



iPSCs

Chimeric synthetic T cell receptor and antigen receptor receptors enable high-sensitivity T cell receptor-negative CD8 $\alpha\beta$ -CD3-positive T-cell development from human-induced pluripotent stem cells

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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, particularly when engineered with chimeric antigen receptors (CARs). Here, we introduce iPSC with synthetic T cell receptor (TCR) and antigen receptor (STAR) structure, which fuses a single-chain variable fragment (scFv) to the constant regions of TCR $\alpha\beta$. 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 CD8 $\alpha\beta$ ⁺ CD3⁺ 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.

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

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 antitumor 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 β and TCR α 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_{235–243}/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_{235–243}/HLA-A*24:02-specific scFv, and evaluated its ability to support iT cell differentiation. We demonstrate that STAR receptors transduction successfully recruit CD3 ϵ expression comparable to that of TCR, thereby enable the maturation of CD8 $\alpha\beta^+$ CD3 $^+$ 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).

WT1-STAR-transduced iPSCs differentiate into CD8 $\alpha\beta^+$ CD3 $^+$ TCR $^-$ 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 hematopoietic progenitor cells (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). Consisted

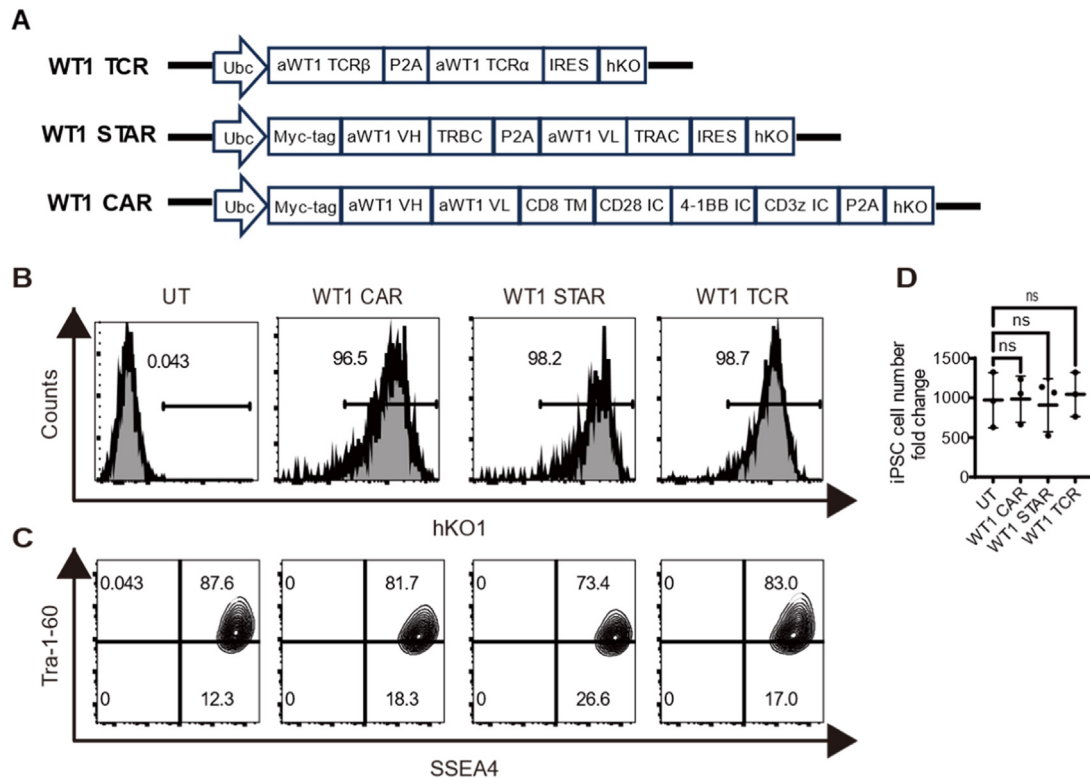


Fig. 1. Generation of WT1-TCR, WT1-STAR and WT1-CAR expressing iPSCs. (A) Schematic representation of lentiviral constructs encoding WT1-TCR, WT1-STAR and WT1-CAR, coexpressed with hKO1 reporter gene. (B) Representative flow cytometry plots for hKO1 expression in untransduced iPSC and WT1-CAR-, -STAR- 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 $\pm$ SD of n independent experiments. One-way ANOVA with Tukey's multiple comparison test. (Color version of figure is available online.)

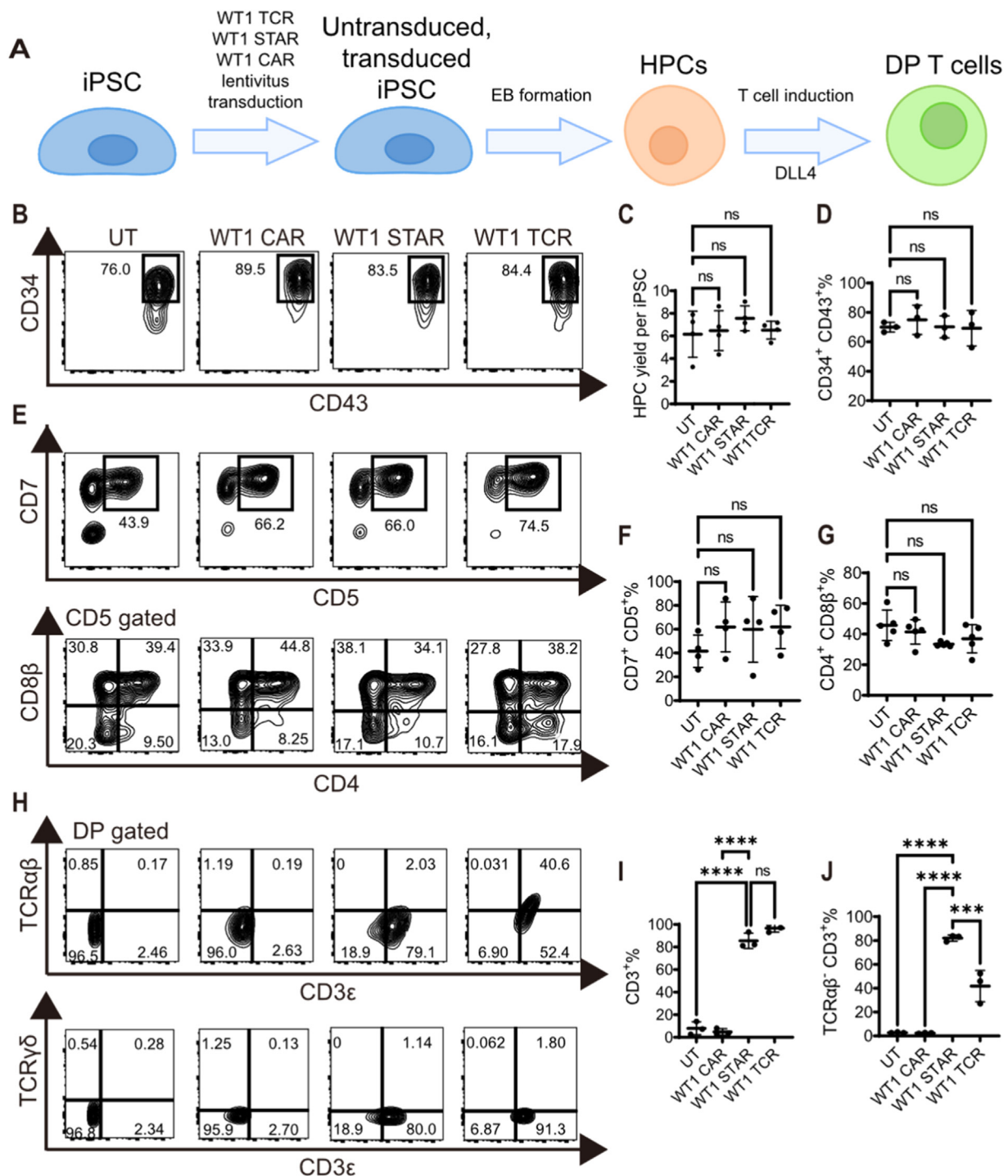


Fig. 2. STAR is compatible with the iPSC platform and recruiting surface CD3 TCR-independently. (A) Schematic illustration of the iPSC production and differentiation process toward CD4⁺ CD8⁺ immature T cells. Lentiviral constructs encoding WT1-CAR, -STAR and -TCR were individually transduced into iPSCs. 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⁺ CD43⁺ 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 CD8β expression patterns within the CD5-positive population were measured. (F) Percentage of CD7⁺ CD5⁺ 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⁺ CD8β⁺ within CD5⁺ 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 TCRαβ, TCRγδ and CD3ε expression on iDP T cells were measured. (I) Percentage of CD3⁺ 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⁺ TCRαβ⁻ 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. *** $P < 0.01$; **** $P < 0.0001$. (Color version of figure is available online.)

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 cocultured 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⁺ CD5⁺ (Figure 2E,F) and CD4⁺ CD8⁺ double-positive (DP) T cells at a frequency similar to that of UT (Figure 2E,G). 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 ζ phosphorylation as a tonic signaling (Figure S4A).

Importantly, STAR-iHPCs generated CD3⁺ DP T cells (iDP T cells) at levels equivalent to TCR-iHPCs (Figure 2H,I), but lacking endogenous TCR $\alpha\beta$ expression (Figure 2H,J and Figure S5A). In contrast, UT- and CAR-iHPCs can only generated CD3⁺ TCR⁺ iDP T cells (Figure 2H,I), consistent with the feeder-free iPSC-T cell differentiation results [22]. To assess whether CD3 ϵ expression on STAR-iDP T cells could support maturation, we stimulated the cells with the anti-CD3 ϵ 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 ϵ expression, underwent significant contraction (Figure 3B). These results indicate that CD3 ϵ 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 T cells (Figure 3C–E). STAR-iPSC-derived T cells (STAR-iT cells) expressed CD3 ϵ at levels comparable to those of TCR-iT cells (Figure 3F and Figure S5B) but lacked CR $\alpha\beta$ or TCR $\gamma\delta$ expression (Figure 3F–G and Figure 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 $\gamma\delta$ ⁺ CD3⁺ cells were more frequent in UT- and CAR-iT cells, likely due to selective expansion of TCR $\gamma\delta$ ⁺ cells in response to OKT3 stimulation during the maturation phase, in contrast to CD3⁺ populations (Figure 3F). These results demonstrate that the STAR/CD3 complex can effectively support CD8 $\alpha\beta$ ⁺ CD3⁺ TCR⁺ iT cell maturation.

To assess cell expansion potential of matured CD8 $\alpha\beta$ iT cells, we used the previously reported PHA/peripheral blood mononuclear cell (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 $\alpha\beta$ ⁺ phenotype (Figure 4D,E) and maintained CD3 ϵ 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 ϵ positive (Figure 4I). These results highlight that STAR structure is superior to CAR and effectively replaces TCR in supporting iPSC-T cells.

STAR-iT cells have higher sensitivity against WT1⁺ tumor cells than CAR-iT cells

To evaluate antigen sensitivity, we cocultured WT1-STAR-, WT1-CAR- and WT1-TCR-iT cells with HLA-A*24:02-positive B-LCLs pulsed with WT1_{235–243} 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 100 ng/mL antigen concentrations, while WT1-CAR-iT cells demonstrated at 10 μ 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 interleukin 2 (IL-2) compared to WT1-CAR-iT cells (Figure 5D). Additionally, interferon- γ (IFN- γ) and tumor necrosis factor- α (TNF- α) 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- γ , TNF- α (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 cocultured 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).

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 ϵ 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 ζ 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 ζ 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 costimulatory 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 ϵ during DP stage, thus can respond to OKT3 stimulation appropriately, while CAR-iT cells lack CD3 ϵ expression at this stage, which may lead to functional

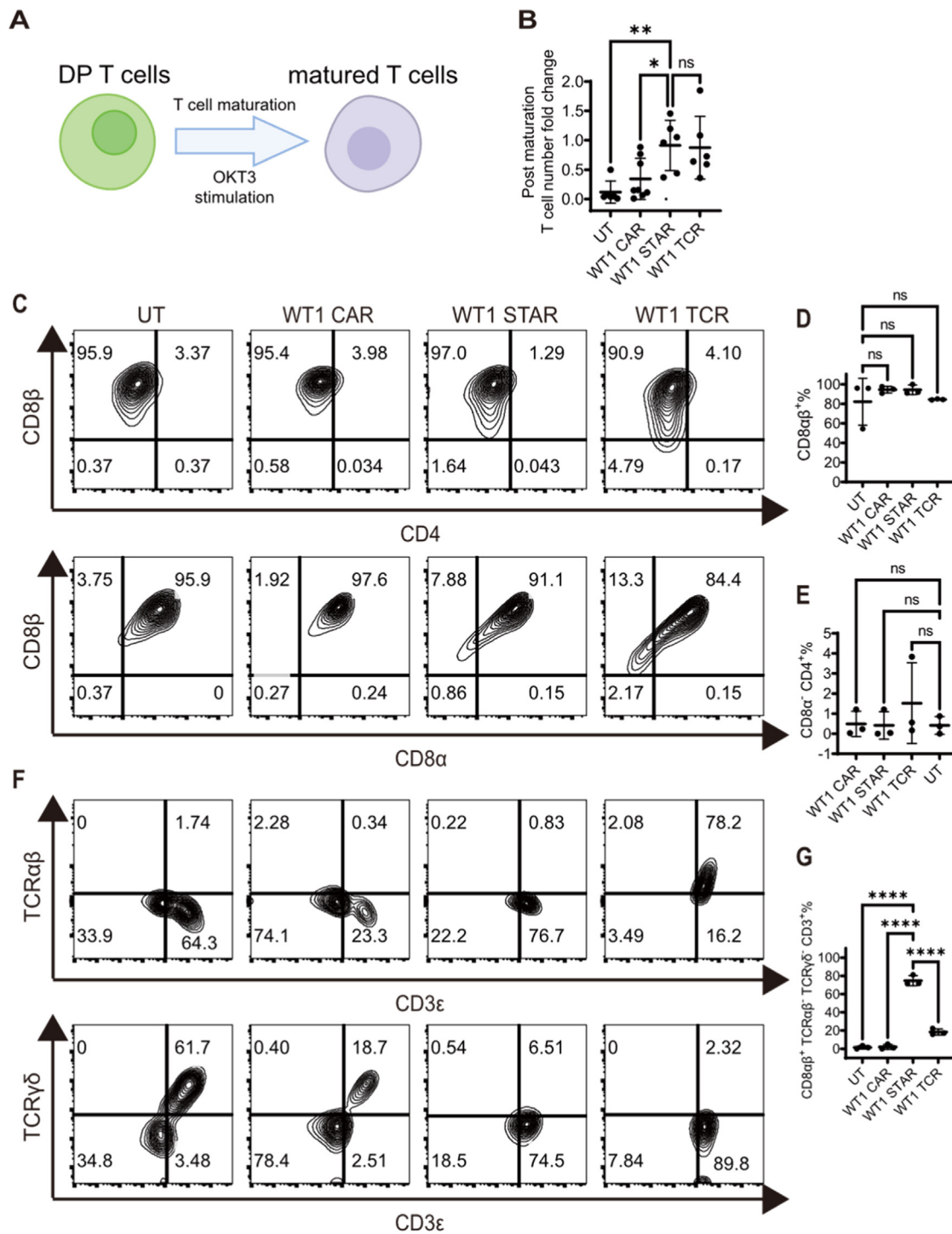


Fig. 3. STAR enables the generation of matured CD8αβ⁺ CD3⁺ TCR⁻ iT cells. (A) Schematic illustration of the maturation process for iDP immature T cells by OKT3 stimulation. (B) Cell proliferation 7 days after OKT3 maturation ($n = 6-8$). Data represent mean $\pm$ SD of n independent experiments. Statistical 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⁺ in cells at day 7 of OKT3 maturation ($n = 3$). Data represent mean $\pm$ SD of n independent experiments. Statistical analysis was performed using one-way ANOVA with Tukey's multiple comparison test. (E) Percentage of CD4⁺ in cells at day 7 of OKT3 maturation ($n = 3$). Data represent mean $\pm$ SD of n independent experiments. Statistical analysis was performed using one-way ANOVA with Tukey's multiple comparison test. (F) Representative flow cytometry plots of CD3ε, TCRαβ and TCRγδ in cells at day 7 of OKT3 maturation. (G) Percentage of CD8αβ⁺ CD3⁺ TCRαβ⁻ and TCRγδ⁻ in cells at day 7 of OKT3 maturation ($n = 3$). Data represent mean $\pm$ SD of n independent experiments. Statistical analysis was performed using by one-way ANOVA with Tukey's multiple comparison test. $^{**}P < 0.01$; $^{****}P < 0.0001$. (Color version of figure is available online.)

maturation failure. Additionally, STAR-iT cells incorporate the constant regions of TCRαβ, which potentially rendering them more similar to conventional TCRαβ-iT cells. In contrast, a proportion of CAR-iT cells are γδ 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

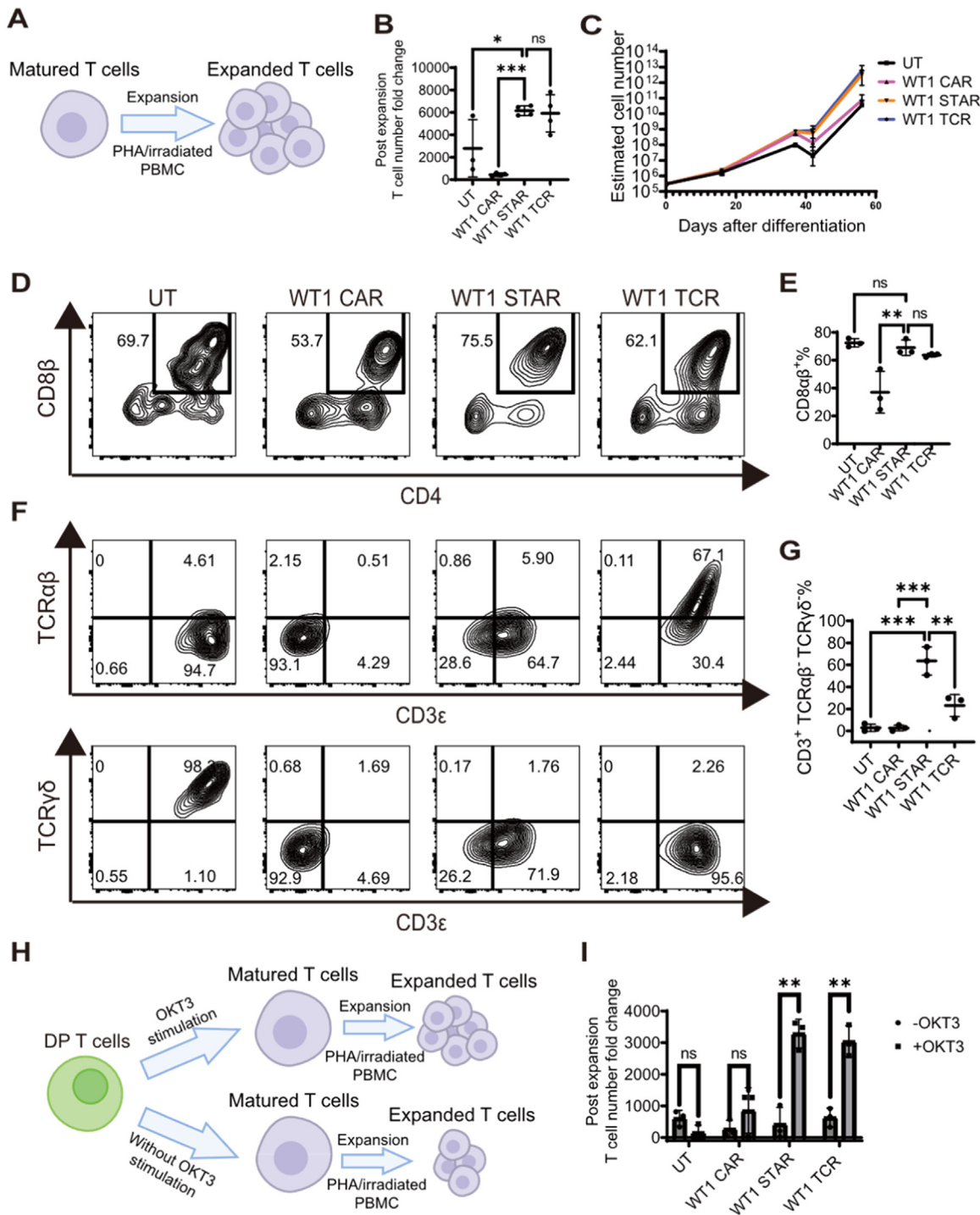


Fig. 4. The CD8αβ⁺ CD3⁺ TCR⁺ phenotype of STAR iT cells was maintained after expansion. (A) Schematic illustration of iPSC-T cell expansion process with irradiated PBMC feeder. (B) Proliferation of UT, WT1-CAR-, WT1-STAR- and WT1-TCR-iT cells at day 14 of PHA/PBMC expansion ($n = 3-4$). Data represent mean $\pm$ 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 iPSC cell number and proliferation fold change ($n = 3$). Data represent mean $\pm$ 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⁺ in cells during PHA/PBMC expansion at day 14 ($n = 3$). Data represent mean $\pm$ SD of n independent experiments. Statistical analysis was performed using one-way ANOVA with Tukey's multiple comparison test. * $P < 0.05$. (F) Representative flow cytometry plots of CD3ε, TCRαβ and TCRγδ expression pattern during PHA/PBMC expansion day 14 cells. (G) Percentage of CD3⁺ TCRαβ⁺ and TCRγδ⁺ during expansion day 14 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. ** $P < 0.01$; *** $P < 0.001$; **** $P < 0.0001$. (H) Schematic illustration of the impact of OKT3 stimulation during maturation on iPSC-T cell expansion capacity. (I) 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 $\pm$ SD of n independent experiments. Statistical analysis was performed using one-way ANOVA with Tukey's multiple comparison test. ** $P < 0.01$. (Color version of figure is available online.)

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

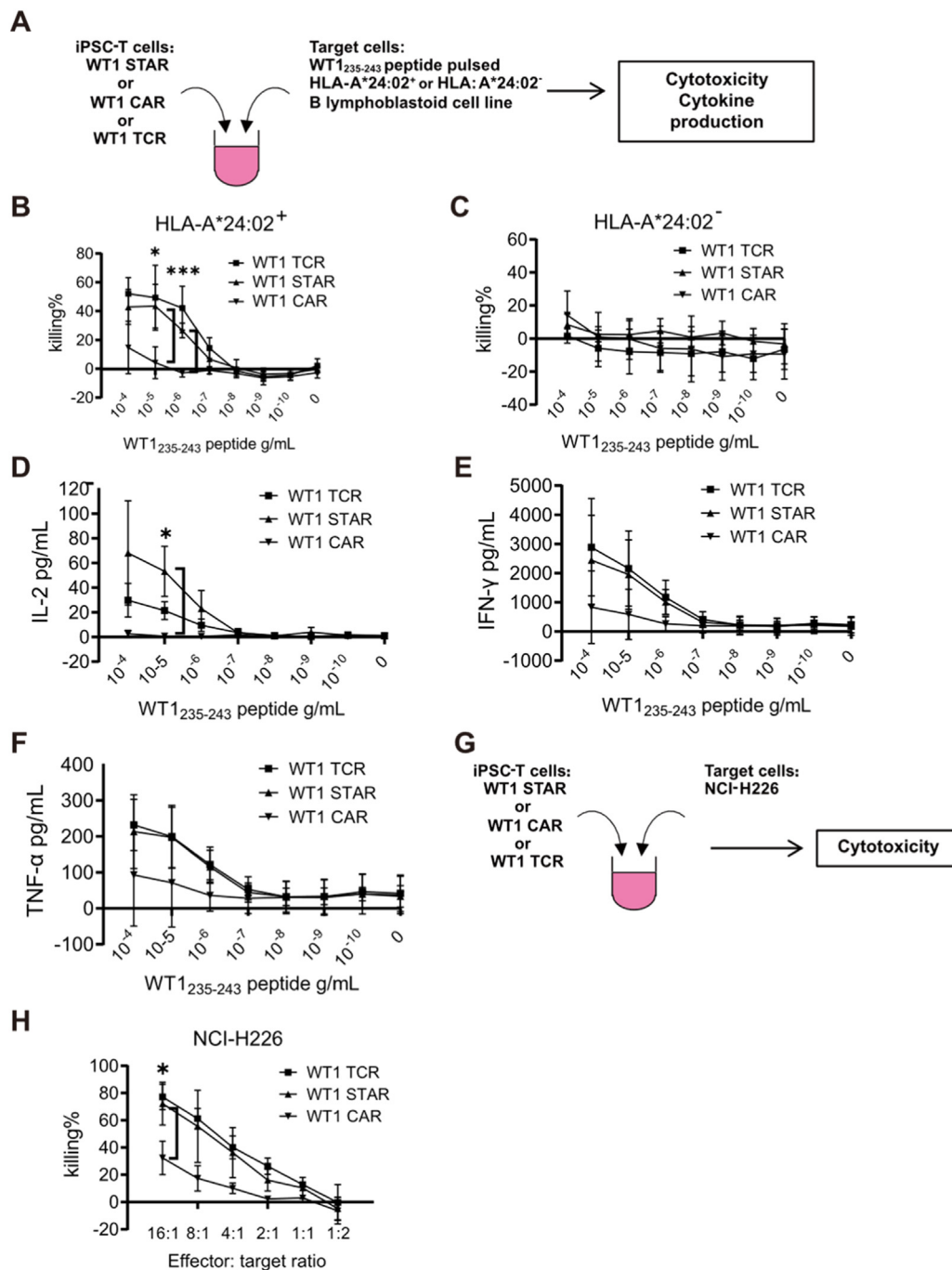


Fig. 5. The STAR-iT cells exhibited higher responsiveness to WT1⁺ tumor cells. (A) Schematic illustration of the killing assay. WT1-CAR-, -STAR- and -TCR-iT cells were cocultured with HLA-A*24:02-positive or -negative B-LCLs. Killing efficiency was evaluated by non-RI killing assay. (B) Non-RI killing results for iT cells against HLA-A*24:02-positive B-LCLs pulsed with serial concentrations of WT1₂₃₅₋₂₄₃ antigen peptide (n = 3). Data represent mean ± 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 WT1₂₃₅₋₂₄₃ antigen peptide (n = 3). Data represent mean ± SD of n independent experiments. (D) Cytometric Bead Array analysis for IL-2 production. Supernatants from cocultures of iT cells with HLA-A*24:02-positive B-LCLs pulsed with a serial of concentrations of WT1₂₃₅₋₂₄₃ were evaluated for IL-2 (n = 3). Data represent mean ± SD of n independent experiments. Statistical analysis was performed using two-tailed t-test. *P < 0.05. (E) Cytometric Bead Array analysis of IFN-γ production. Supernatants of iT cells with HLA-A*24:02-positive B-LCLs pulsed with a serial of concentrations of WT1₂₃₅₋₂₄₃ were evaluated for IFN-γ (n = 3). Data represent mean ± SD of n independent experiments. (F) Cytometric Bead Array analysis of TNF-α production. Supernatants from iT cells with HLA-A*24:02-positive B-LCLs pulsed with a serial of concentrations of WT1₂₃₅₋₂₄₃ were evaluated for TNF-α (n = 3). Data represent mean ± SD of n independent experiments. (G) Schematic illustration of killing assay against NCI-H226. WT1-CAR, -STAR and -TCR-iT cells were cocultured 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 ± SD of n independent experiments. Statistical analysis was performed using two-tailed t-test. *P < 0.05. (Color version of figure is available online.)

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 TAAs 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 TCR signaling machinery for targeted antitumor 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 coreceptor for membrane anchoring, while TRuCs directly fuse a scFv to CD3 ϵ chain. Although both structures adopt native TCR signaling machinery and exhibited enhanced antitumor 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 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 antitumor activity [5]. After 42 days of differentiation, approximately 10^9 STAR-iT cells can be produced from 3×10^5 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⁺ phenotype rather than CD4⁺. 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⁺ and CD8⁺ T cells, CD4⁺ T-cell commitment needs sustained and relatively strong TCR–MHC class II signaling. In contrast, CD8⁺ 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⁺ lineage specification. Moreover, our current system does not provide finely tuned TCR signaling, which may be suboptimal for CD4⁺ 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⁺ 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⁺ 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⁺ and CD4⁺ iT cells, whether these differences are coreceptor–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-CD3 ζ) 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 pLV SIN lentiviral vector using the NEBuilder HiFi Assembly kit (New England Biolabs, Ipswich, MA, USA). The resulting pLV SIN anti-WT1-28BB ζ 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 pLV SIN anti-WT1VH-TRBC-WT1VL-TRAC (STAR) vector. The WT1_{235–243} 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 iPSC 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⁺, SSEA4⁺ and hK01⁺ 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 1×10^4 α -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 1×10^4 α -Glutamine–Penicillin–Streptomycin.

Flow cytometry and antibodies

All flow cytometry staining was 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 was added to all samples for viability analysis prior to flow cytometry analysis and sorting by FACS Aria II (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 ϵ (UCHT1), CD4 (OKT4), CD5 (UCHT2), CD7 (CD7-6B7), CD8 (SK1), CD14 (HCD14), CD235a (GA-R2(HIR2)), IFN- γ (B27), IL-2 (MQ1-17H12), TNF- α (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 β (SID18BEE).

HPC induction from primed iPSCs

T cell differentiation from iPSCs was initiated by inducing HPCs. iPSCs were maintained for 7 days and dissociated into single cells. Cells were cultured in AK02 medium containing $10 \mu\text{M}$ Y-27632 (FujiFilm Wako, Osaka, Japan) and $10 \mu\text{M}$ CHIR99021 (Tocris Bioscience) in 6-well ultra-low attachment plates (Corning) for 24 h to form EBs. After 24 h, EBs were collected and resuspended in StemPro-34 (Thermo Fisher) supplemented with 1×10^4 α -Glutamine–Penicillin–Streptomycin, 2 mM Glutamax (Thermo Fisher), 50 $\mu\text{g}/\text{mL}$ ascorbic acid-2-phosphate (Sigma), 400 μM monothioglycerol (MTG, Nacalai, Kyoto, Japan) and 1×10^4 Insulin-Transferrin-Selemin 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 h, $6 \mu\text{M}$ 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 2 days. Cultures were maintained in a 5% CO_2 , 5% O_2 environment for the first 6 days, and in a 5% CO_2 environment thereafter. CD235a $^+$ CD14 $^-$ CD34 $^{\text{hi}}$ CD43 $^+$ 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 $^-$ CD14 $^-$ CD34 $^{\text{hi}}$ CD43 $^+$ EB cells on 48-well plates coated with recombinant human Fc-DLL4 (10 $\mu\text{g}/\text{mL}$, Sino Biological) and retronectin (10 $\mu\text{g}/\text{mL}$, TAKARA, Japan). Cells were cultured in T cell differentiation medium composed of α MED (Thermo Fisher) supplemented with 15% FBS, 1×10^4 α -Glutamine–Penicillin–Streptomycin, 1×10^4 ITS-G, 55 μM 2-Mercaptoethanol (Thermo Fisher), 50 $\mu\text{g}/\text{mL}$ ascorbic acid-2-phosphate, 2 mM Glutamax, 10 ng/mL rhSCF, 5 ng/mL rhIL-7, 5 ng/mL FLT3L, 10 nM rhSDF-1 α (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, 1×10^5 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, $1 \sim 5 \times 10^4$ DP cells were sorted and seeded onto retronectin-coated plate in α MED supplemented with 15% FBS, 1×10^4 α -Glutamine–Penicillin–Streptomycin, 1×10^4 ITS, 2 mM Glutamax, 50 $\mu\text{g}/\text{mL}$ ascorbic acid 2-phosphate, 5 ng/mL IL-7, 20 ng/mL IL-21, 10 nM rhSDF-1 α (PeproTech) and 15 μM SB203580 (Tocris Bioscience). For the first 2 days, 1 $\mu\text{g}/\text{mL}$ 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]. 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 α MED supplemented with 15% FBS, 1×10^4 α -Glutamine–Penicillin–Streptomycin, 1×10^4 ITS, ascorbic acid 2-phosphate, 2 $\mu\text{g}/\text{mL}$ PHA, 5 ng/mL IL-7, 5 ng/mL IL-15 (PeproTech). Cultures were split and replenished with the fresh medium every 2 or 3 days to maintain sub-confluent cell density.

Nonradioactive cellular cytotoxicity assay

The nonradioactive cellular cytotoxicity assay (Techno Suzuta Co.) was used to measure cytotoxicity. $10^6/\text{mL}$ target cells were cocultured with 2.5 μL BM-HT in 37°C for 15 min, then washed three times at 4°C, 600 g for 5 min. Target cells were cocultured with effector cells to conduct the cytotoxicity assay. 2% Triton PBS was used to lyse target cells for evaluating the maximal killing. After coculture, 20 μL of culture supernatant was incubated with 200 μL Europium solution for 15 min at room temperature. Time-resolved fluorescence was measured by using Multimode Plate Reader NIVO (PerkinElmer). %lysis = (experimental lysis – spontaneous lysis)/(maximal lysis – spontaneous lysis) $\times 100\%$.

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_{235–243} peptide for 1 h, then cocultured with iPSC-T cells at 2:1 effector-to-target ratio for 3 h [5,38]. Additionally, the mesothelioma cell line NCI-H226, which is endogenously expressing WT1, was cocultured with iPSC-T cell at effector-to-target ratio ranging from 1:2 to 16:1 for 3 h.

Cytokine production

The BD Cytometric Bead Array Flex Set System (BD Biosciences) was used to measure human IL-2, IFN- γ and TNF- α levels [6]. iPSC-T cells were cocultured with WT1_{235–243} peptide-pulsed HEV60 cells. After 24 h, 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 α MED supplemented with PMA, ionomycin and monensin at 37°C for 3 h [5]. Cells were washed at 4°C, 600 g for 5 min 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 min at room temperature. Cells were washed twice with intracellular staining perm wash

buffer at 4°C, 600 g for 5 min and blocked with 2% FBS in PBS for 1 h at room temperature. Finally, the fixed cells were stained with either isotype controls or antibodies against IL-2, IFN- γ and TNF- α in the dark for 20 min at room temperature. Cells were washed and analyzed by FACS Aria II (BD Biosciences).

CD247 phosflow

iPSC-derived HPCs were seeded on FcDLL4 and cultured for 3 days. Cells were harvested, washed and fixed with -20°C methanol on ice for 15 min, then wash twice with 2% FBS in PBS at 4°C, 600 g for 5 min. Cells were blocked with 2% FBS in PBS for 1 h at room temperature. Cells were stained with either isotype control or antibody against CD247 (pY142) in the dark at 4°C for 20 min [8]. Cells were washed and analyzed by FACS Aria II (BD Biosciences).

Statistical analysis

Number of independent experiments is 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.

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.

Declaration of Competing 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. Other authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this article.

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Supplementary materials

Supplementary material associated with this article can be found in the online version at doi:10.1016/j.jcyt.2025.102001.

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