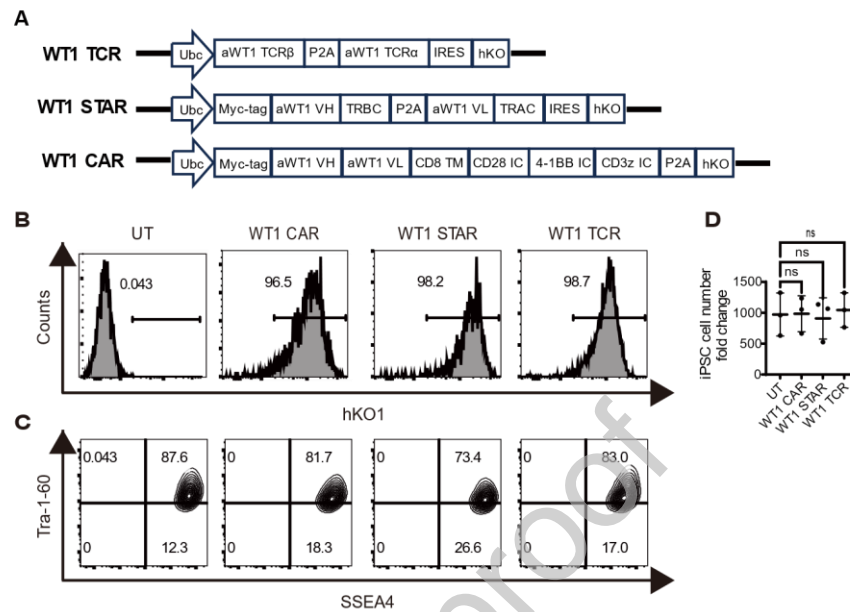
In this research, we engineered a STAR receptor incorporating the WT1<sub>235–243</sub>/HLA-

A\*24:02-specific scFv, and evaluated its ability to support iT cell differentiation. We demonstrate that STAR receptors transduction successfully recruit CD3 $\epsilon$  expression comparable to that of TCR, thereby enable the maturation of CD8 $\alpha\beta$ <sup>+</sup> CD3<sup>+</sup> iPSC-T cells in a TCR-independent manner. STAR-iT cells exhibited HLA-restricted cytotoxicity, cytokine production and demonstrated comparable sensitivity to TCR-iT cells, while outperforming CAR-T cells against WT1-expressing tumor cells. Our findings demonstrated STAR receptors resolve the structural incompatibility of scFv with iPSC-T cell differentiation, targeting intracellular tumor antigen, and substantially boosts their antigen responsiveness, which expand the toolkit for iPSC-T cell-based immunotherapy.

## Results

### Generation of WT1 STAR-Expressing iPSCs

We first constructed WT1-specific TCR, STAR, and CAR expression cassettes (Figure 1A). To confirm STAR's ability to engage the CD3 complex, we transduced the constructs into a TRAC/TRBC double-knockout Jurkat cell line(22). STAR, but not CAR, restored surface CD3 expression, indicating successful recruitment of the CD3 complex (Figure S1A, B). Next, we introduced each construct into an HLA-A\*24:02-positive iPSC clone (MH15s01) to generate WT1-TCR-, WT1-STAR-, and WT1-CAR-expressing iPSCs (Figure 1B). All engineered iPSCs maintained typical colony morphology (Figure S2A) and expressed pluripotency markers TRA-1-60 and SSEA4 (Figure 1C). Proliferation rates were comparable to untransduced (UT) iPSCs (Figure 1D)



**Figure 1 Generation of WT1-TCR, WT1-STAR and WT1-CAR expressing iPSCs**

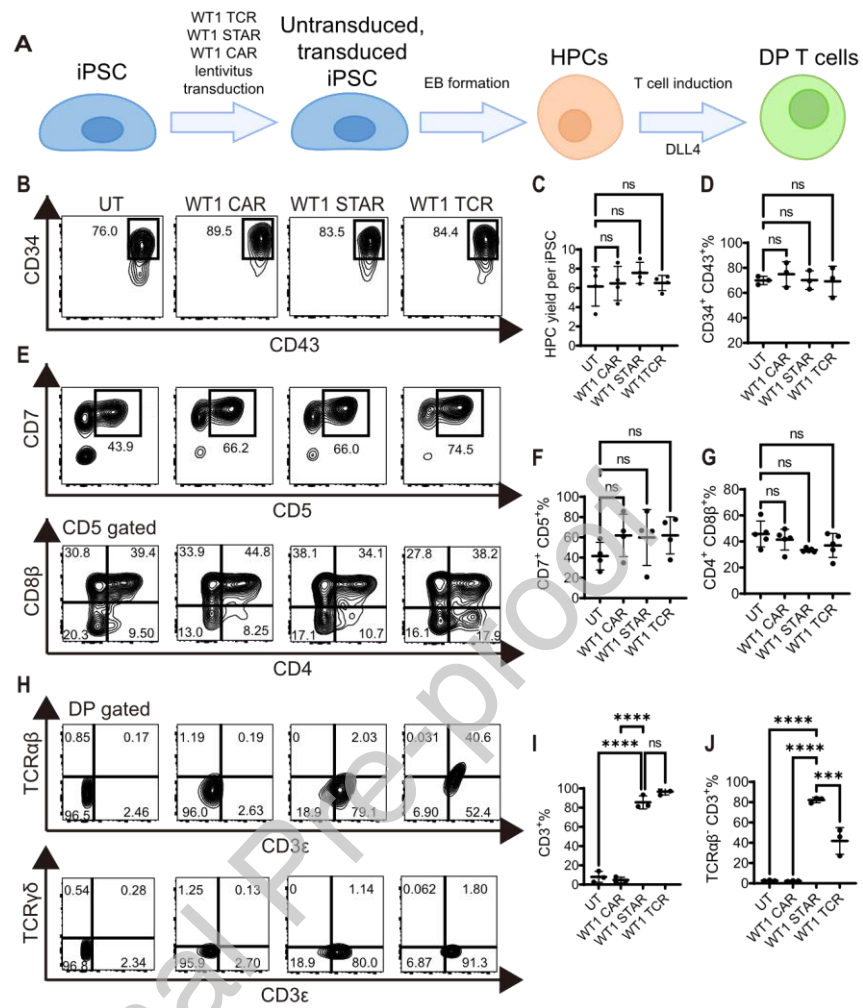
(A) Schematic representation of lentiviral constructs encoding WT1-TCR, WT1-STAR, and WT1-CAR, co-expressed with hKO1 reporter gene. (B) Representative flow cytometry plots for hKO1 expression in untransduced iPSC and WT1-CAR-, -STAR-, and -TCR-iPSCs. (C) Representative flow cytometry plots for pluripotency markers Tra-1-60 and SSEA4 in untransduced and transduced iPSCs. (D) Proliferation capacity of untransduced and transduced iPSCs. (n=3) Data represent mean ± SD of n independent experiments. One-way ANOVA with Tukey's multiple comparison test.

## WT1-STAR-transduced iPSCs differentiate into CD8αβ<sup>+</sup> CD3<sup>+</sup> TCR<sup>-</sup> iPSC-T cells

To assess whether WT1-STAR can support T cell development from iPSCs, we first initiated hematopoietic differentiation via the embryoid body (EB) method (Figure 2A).

STAR-iPSCs formed EBs with normal morphology (Figure S3A), and maintained hKO1 expression throughout differentiation, indicating stable transgene expression and no apparent growth disadvantage (Figure S3B). STAR-iPSC also generated CD34 positive HPCs at comparable amounts to UT (Figure 2B-D). Additionally, the STAR-iPSC-derived HPCs (STAR-iHPCs) exhibited low lineage marker expression, consistent with that of UT iPSC (Figure S3C-E). Consistent with previous studies that TCR or CAR transduction into iPSCs does not interfere with hematopoietic differentiation of iPSCs(8, 15, 23).

To induce T cell specification, we co-cultured iHPCs with DLL4-coated plate to activate Notch signaling, a pathway essential for T cell lineage commitment(5, 24). WT1-STAR-, TCR-, and CAR-iHPCs successfully differentiated into CD7<sup>+</sup> CD5<sup>+</sup> (Figure 2E-F) and CD4<sup>+</sup> CD8<sup>+</sup> double-positive (DP) T cells at a frequency similar to that of UT (Figure 2E, 2G). Consistent with previous studies(8, 16), the third-generation CAR used in this study did not impair T cell progenitor differentiation, because it did not induce CD3 $\zeta$  phosphorylation as a tonic signaling (Figure S4A).



**Figure 2 STAR is compatible with the iPSC platform and recruiting surface CD3 TCR-**

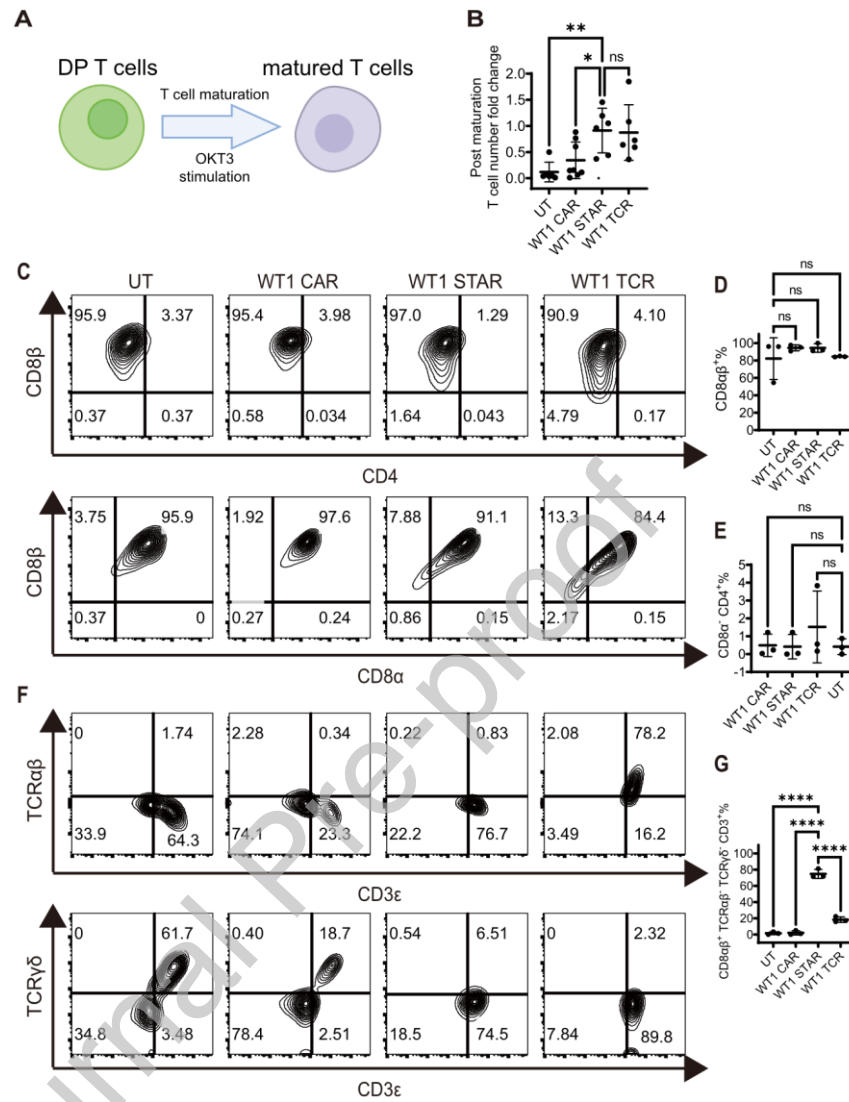
**independently**

**(A)** Schematic illustration of the iPSC production and differentiation process toward CD4<sup>+</sup> CD8<sup>+</sup> immature T cells. Lentiviral constructs encoding WT1-CAR, -STAR, and -TCR were individually transduced into iPSCs. These transduced iPSCs were differentiated into HPCs by EB method, followed by differentiation into immature T cells by feeder-free protocol. Transduced iPSCs: WT1-CAR-, -STAR-, and -TCR-iPSCs; EB: embryoid body differentiation method; HPCs: hematopoietic progenitor cells; DP: CD4, CD8 double positive immature T cells. **(B)** HPC produced per iPSC at EB day 14 differentiation. (n=3) Data represent mean  $\pm$  SD of n independent experiments. Statistical analysis was performed using one-way ANOVA with Tukey's multiple comparison test. **(C)** Percentage of CD34<sup>+</sup> CD43<sup>+</sup> at EB day 14 HPCs derived from untransduced iPSC, WT1-CAR-, -STAR-, and -TCR-iPSC. (n=3) Data represent mean  $\pm$  SD of n independent experiments. Statistical analysis was performed using one-way ANOVA with Tukey's multiple comparison test. **(D)** Representative flow cytometry plots of CD34 and CD43 expression at EB day 14 HPCs. **(E)** Representative flow cytometry plots of T cell markers on iHPCs after 21 days of FcDLL4 culture. CD7 and CD5 expression were detected, and CD4 and CD8 $\beta$  expression patterns within the CD5 positive population were measured. **(F)** Percentage of CD7<sup>+</sup> CD5<sup>+</sup> in FcDLL4 D21 cells. (n=3) Data represent mean  $\pm$  SD of n independent experiments. Statistical analysis was performed using one-way ANOVA with Tukey's multiple comparison test. **(G)** Percentage of CD4<sup>+</sup> CD8b<sup>+</sup> within CD5<sup>+</sup> cells at FcDLL4 day 21 cells. (n=3) Data represent mean  $\pm$  SD of n independent experiments. Statistical analysis was performed using one-way ANOVA with Tukey's multiple comparison test. **(H)** Representative flow cytometry plots of TCR $\alpha\beta$ , TCR $\gamma\delta$ , and CD3 $\epsilon$  expression on iDP T cells were measured. **(I)** Percentage of CD3<sup>+</sup> in iDP T cells. (n=3) Data represent mean  $\pm$  SD of n independent experiments. Statistical analysis was performed using one-way ANOVA with Tukey's multiple comparison test. \*\*\*\*p<0.0001 **(J)** Percentage of CD3<sup>+</sup> TCR $\alpha\beta$ <sup>+</sup> iDP T cells. (n=3) Data represent mean  $\pm$  SD of n independent experiments. Statistical analysis was performed using one-way ANOVA with Tukey's multiple comparison test. \*\*\*p<0.01; \*\*\*\*p<0.0001

Importantly, STAR-iHPCs generated CD3<sup>+</sup> DP T cells (iDP T cells) at levels equivalent to TCR-iHPCs (Figure 2H-I), but lacking endogenous TCR $\alpha\beta$  expression (Figure 2H, 2J, S5A). In contrast, UT- and CAR-iHPCs can only generated CD3<sup>+</sup> TCR<sup>-</sup> iDP T cells (Figure

2H-I), consistent with the feeder-free iPSC-T cell differentiation results(22). To assess whether CD3 $\epsilon$  expression on STAR-iDP T cells could support maturation, we stimulated the cells with the anti-CD3 $\epsilon$  agonist antibody, OKT3(5) (Figure 3A). Both STAR- and TCR-iDP T cells persisted, indicating they responded to CD3 agonist antibody; whereas UT- and CAR-iDP, T cells, which lacked CD3 $\epsilon$  expression, underwent significant contraction (Figure 3B). These results indicate that CD3 $\epsilon$  recruited by STAR is functional and can support T cell maturation.

Following OKT3 stimulation, STAR-iDP T cells matured into CD8 $\alpha\beta$  single positive (SP) T cells (Figure 3C-E). STAR-iPSC-derived T cells (STAR-iT cells) expressed CD3 $\epsilon$  at levels comparable to those of TCR-iT cells (Figure 3F, S5B) but lacked CR $\alpha\beta$  or TCR $\gamma\delta$  expression (Figure 3F-G, S5C-D), confirming that STAR is sufficient to replace TCR during CD8 $\alpha\beta$  iT cell maturation. Further phenotypic characterization revealed that STAR-iT cells expressed CCR7, CD62L, and CD45RA (Figure S6A), markers associated with memory-like T cells. These markers are positively correlated with enhanced proliferative capacity and antitumor efficacy(6, 25). We observed that TCR $\gamma\delta$ <sup>+</sup> CD3<sup>+</sup> cells were more frequent in UT- and CAR-iT cells, likely due to selective expansion of TCR $\gamma\delta$ <sup>+</sup> cells in response to OKT3 stimulation during the maturation phase, in contrast to CD3<sup>-</sup> populations (Figure 3F). These results demonstrate that the STAR/CD3 complex can effectively support CD8 $\alpha\beta$ <sup>+</sup> CD3<sup>+</sup> TCR<sup>-</sup> iT cell maturation.

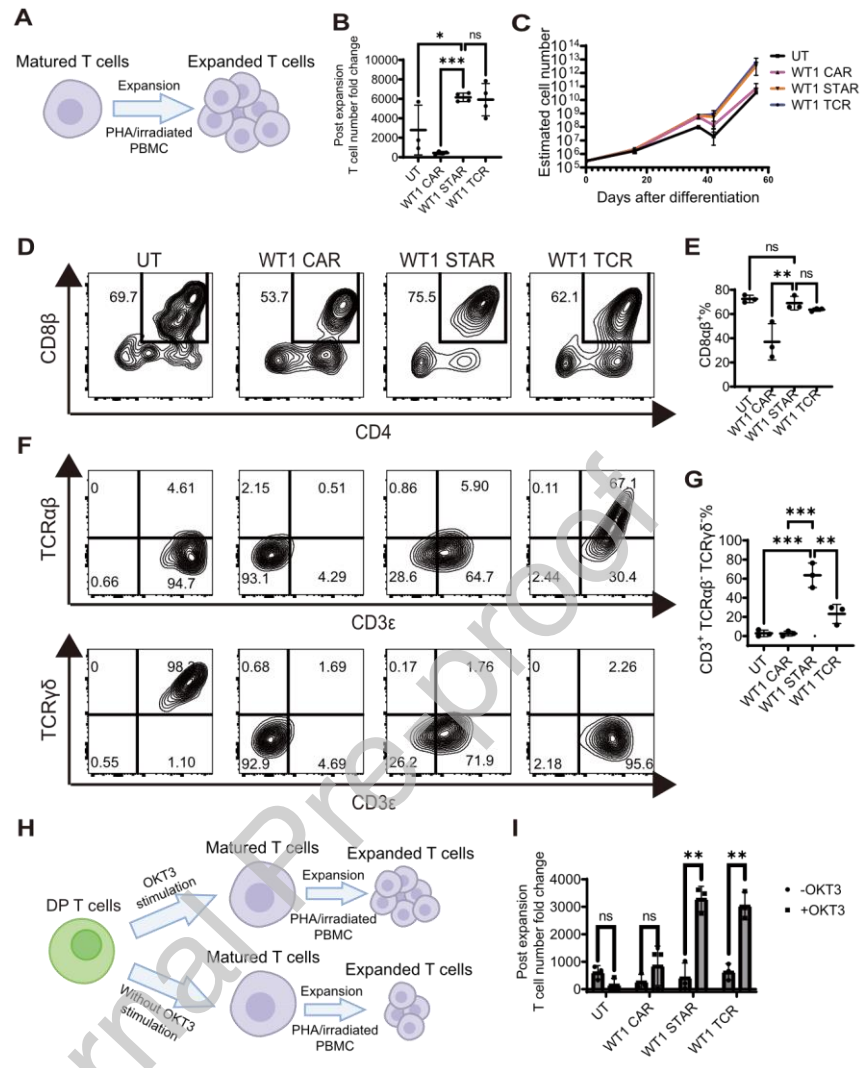


**Figure 3 STAR enables the generation of matured CD8αβ<sup>+</sup> CD3<sup>+</sup> TCR<sup>+</sup> iT cells**

(A) Schematic illustration of the maturation process for iDP immature T cells by OKT3 stimulation. (B) Cell proliferation 7 days after OKT3 maturation. (n=6-8) Data represent mean ± SD of n independent experiments. Statistical analysis was performed using one-way ANOVA with Tukey's multiple comparison test. \*\*p<0.01 (C) Representative flow cytometry plots of CD4, CD8α, and CD8β expression patterns in cells at day 7 of OKT3 maturation. (D) Percentage of CD8αβ<sup>+</sup> in cells at day 7 of OKT3 maturation. (n=3) Data represent mean ± SD of n independent experiments. Statistical analysis was performed using one-way ANOVA with Tukey's multiple comparison test. (E) Percentage of CD4<sup>+</sup> in cells at day 7 of OKT3 maturation. (n=3) Data represent mean ± SD of n independent experiments. Statistical analysis was performed using one-way ANOVA with Tukey's multiple comparison test. (F) Representative flow cytometry plots of CD3ε, TCRαβ, and TCRγδ in cells at day 7 of OKT3 maturation. (G) Percentage of CD8αβ<sup>+</sup> CD3<sup>+</sup> TCRαβ<sup>+</sup> and TCRγδ<sup>+</sup> in cells at day 7 of OKT3 maturation. (n=3) Data represent mean ± SD of n independent experiments. Statistical analysis was performed using by one-way ANOVA with Tukey's multiple comparison test. \*\*p<0.01; \*\*\*\*p<0.0001

To assess cell expansion potential of matured CD8αβ iT cells, we used the previously reported PHA/PBMC expansion protocol(23) (Figure 4A). STAR-iT cells exhibited robust expansion comparable to TCR-iT cells (Figure 4B). Notably, STAR-iT cells expanded

approximately 10 fold more than CAR-iT cells under the same conditions (Figure 4B). At the clonal level, each STAR-iPSC generated nearly 100-fold more iT cells than CAR-iPSCs. (Figure 4C). Throughout expansion, STAR-iT cells retained a CD8 $\alpha\beta$ <sup>+</sup> phenotype (Figure 4D-E) and maintained CD3 $\epsilon$  expression (Figure S5E) while lacking both TCR $\alpha\beta$  (Figure S5F) and TCR $\gamma\delta$  (Figure S5G) expression (Figure 4F-G). We hypothesized that the inferior proliferation of WT1 CAR iT cells compared to WT1 STAR iT cells is due to the lack of CD3 signaling during their maturation. Therefore, we examined the impact of OKT3 stimulation during iT cells maturation on their subsequent expansion ability. iDP T cells were matured with or without OKT3 stimulation and subsequently expanded (Figure 4H). OKT3-stimulated WT1-STAR- and -TCR-iDP T cells expanded robustly, whereas cells matured without OKT3 exhibited poor expansion, although they are CD3 $\epsilon$  positive (Figure 4J). These results highlight that STAR structure is superior to CAR and effectively replaces TCR in supporting iPSC-T cells.



**Figure 4 The CD8αβ<sup>+</sup> CD3<sup>+</sup> TCR<sup>-</sup> phenotype of STAR iT cells was maintained after expansion**

(A) Schematic illustration of iPSC-T cell expansion process with irradiated PBMC feeder. (B) Proliferation of UT, WT1-CAR-, WT1-STAR- and WT1-TCR-iT cells at day 14 of PHA/PBMC expansion. (n=3-4) Data represent mean ± SD of n independent experiments. Statistical analysis was performed using one-way ANOVA with Tukey's multiple comparison test. \*p<0.05 (C) Estimated cell yield per iPSC. Cell yield was calculated based on seeded iPS cell number and proliferation fold change. (n=3) Data represent mean ± SD of n independent experiments. (D) Representative flow cytometry plots of CD8α and CD8β expression during expansion at day 14 cells. (E) Percentage of CD8αβ<sup>+</sup> in cells during PHA/PBMC expansion at day 14. (n=3) Data represent mean ± SD of n independent experiments. Statistical analysis was performed using one-way ANOVA with Tukey's multiple comparison test. \*p<0.05 (F) Representative flow cytometry plots of CD3ε, TCRαβ, and TCRγδ expression pattern during PHA/PBMC expansion day 14 cells. (G) Percentage of CD3<sup>+</sup> TCRαβ<sup>-</sup> and TCRγδ<sup>-</sup> during expansion day 14 cells. (n=3) Data represent mean ± SD of n independent experiments. Statistical analysis was performed using one-way ANOVA with Tukey's multiple comparison test. \*\*p<0.01; \*\*\*p<0.001; \*\*\*\*p<0.0001 (H) Schematic illustration of the impact of OKT3 stimulation during maturation on iPSC-T cell expansion capacity. (J) Proliferation on day 8 of PHA/PBMC expansion for UT, WT1-CAR-, WT1-STAR- and WT1-TCR-iT cells with or without OKT3 stimulation during maturation. (n=3) Data represent mean ± SD of n independent experiments. Statistical analysis was performed using one-way ANOVA with Tukey's multiple comparison test. \*\*p<0.01

## STAR-iT cells have higher sensitivity against WT1<sup>+</sup> tumor cells than CAR-iT cells

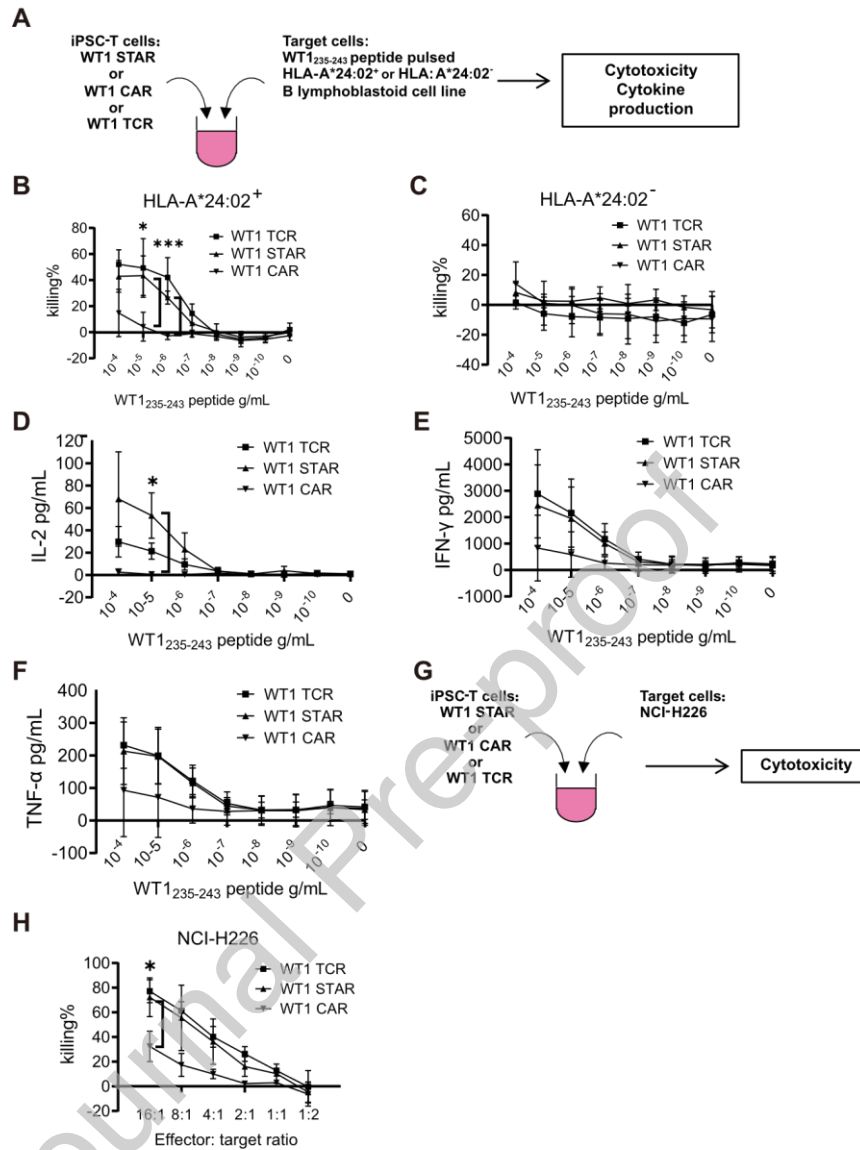
To evaluate antigen sensitivity, we co-cultured WT1-STAR-, WT1-CAR-, and WT1-TCR-iT cells with HLA-A\*24:02-positive B-LCLs pulsed with WT1<sub>235-243</sub> peptide to assess

cytotoxicity and cytokine production(6) (Figure 5A). WT1-STAR-iT cells exhibited similar killing efficiency to WT1-TCR-iT cells and significantly outperformed WT1-CAR-iT cells (Figure 5B). In addition, WT1-STAR-iT cells did not exhibit cytotoxicity against HLA-A\*24:02-negative B-LCLs, confirming their HLA-restriction (Figure 5C). Notably, WT1-STAR-iT cells demonstrated cytotoxicity at 100ng/mL antigen concentrations, while WT1-CAR-iT cells demonstrated at 10 $\mu$ g/mL, indicating a 100-fold difference in sensitivity. These results suggest that the STAR-iT cells possess greater sensitivity in antigen recognition.

The enhanced sensitivity of WT1-STAR-iT cells was further confirmed by cytokine production assays. Upon peptide stimulation, WT1-STAR-iT cells secreted significantly higher levels of IL-2 compared to WT1-CAR-iT cells (Figure 5D). Additionally, IFN- $\gamma$  and TNF- $\alpha$  secretion levels in WT1-STAR-iT cells were comparable to WT1-TCR-iT cells and higher than in WT1-CAR-iT cells (Figure 5E-F). We also stimulated iT cells with PMA plus ionomycin to bypass receptor-mediated activation and directly activate T cells. WT1-CAR-iT cells exhibited robust IFN- $\gamma$ , TNF- $\alpha$  (Figure S7C-D), and IL-2 production, comparable to WT1-TCR- and -STAR-iT cells (Figure S7E-F), indicating that WT1-CAR-iT cells remain functional. Therefore, we imply that the poor antigen sensitivity of CAR-iT cells were likely due to the antigen recognition process instead of functional deficiency.

To determine whether increased sensitivity of STAR-iT cells was due to differences in surface receptor expression, we assessed Myc-tag expression of STAR- and CAR-iT cells. Flow cytometry analysis confirmed that STAR and CAR were expressed at comparable levels (Figure S7A-B), indicating that the superior responsiveness of STAR-iT cells was not due to receptor expression differences.

To assess the functionality of STAR-iT cells against endogenously WT1-expressing tumor cells, iT cells were co-cultured with NCI-H226, a mesothelioma cell line naturally expressing WT1 and HLA-A\*24:02 (Figure 5G). WT1-STAR-iT cells exhibited similar killing capacity to WT1-TCR-iT cells and significantly outperformed WT1-CAR-iT cells (Figure 5H).



### Figure 5 The STAR-iT cells exhibited higher responsiveness to WT1<sup>+</sup> tumor cells

(A) Schematic illustration of the killing assay. WT1-CAR-, -STAR-, and -TCR-iT cells were co-cultured with HLA-A\*24:02 positive or negative B-LCLs. Killing efficiency was evaluated by Non-RI killing assay. (B) Non-RI killing results for iT cells against HLA-A\*24:02 positive B-LCLs pulsed with serial concentrations of WT1<sub>235-243</sub> antigen peptide. (n=3) Data represent mean  $\pm$  SD of n independent experiments. Statistical analysis was performed using two tailed t-test. \*p<0.05; \*\*\*p<0.001 (C) Non-RI killing results for iT cells against HLA-A\*24:02 negative B-LCLs pulsed with a serial of concentrations of WT1<sub>235-243</sub> antigen peptide. (n=3). Data represent mean  $\pm$  SD of n independent experiments. (D) Cytometric Bead Array analysis for IL-2 production. Supernatants from co-cultures of iT cells with HLA-A\*24:02 positive B-LCLs pulsed with a serial of concentrations of WT1<sub>235-243</sub> were evaluated for IL-2. (n=3) Data represent mean  $\pm$  SD of n independent experiments. Statistical analysis was performed using two tailed t-test. \*p<0.05 (E) Cytometric Bead Array analysis of IFN- $\gamma$  production. Supernatants of iT cells with HLA-A\*24:02 positive B-LCLs pulsed with a serial of concentrations of WT1<sub>235-243</sub> were evaluated for IFN- $\gamma$ . (n=3) Data represent mean  $\pm$  SD of n independent experiments. (F) Cytometric Bead Array analysis of TNF- $\alpha$  production. Supernatants from iT cells with HLA-A\*24:02 positive B-LCLs pulsed with a serial of concentrations of WT1<sub>235-243</sub> were evaluated for TNF- $\alpha$ . (n=3) Data represent mean  $\pm$  SD of n independent experiments. (G) Schematic illustration of killing assay against NCI-H226. WT1-CAR-, -STAR-, and -TCR-iT cells were co-cultured with NCI-H226 cells. The killing efficiency was evaluated by Non-RI killing assay. (H) Non-RI killing results for iT cells against NCI-H226 at various effector to target cell ratio. (n=3) Data represent mean  $\pm$  SD of n independent experiments. Statistical analysis was performed using two tailed t-test. \*p<0.05

## Discussion

Here, we introduce STAR-iT cells as a novel platform for iPSC-derived T cell therapy.

We demonstrate that the STAR construct restores CD3 $\epsilon$  expression and supports iT cell

maturation. In addition, STAR-iT cells possess superior antigen sensitivity and cytotoxicity compared to CAR-iT cells. These findings suggest that STAR-iT cells could overcome key limitations of conventional CAR-iT cells.

Unlike CARs, STAR receptors do not appear to impair T cell differentiation. Previous studies have reported that CD28 $\zeta$  CAR constructs can induce strong tonic signaling in T cells resulting in T cell exhaustion(26). Moreover, sustained tonic signaling from CAR has shown to impair iPSC-T cell differentiation(8, 15, 16). This effect is primarily attributed to the dimerization of the scFv region in CARs, which leads to the constitutive phosphorylation of CD3 $\zeta$  in an antigen-independent manner(27). In contrast, TCR forms a complex with CD3, in which spatial constraints prevent receptor dimerization(27). Given STAR receptor shares structural similarity with the TCR and also associates with CD3 to form a physiological complex, it likely avoids scFv-mediated dimerization despite using the same scFv domain as CAR(17). Besides, STAR does not contain CD28 co-stimulatory domain, which is known to induce strong tonic signals. As a result, STAR does not trigger excessive tonic signaling, and thus supports proper iPSC-T cell differentiation.

Our study demonstrated that STAR-iT cells have higher antigen sensitivity than CAR-iT cells, which may be partially attributed to the differentiation process. STAR iT cells express CD3 $\epsilon$  during DP stage, thus can respond to OKT3 stimulation appropriately, while CAR-iT cells lack CD3 $\epsilon$  expression at this stage, which may lead to functional maturation failure. Additionally, STAR-iT cells incorporate the constant regions of TCR $\alpha\beta$ , which potentially rendering them more similar to conventional TCR $\alpha\beta$ -iT cells. In contrast, a proportion of CAR-iT cells are  $\gamma\delta$  T cells. This intrinsic difference in T cell identity may also

contribute to the observed differential antigen sensitivity. These factors, combined with the structural advantages of the STAR receptor, may contribute to the enhanced antigen sensitivity observed in STAR-iT cells compared to CAR-iT cells. Moreover, we observed that STAR-iT cells exhibited superior expansion compared to CAR-iT cells under PHA-based stimulation. This difference may be partly attributed to the expansion method itself, which may not be optimal for CAR-iT cells. CAR-iT cell populations contain a proportion of  $\gamma\delta$  T cells, and previous studies have shown that ConA is more effective at expanding  $\gamma\delta$  T cells (28). Therefore, the superior proliferation of STAR-iT cells may result from better compatibility with the PHA-based expansion protocol.

Based on our findings and prior results in primary T cells(17, 18), we anticipate that STAR-iT cells may also exhibit superior antitumor activity in vivo compared to CAR-iT cells. Previous studies have demonstrated that STAR receptors confer enhanced antigen sensitivity and improved tumor control in primary T cell models, even when utilizing the same scFv domain as CAR. This improved functionality is thought to arise from the integration of STAR into the native TCR-CD3 signaling machinery, allowing more physiological signal transduction(17, 18). Given that our in vitro results revealed comparable sensitivity and cytokine production between STAR-iT and TCR-iT cells and significantly higher than that of CAR-iT cells, it is reasonable to hypothesize that STAR-iT cells will similarly outperform CAR-iT cells in preclinical in vivo models. Further studies are warranted to directly assess the persistence, tumor infiltration, and therapeutic efficacy of STAR-iT cells in vivo.

The ability of TCRs to recognize intracellular antigens is another inherent advantage.

STAR-iT cells, by combining an HLA-restricted scFv with STAR technology, can similarly target intracellular antigens. At the same time, STAR offers increased flexibility over traditional TCRs, enabling the use of either HLA-dependent or HLA-independent scFvs. While natural TCRs generally exhibit low affinity for tumor-associated antigens due to negative selection, STAR bypasses the need to laboriously screen for optimal TCR affinity, offering a more efficient solution.

While other studies have attempted to generate TCR negative iPSC-T cells using 1XX CAR(15), our work is the first to report that STAR-iT cells maintain high levels of CD3 expression. Since the TCR/CD3 is essential for both T cell maturation and *in vivo* persistence(29), the ability of STAR to sustain CD3 expression is likely to improve long-term therapeutic efficacy.

Compared to other TCR-like chimeric receptors, such as T cell antigen coupler (TAC) and TCR fusion constructs (TRuCs)(30, 31), STAR is more suitable for iPSC platforms. TAC and TRuCs are novel synthetic receptors designed to harness the native T cell receptor (TCR) signaling machinery for targeted anti-tumor responses. Structurally, TAC contains three domains, a scFv to recognize antigen, a CD3-binding domain that engages the endogenous TCR complex, and a hinge-transmembrane domain of co-receptor for membrane anchoring, while TRuCs directly fuse a scFv to CD3 $\epsilon$  chain. Although both structures adopt native TCR signaling machinery and exhibited enhanced anti-tumor efficiency than CAR-T cells, they still rely on an intact TCR structure, which still necessitates selecting a receptor with appropriate affinity and minimal cross-reactivity. In contrast, STAR receptors constructed with only TCR constant regions and validated scFvs

circumvent these issues.

Many previously reported TCR-like engineered receptors have been applied to autologous primary T cells and be expected to improve the therapeutic potential(17, 18, 30-34); however, in case of allogeneic use of primary T cells as a platform, retained endogenous TCRs of the polyclonal T cell may trigger graft-versus-host disease (GVHD) at injection, thereby limiting their broader clinical applicability. iPSC-derived T lymphocytes could be cancelled TCR-dependent antigen specificity by knocking-out of TRA gene(15) or transducing V(D)J region-missing “mini” TCR(22), both at iPSC stage. Another potential concern with TCR-like constructs is mispairing with endogenous TCR chains, which can lead to off-target toxicity. Although strategies like endogenous TCR knock-outs, extra disulfide bridge insertion or replacement of murine transmembrane domains have been explored, they do not fully reduce immunogenicity(17). However, STAR-iT cells uniformly lacked TCR expression while maintaining STAR expression, mitigating these issues and improving uniformity compared to primary T cells.

Our study demonstrates that previously reported clinically scalable CD8 iT-cell manufacturing protocols can be applied to generate STAR-iT cells with potent anti-tumor activity(5). After 42 days of differentiation, approximately  $10^9$  STAR-iT cells can be produced from  $3 \times 10^5$  STAR-iPSCs, followed by further thousands-fold expansion during subsequent culture. Considering that current CAR-T cell therapies typically require  $10^9$  cells per patient, these results suggest that our approach has the potential to generate therapeutic products for multiple patients. Moreover, the use of larger-scale apparatus, such as automated stirred-tank bioreactor systems, is expected to further increase yield,

and efforts in this direction are currently underway. Therefore, when combined with the iPSC platform, large-scale expansion has the potential to markedly reduce manufacturing costs and improve clinical applicability. Future studies will investigate the safety concerns of STAR-iT cells by in vivo tumor-bearing mouse models and preclinical safety assessments to determine appropriate dosing strategies. Furthermore, incorporation of suicide genes into STAR-iPSC platform may help mitigate potential toxicities and enhance their clinical safety.(35).

The differentiated STAR-iT cells predominantly exhibited a CD8<sup>+</sup> phenotype rather than CD4<sup>+</sup>. We hypothesized that this lineage bias may result from the current in vitro feeder free differentiation system, which cannot fully recapitulate the thymic microenvironment. In the lineage choice between CD4<sup>+</sup> and CD8<sup>+</sup> T cells, CD4<sup>+</sup> T-cell commitment needs sustained and relatively strong TCR–MHC class II signaling. In contrast, CD8<sup>+</sup> T-cell specification relies more heavily on IL-7(36). To enhance progenitor viability under feeder-free conditions, we employed a relatively high concentration of IL-7, which may have favored CD8<sup>+</sup> lineage specification. Moreover, our current system does not provide finely tuned TCR signaling, which may be suboptimal for CD4<sup>+</sup> iT-cell differentiation. Previous studies have shown that artificial thymic organoids (ATOs), generated by coculturing iPSC-HPCs with human DLL4-overexpressing MS5 stromal cells, can induce CD4<sup>+</sup> iT cells differentiation(37). The ATO exhibits a thymus-like 3D differentiation structure, suggesting that such architecture may enable fine-tuned regulation of differentiation signals, which could be important for CD4<sup>+</sup> iT-cell development. In future studies, we plan to differentiate STAR-iT cells using the ATO system and investigate whether the same

STAR construct exhibits functional differences between CD8<sup>+</sup> and CD4<sup>+</sup> iT cells, whether these differences are co-receptor-dependent, and whether cooperative interactions exist between the two subsets.

In conclusion, our findings demonstrate that combining STAR technology with the iPSC platform could enhance the development of iPSC-T cell-based immunotherapies. The STAR-iT cells could circumvent the limitations of conventional CAR- and TCR-iT cells. These results lay the groundwork for future research aimed at translating STAR-iT cells into effective cancer immunotherapies.

## **Material and method**

### **Constructions of the lentiviral vectors**

The codon-optimized, and Myc-tagged anti-WT1-scFv sequence was synthesized by GeneArt (Thermo Fisher Japan). The hinge, CD8 transmembrane domain, and intracellular domains (CD28-4-1BB-CD3zeta) were amplified with KOD One master mix blue (TOYOBO, Osaka, Japan) according to the manufacturer's protocol. The amplified gene fragments were inserted into the pLV5IN lentiviral vector using the NEBuilder HiFi Assembly kit (New England Biolabs, Ipswich, MA, USA). The resulting pLV5IN anti-WT1-28BBz vector was used to PCR-amplify the WT1-VL and -VH sequences, which were assembled with amplified TRAC- and TRBC-sequences in another HIFI-reaction to obtain the pLV5IN anti-WT1VH-TRBC-WT1VL-TRAC (STAR) vector. The WT1<sub>235-243</sub> peptide/HLA A\*24:02 restricted TCR was derived from our laboratory's prior study(38). The scFv sequence and primers are listed in Table S1.

### **Human induced pluripotent stem cell lines**

An HLA homozygous iPSC line Ff-MH15s01, was established at the Center for iPS Cell Research and Application (CiRA), Kyoto University and distributed with informed consent and permission of the Ethical Review Board at CiRA. This iPSC clone was transduced separately with lentiviral vectors encoding WT1-TCR, WT1-STAR or WT1-CAR. 2000 Tra-1-60<sup>+</sup>, SSEA4<sup>+</sup> and hKO1<sup>+</sup> iPSCs expressing WT1-TCR, WT1-STAR or WT1-CAR were sorted and seeded onto Easy iMatrix-511 silk (Matrixome, Japan) coated 6-well plates and cultured in AK02 medium (Ajinomoto, Japan).

### Cell lines

The B-lymphoblastoid cell lines (B-LCLs) HEV60 (HLA-A\*24:02-positive) and HEV277 (HLA-A\*33:03-positive) (RIKEN BRC, Japan) were used as antigen-presenting cells. These cells were maintained in RPMI supplemented with 20% FBS and 1x L-Glutamine–Penicillin–Streptomycin (Sigma). An HLA-A24:02-expressing mesothelioma cell line NCI-H226, which naturally expresses WT1 antigen, was maintained in RPMI supplemented with 10% FBS and 1x L-Glutamine–Penicillin–Streptomycin.

### Flow cytometry and antibodies

All flow cytometry staining were performed in FACS buffer (2% FBS in PBS) for 30 min on ice. Antibodies were added to cells at a 1:100 final dilution and stained for 30 min on ice. Following washes, propidium iodide (PI) was added to all samples for viability analysis prior to flow cytometry analysis and sorting by FACS AriaII (BD Biosciences). Flow cytometry data were processed and analyzed with FlowJo software (Tree Star Inc.).

Monoclonal antibodies used for surface and intracellular staining were obtained from the following suppliers: BioLegend: CD3 $\epsilon$  (UCHT1), CD4 (OKT4), CD5 (UCHT2), CD7

(CD7-6B7), CD8 (SK1), CD14 (HCD14), CD235a (GA-R2(HIR2), IFN- $\gamma$  (B27), IL-2 (MQ1-17H12), TNF- $\alpha$  (Mab11), TCR $\alpha\beta$  (IP26); BD Biosciences: CD34 (581), CD43 (IM7), CD247(pY142) (K25-407.69), SSEA4 (MC813-70), and Tra-1-60 (Tra-1-60); Thermo Fisher: TCR $\gamma\delta$  (5A6.E9); Cell Signaling: Myc-tag (9B11); Invitrogen: CD8 $\beta$  (SID18BEE).

### **HPC induction from primed iPSCs**

T cell differentiation from iPSCs was initiated by inducing hematopoietic progenitors cells (HPCs). iPSCs were maintained for seven days and dissociated into single cells. Cells were cultured in AK02 medium containing 10  $\mu$ M Y-27632 (FujiFilm Wako, Osaka, Japan) and 10  $\mu$ M CHIR99021 (Tocris Bioscience) in 6-well ultra-low attachment plates (Corning) for 24 hours to form embryoid bodies (EBs). After 24 hours, EBs were collected and resuspended in StemPro-34 (Thermo Fisher) supplemented with 1x L-Glutamine–Penicillin–Streptomycin, 2 mM Glutamax (Thermo Fisher), 50  $\mu$ g/mL ascorbic acid-2-phosphate (Sigma), 400  $\mu$ M monothioglycerol (MTG, Nacalai, Kyoto, Japan), and 1 $\times$  Insulin-Transferrin-Selenium solution (ITS-G, Thermo Fisher) (hereafter “EB basal medium”), plus 50 ng/mL recombinant human (rh) BMP-4 (R&D Systems), 50 ng/mL rhVEGF (R&D Systems), and 50 ng/mL bFGF (FujiFilm Wako). After 24 hours, 6  $\mu$ M SB431542 (FujiFilm Wako) was added. At day 4, EBs were collected and resuspended in EB basal medium supplemented with 50 ng/mL rhVEGF, 50 ng/mL rhbFGF, and 50 ng/mL rhSCF (R&D Systems). At day 6, EBs were collected and resuspended in EB basal medium supplemented with 50 ng/mL rhVEGF, 50 ng/mL rhbFGF, 50 ng/mL rhSCF, 30 ng/mL rhTPO (PeproTech), and 10 ng/mL FLT3L (PeproTech). From day 7, the medium was replaced every two days. Cultures were maintained in a 5% CO<sub>2</sub>, 5% O<sub>2</sub> environment

for the first six days, and in a 5% CO<sub>2</sub> environment thereafter. CD235a<sup>-</sup> CD14<sup>-</sup> CD34<sup>hi</sup> CD43<sup>+</sup> EB cells were sorted by flow cytometry at day 14 or 16.

### **Differentiation of the iPSC-derived T cell progenitors**

T cell lineage commitment was induced by seeding 2000 CD235a<sup>-</sup> CD14<sup>-</sup> CD34<sup>hi</sup> CD43<sup>+</sup> EB cells on 48-well plates coated with recombinant human Fc-DLL4 (10 µg/mL, Sino Biological) and retronectin (10 µg/mL, TAKARA, Japan). Cells were cultured in T cell differentiation medium composed of αMEM (Thermo Fisher) supplemented with 15% FBS, 1x L-Glutamine–Penicillin–Streptomycin, 1x ITS-G, 55 µM 2-Mercaptoethanol (Thermo Fisher), 50 µg/mL ascorbic acid-2-phosphate, 2 mM Glutamax, 10 ng/mL rhSCF, 5 ng/mL rhIL-7, 5 ng/mL FLT3L, 10 nM rhSDF-1α (PeproTech), and 15 µM SB203580 (Tocris Bioscience). The medium was replenished every 2-3 days. Differentiating cells were transferred into a new coated plate every week. At day 14, 1×10<sup>5</sup> cells per well were transferred to a new Fc-DLL4-coated well. The differentiating cells were analyzed by flow cytometry at week 3.

### **Generation of mature CD8 single-positive T cells**

To mature DP T cells, 1~5×10<sup>4</sup> DP cells were sorted and seeded onto retronectin-coated plate in αMEM supplemented with 15% FBS, 1x L-Glutamine–Penicillin–Streptomycin, 1x ITS, 2 mM Glutamax, 50 µg/mL ascorbic acid 2-phosphate, 5 ng/mL IL-7, 20 ng/mL IL-21, 10 nM rhSDF-1α (PeproTech), and 15 µM SB203580 (Tocris Bioscience). For the first two days, 1 µg/mL OKT3 (CD3 agonist antibody) was added to the culture medium, and it was removed in the subsequent culture. Matured T cells were analyzed by flow cytometry around day 7.

### **iPSC-T cells expansion**

iPSC-T cells were expanded as published previously (23). Peripheral blood mononuclear cells (PBMC) samples were obtained from healthy volunteers who provided written informed consent. iPSC-T cells were mixed with 40 Gy-irradiated PBMCs at a responder to feeder ratio of 1/10 in  $\alpha$ MEM supplemented with 15% FBS, 1x L-Glutamine–Penicillin–Streptomycin, 1x ITS, ascorbic acid 2-phosphate, 2 $\mu$ g/mL PHA, 5ng/mL IL-7, 5ng/mL IL-15 (PeproTech). Cultures were split and replenished with the fresh medium every 2 or 3 days to maintain sub-confluent cell density.

### **Non-Radioactive cellular cytotoxicity assay**

The Non-Radioactive cellular cytotoxicity assay (Techno suzuta co.) was used to measure cytotoxicity. 10<sup>6</sup>/mL target cells were co-cultured with 2.5  $\mu$ L BM-HT in 37°C for 15 minutes, then washed three times at 4°C, 600g for 5 minutes. Target cells were co-cultured with effector cells to conduct the cytotoxicity assay. 2% Triton PBS was used to lyse target cells for evaluating the maximal killing. After co-culture, 20  $\mu$ L of culture supernatant was incubated with 200  $\mu$ L Europium solution for 15 minutes at room temperature. Time-resolved fluorescence was measured by using Multimode Plate Reader NiVO (PerkinElmer). %lysis = (experimental lysis – spontaneous lysis) / (maximal lysis – spontaneous lysis) x 100%.

The B lymphoblastoid cell lines (B-LCLs), HEV60 (HLA-A\*24:02-positive) and HEV277 (HLA-A\*33:03-positive), were used to evaluate iPSC-T cells cytotoxicity. B-LCLs were pulsed with varying concentrations of WT1<sub>235–243</sub> peptide for 1 hour, then co-cultured with iPSC-T cells at 2:1 effector-to-target ratio for 3 hours(5, 38). Additionally, the

mesothelioma cell line NCI-H226, which is endogenously expressing WT1, was co-cultured with iPSC-T cell at effector-to-target ratio ranging from 1:2 to 16:1 for 3 hours.

### **Cytokine production**

The BD Cytometric Bead Array Flex Set System (BD Biosciences) was used to measure human interleukin 2 (IL-2), interferon- $\gamma$  (IFN- $\gamma$ ), and tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) levels (6). iPSC-T cells were co-cultured with WT1<sub>235-243</sub> peptide-pulsed HEV60 cells. After 24 hours, cells were centrifuged and the supernatants were collected. The concentration of these cytokines was determined according to the manufacturer's instructions.

### **PMA and ionomycin stimulation**

iPSC-T cells were cultured in  $\alpha$ MEM supplemented with PMA, ionomycin and monensin at 37°C for 3 hours (5). Cells were washed at 4°C, 600g for 5 minutes with 2% FBS in PBS. iPSC-T cells were stained with LIVE/DEAD™ Viability/Cytotoxicity Kit (Invitrogen) in the dark for 20 min at 4°C to determine the alive cells, followed by fixation with Fixation Buffer (BioLegend) in the dark for 20 minutes at room temperature. Cells were washed twice with intracellular staining perm wash buffer at 4°C, 600g for 5 minutes and blocked with 2% FBS in PBS for 1 hour at room temperature. Finally, the fixed cells were stained with either isotype controls or antibodies against IL-2, IFN- $\gamma$ , and TNF- $\alpha$  in the dark for 20 min at room temperature. Cells were washed and analyzed by FACS Arianal (BD Biosciences)

### **CD247 phosflow**

iPSC-derived HPCs were seeded on FcDLL4 and cultured for three days. Cells were harvested, washed and fixed with  $-20^{\circ}\text{C}$  methanol on ice for 15 minutes, then wash twice with 2% FBS in PBS at  $4^{\circ}\text{C}$ , 600g for 5 minutes. Cells were blocked with 2% FBS in PBS for 1 hour at room temperature. Cells were stained with either isotype control or antibody against CD247 (pY142) in the dark at  $4^{\circ}\text{C}$  for 20 minutes (8). Cells were washed and analyzed by FACS Arianall (BD Biosciences).

### **Statistical analysis**

Number of independent experiments are listed in the figure legends. All Data is presented as mean  $\pm$  SD. Statistics were performed using Excel (Microsoft) and Prism (GraphPad software). p-values were calculated by either two-tailed t-test for comparing two groups or one-way ANOVA with Tukey's multiple comparison test for more than three groups. \* $p < 0.05$  was considered significant.

## Acknowledgments

We thank Dr. Atsutaka Minagawa for providing WT1 TCR vector; Mr. Munehiro Yoshida for their technical assistance; Ms. Katsura Noda, and Mrs. Akiko Washimi for their administrative assistance.

## Author contribution

C.Z., M.R., and S.K. wrote the manuscript; C.Z., M.R., Y.K., and S.K. designed this study; C.Z., M.R., Q.G., B.W., Y.K., and S.K. interpreted data; C.Z., M.R., Q.G., and Y.K. performed experiments, and analyzed data; M.R., Q.G., A.I., B.W., and Y.K. provided critical materials, protocols or advice for the experiments; S.K. supervised the study.

†These authors contributed equally to this work and share first authorship: Chaoqi Zhang, Michael Rassner, Yohei Kawai.

## Funding

This work was supported by the Japan Agency for Medical Research and Development (grant numbers: JP21am0101078, JP21am0401013, and JP21bm1004001).

M. Rassner was supported by a research grant from the German Research Foundation (DFG RA3488-1/1).

## Conflict of interest

S. Kaneko is a founder, shareholder, and chief scientific officer at ShinObi Co., Ltd., and received research funding from Takeda Pharmaceutical Co. Ltd., Kirin Co., Ltd., Astellas Co., Ltd., Tosoh Co. Ltd., and ShinObi Co., Ltd.

## Reference

1. June CH, O'Connor RS, Kawalekar OU, Ghassemi S, Milone MC. CAR T cell immunotherapy for human cancer. *Science*. 2018;359(6382).  
<http://doi.org/10.1126/science.aar6711>
2. Yun K, Siegler EL, Kenderian SS. Who wins the combat, CAR or TCR? *Leukemia*. 2023;37(10). <http://doi.org/10.1038/s41375-023-01976-z>
3. Kohl U, Arsenieva S, Holzinger A, Abken H. CAR T Cells in Trials: Recent Achievements and Challenges that Remain in the Production of Modified T Cells for Clinical Applications. *Hum Gene Ther*. 2018;29(5). <http://doi.org/10.1089/hum.2017.254>
4. Depil S, Duchateau P, Grupp SA, Mufti G, Poirot L. 'Off-the-shelf' allogeneic CAR T cells: development and challenges. *Nat Rev Drug Discov*. 2020;19(3).  
<http://doi.org/10.1038/s41573-019-0051-2>
5. Iriguchi S, Yasui Y, Kawai Y, Arima S, Kunitomo M, Sato T, et al. A clinically applicable and scalable method to regenerate T-cells from iPSCs for off-the-shelf T-cell immunotherapy. *Nat Commun*. 2021;12(1). <http://doi.org/10.1038/s41467-020-20658-3>
6. Kawai Y, Kawana-Tachikawa A, Kitayama S, Ueda T, Miki S, Watanabe A, et al. Generation of highly proliferative, rejuvenated cytotoxic T cell clones through pluripotency reprogramming for adoptive immunotherapy. *Mol Ther*. 2021;29(10).  
<http://doi.org/10.1016/j.ymthe.2021.05.016>
7. Wang B, Iriguchi S, Waseda M, Ueda N, Ueda T, Xu H, et al. Generation of hypoinmunogenic T cells from genetically engineered allogeneic human induced pluripotent stem cells. *Nat Biomed Eng*. 2021;5(5). <http://doi.org/10.1038/s41551-021-00730-z>

8. Ueda T, Shiina S, Iriguchi S, Terakura S, Kawai Y, Kabai R, et al. Optimization of the proliferation and persistency of CAR T cells derived from human induced pluripotent stem cells. *Nat Biomed Eng.* 2023;7(1). <http://doi.org/10.1038/s41551-022-00969-0>
9. Woan KV, Kim H, Bjordahl R, Davis ZB, Gaidarova S, Goulding J, et al. Harnessing features of adaptive NK cells to generate iPSC-derived NK cells for enhanced immunotherapy. *Cell Stem Cell.* 2021;28(12). <http://doi.org/10.1016/j.stem.2021.08.013>
10. Iriguchi S, Kaneko S. Toward the development of true "off-the-shelf" synthetic T-cell immunotherapy. *Cancer Sci.* 2019;110(1). <http://doi.org/10.1111/cas.13892>
11. Poorebrahim M, Mohammadkhani N, Mahmoudi R, Gholizadeh M, Fakhr E, Cid-Arregui A. TCR-like CARs and TCR-CARs targeting neoepitopes: an emerging potential. *Cancer Gene Ther.* 2021;28(6). <http://doi.org/10.1038/s41417-021-00307-7>
12. Weber EW, Maus MV, Mackall CL. The Emerging Landscape of Immune Cell Therapies. *Cell.* 2020;181(1). <http://doi.org/10.1016/j.cell.2020.03.001>
13. Shafer P, Kelly LM, Hoyos V. Cancer Therapy With TCR-Engineered T Cells: Current Strategies, Challenges, and Prospects. *Front Immunol.* 2022;13<http://doi.org/10.3389/fimmu.2022.835762>
14. Teppert K, Wang XT, Anders K, Evaristo C, Lock D, Künkele A. Joining Forces for Cancer Treatment: From "TCR versus CAR" to "TCR and CAR". *Int J Mol Sci.* 2022;23(23). <http://doi.org/ARTN 14563>  
[10.3390/ijms232314563](https://doi.org/10.3390/ijms232314563)
15. van der Stegen SJC, Lindener PL, Petrovic RM, Xie H, Diop MP, Alexeeva V, et al. Generation of T-cell-receptor-negative CD8 $\alpha$ beta-positive CAR T cells from T-cell-derived

induced pluripotent stem cells. *Nat Biomed Eng.* 2022;6(11). [http://doi.org/10.1038/s41551-](http://doi.org/10.1038/s41551-022-00915-0)

[022-00915-0](http://doi.org/10.1038/s41551-022-00915-0)

16. Li S, Wang CS, Montel-Hagen A, Chen HC, Lopez S, Zhou O, et al. Strength of CAR signaling determines T cell versus ILC differentiation from pluripotent stem cells. *Cell Rep.* 2023;42(3). <http://doi.org/10.1016/j.celrep.2023.112241>
17. Liu Y, Liu G, Wang J, Zheng ZY, Jia L, Rui W, et al. Chimeric STAR receptors using TCR machinery mediate robust responses against solid tumors. *Sci Transl Med.* 2021;13(586). <http://doi.org/10.1126/scitranslmed.abb5191>
18. Mansilla-Soto J, Eyquem J, Haubner S, Hamieh M, Feucht J, Paillon N, et al. HLA-independent T cell receptors for targeting tumors with low antigen density. *Nature Medicine.* 2022;28(2). <http://doi.org/10.1038/s41591-021-01621-1>
19. Kuhns MS, Davis MM. Disruption of extracellular interactions impairs T cell receptor-CD3 complex stability and signaling. *Immunity.* 2007;26(3). <http://doi.org/10.1016/j.immuni.2007.01.015>
20. Akahori Y, Wang LN, Yoneyama M, Seo N, Okumura S, Miyahara Y, et al. Antitumor activity of CAR-T cells targeting the intracellular oncoprotein WT1 can be enhanced by vaccination. *Blood.* 2018;132(11). <http://doi.org/10.1182/blood-2017-08-802926>
21. Ochi T, Nakatsugawa M, Chamoto K, Tanaka S, Yamashita Y, Guo TX, et al. Optimization of T-cell Reactivity by Exploiting TCR Chain Centricity for the Purpose of Safe and Effective Antitumor TCR Gene Therapy. *Cancer Immunol Res.* 2015;3(9). <http://doi.org/10.1158/2326-6066.Cir-14-0222>

22. Takayanagi SI, Wang B, Hasegawa S, Nishikawa S, Fukumoto K, Nakano K, et al. Mini-TCRs: Truncated T cell receptors to generate T cells from induced pluripotent stem cells. *Mol Ther Methods Clin Dev.* 2023;31<http://doi.org/10.1016/j.omtm.2023.101109>
23. Nishimura T, Kaneko S, Kawana-Tachikawa A, Tajima Y, Goto H, Zhu D, et al. Generation of rejuvenated antigen-specific T cells by reprogramming to pluripotency and redifferentiation. *Cell Stem Cell.* 2013;12(1). <http://doi.org/10.1016/j.stem.2012.11.002>
24. Amsen D, Helbig C, Backer RA. Notch in T Cell Differentiation: All Things Considered. *Trends Immunol.* 2015;36(12). <http://doi.org/10.1016/j.it.2015.10.007>
25. Gattinoni L, Lugli E, Ji Y, Pos Z, Paulos CM, Quigley MF, et al. A human memory T cell subset with stem cell-like properties. *Nat Med.* 2011;17(10). <http://doi.org/10.1038/nm.2446>
26. Smirnov S, Mateikovich P, Samochernykh K, Shlyakhto E. Recent advances on CAR-T signaling pave the way for prolonged persistence and new modalities in clinic. *Frontiers in Immunology.* 2024;15<http://doi.org/ARTN 1335424>  
10.3389/fimmu.2024.1335424
27. Kouro T, Himuro H, Sasada T. Exhaustion of CAR T cells: potential causes and solutions. *J Transl Med.* 2022;20(1). <http://doi.org/ARTN 239>  
10.1186/s12967-022-03442-3
28. Capsomidis A, Benthall G, Van Acker HH, Fisher J, Kramer AM, Abeln Z, et al. Chimeric Antigen Receptor-Engineered Human Gamma Delta T Cells: Enhanced Cytotoxicity with Retention of Cross Presentation. *Molecular Therapy.* 2018;26(2).  
<http://doi.org/10.1016/j.ymthe.2017.12.001>

29. Stenger D, Stief TA, Kaeuferle T, Willier S, Rataj F, Schober K, et al. Endogenous TCR promotes in vivo persistence of CD19-CAR-T cells compared to a CRISPR/Cas9-mediated TCR knockout CAR. *Blood*. 2020;136(12). <http://doi.org/10.1182/blood.2020005185>
30. Helsen CW, Hammill JA, Lau VWC, Mwawasi KA, Afsahi A, Bezverbnaya K, et al. The chimeric TAC receptor co-opts the T cell receptor yielding robust anti-tumor activity without toxicity. *Nature Communications*. 2018;9<http://doi.org/ARTN 3049>  
10.1038/s41467-018-05395-y
31. Baeuerle PA, Ding J, Patel E, Thoraus N, Horton H, Gierut J, et al. Synthetic TRuC receptors engaging the complete T cell receptor for potent anti-tumor response. *Nature Communications*. 2019;10<http://doi.org/ARTN 2087>  
10.1038/s41467-019-10097-0
32. Dao T, Xiong GY, Mun SS, Meyerberg J, Korontsvit T, Xiang JY, et al. A dual-receptor T-cell platform with Ab-TCR and costimulatory receptor achieves specificity and potency against AML. *Blood*. 2024;143(6). <http://doi.org/10.1182/blood.2023021054>
33. Xu Y, Yang Z, Horan LH, Zhang P, Liu L, Zimdahl B, et al. A novel antibody-TCR (AbTCR) platform combines Fab-based antigen recognition with gamma/delta-TCR signaling to facilitate T-cell cytotoxicity with low cytokine release. *Cell Discov*. 2018;4<http://doi.org/10.1038/s41421-018-0066-6>
34. He PC, Liu HB, Zimdahl B, Wang J, Luo MN, Chang Q, et al. A novel antibody-TCR (AbTCR) T-cell therapy is safe and effective against CD19-positive relapsed/refractory B-cell lymphoma. *J Cancer Res Clin*. 2023;149(7). <http://doi.org/10.1007/s00432-022-04132-9>

35. Shi ZD, Tchao J, Wu L, Carman AJ. Precision installation of a highly efficient suicide gene safety switch in human induced pluripotent stem cells. *Stem Cells Transl Med.* 2020;9(11). <http://doi.org/10.1002/sctm.20-0007>
36. Singer A, Adoro S, Park JH. Lineage fate and intense debate: myths, models and mechanisms of CD4- versus CD8-lineage choice. *Nat Rev Immunol.* 2008;8(10). <http://doi.org/10.1038/nri2416>
37. Montel-Hagen A, Seet CS, Li S, Chick B, Zhu Y, Chang P, et al. Organoid-Induced Differentiation of Conventional T Cells from Human Pluripotent Stem Cells. *Cell Stem Cell.* 2019;24(3). <http://doi.org/10.1016/j.stem.2018.12.011>
38. Minagawa A, Yoshikawa T, Yasukawa M, Hotta A, Kunitomo M, Iriguchi S, et al. Enhancing T Cell Receptor Stability in Rejuvenated iPSC-Derived T Cells Improves Their Use in Cancer Immunotherapy. *Cell Stem Cell.* 2018;23(6). <http://doi.org/10.1016/j.stem.2018.10.005>