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Soluble and Immobilized Anti-CD3/28 Distinctively Expand and Differentiate Primary Human T Cells: An Implication for Adoptive T Cell Therapy

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ABSTRACT

Cell-based cancer therapies have led to a paradigm shift in the treatment of patients with various cancers. To date, a vast majority of cancer immunotherapies have used genetically engineered T cells to target tumors. Stimulation and ex vivo expansion of T cells, as one of the crucial starting materials for T cell manufacturing, have always been a critical part of adoptive T-cell therapy (ACT). Typically, anti-CD3 and anti-CD28 monoclonal antibodies (mAbs) along with interleukin-2 (IL-2), through transducing signals one, two, and three, respectively, are essential for in vitro T cell activation. Terminal differentiation and replicative senescence are the main barriers of the ACTs during the manufacturing of engineered T cells ex vivo.

In this study, we aimed to compare the T cell activation protocol that we developed in our lab (soluble anti-CD3/28 mAbs) with a common T cell activation protocol (immobilized anti-CD3/soluble anti-CD28) in terms of T cell expansion, activation, immunophenotype, and cellular fate.

We observed that T cells were equally expanded in both protocols. Notably, our modified protocol promoted the outgrowth of $CD8^+$ T cells postactivation. Concerning the low concentrations of both soluble anti-CD3 and anti-CD28, the modified protocol could significantly enrich memory T cell subsets.

In conclusion, our data demonstrated that the soluble CD3/28 mAbs protocol is cost-effective and more efficient for generating more potent T cells, thereby expecting a better therapeutic outcome.

Keywords: Adoptive cellular therapy; Immobilized antibodies; Lymphocyte activation; Memory phenotype

INTRODUCTION

Adoptive cellular therapy (ACT) is considered as one of the main treatment modalities of cancer immunotherapy. ACT is defined as the infusion of functional autologous or allogeneic immune cells into cancer patients to target cancer.[1] There are three major pillars for ACT: tumor-infiltrating lymphocytes (TIL)-, T-cell receptor (TCR)-engineered T cell- and chimeric antigen receptor (CAR)-engineered T-cells–based

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therapies.[2] The most promising and effective forms of ACTs are TCR-engineered and chimeric antigen receptor (CAR) T cells. Engineering of T cells mainly involves the isolation of peripheral blood mononuclear cells (PBMCs) from patients, activation of T cells via the endogenous T-cell receptor/CD3 complex and CD28 costimulatory molecules followed by CAR-encoding viral transduction, ex vivo expansion of engineered T cells, and infusion of T-cell product.

Upon T-cell activation, T cells undergo expansion and primarily differentiate into specific cell subsets, including central memory T cells (Tcm), effector memory T cells (Tem), and terminally differentiated effector T cells (Tte).[3] Various studies have revealed that Tcm cells have a superior capacity to fight tumors and persist longer in vivo compared to Tem and Tte cells.[4, 5] Thus, activation of T cells prior to viral transduction can promote differentiation of engineered T cells toward terminally differentiated T cells with inferior antitumor potency.[6]

A recent study has attempted to develop a protocol to virally transduce nonactivated T cells for the production of engineered T cells.[7] However, in cancer immunotherapy, an adequate number of engineered T cells (a few hundred billion in some cases) is critical for mounting an effective antitumor response,[8] and isolating this number of T cells from cancer patients is challenging. In this study, we aimed to develop a T-cell activation protocol that is not only cost-effective but also yields an adequate number of central and effector memory T cells with superior antitumor activity.

In clinical settings, T-cell stimulation is mostly performed by immobilized anti-CD3/28 monoclonal antibodies (mAbs).[8] Compared to soluble anti-CD3/28, immobilized anti-CD3/28 mAbs induce higher rates of proliferation and differentiation.[8] Based on our previous study,[9] we hypothesized that reducing the concentration of T cell stimulators, in this case, anti-CD3/28 mAbs, and utilizing soluble mAbs instead of immobilized mAbs might decrease terminal differentiation of T cells and enrich memory T cell subsets.

We also decreased the concentration of anti-CD3/28 by 8 to 10 folds. We further compared T cell expansion, activation markers, and memory T cell subsets between two different protocols: modified T cell activation protocol (soluble anti-CD3/28 mAbs) and conventional T-cell activation protocol (immobilized anti-CD3/soluble anti-CD28); the latter has previously been described.[10]

MATERIALS AND METHODS

Antibodies and Reagents

Recombinant human interleukin-2 (IL-2) (#130-097-746), human T-cell activators, anti-human CD3 (clone: OKT3, #130-093-387), and anti-human CD28 (clone: 15E8, #130-093-375) were purchased from Miltenyi Biotec, Germany. RPMI 1640 and fetal bovine serum (FBS) were obtained from Gibco, USA. PE anti-human CD4 (clone: PRA-T4, #300508), APC anti-human CD8 (clone: PRA-T8, #301014), FITC anti-human CD3 (clone: UCHT1, #300406), and PE anti-human CD45RO (clone: UCHL1, #C4205) antibodies were purchased from Biolegend, USA. PE/FITC conjugated anti-CD25/CD4 (clone: RPA-T4 and BC96, #22-8425-71) cocktail antibody was obtained from eBioscience, USA. Alexa fluor-647 anti-human CCR7 (clone: 3D12, #557734) was obtained from BD, USA.

PBMC Isolation

Whole blood was drawn from 3 healthy donors with no history of disease in the last 3 months. The participants gave written informed consent for a study protocol approved by the Institutional Research Ethics Committee of Tehran University of Medical Sciences (IR.TUMS.MEDICINE.REC.1399.715). PBMCs were then isolated from peripheral blood using Histopaque-1077 (Sigma Aldrich, USA). Briefly, whole blood was diluted 2x with phosphate-buffered saline (PBS). Then 20 mL of diluted blood was placed over 10 mL of Histopaque in a 50 mL conical tube. Centrifugation was performed at 800 *g* for 20 minutes with the brake off. The buffy coat was then collected, transferred to a new 15 mL conical tube, and washed twice with PBS (at 400 *g* for 5 min). After platelet removal (centrifugation at 100 *g*, for 4 min), PBMCs were counted using a hemocytometer and stored in cryovials containing cryoprotective media made of 10% dimethyl sulfoxide (Sigma Aldrich, USA) and 90% heat-inactivated FBS (Gibco, USA) for long-term storage in a nitrogen tank.

T-Cell Activation

T cells were activated with soluble or coated anti-human CD3 mAb (Miltenyi Biotec, Germany) and soluble anti-human CD28 mAb (Miltenyi Biotec, Germany). For T-cell activation using coated anti-human CD3, $2x10^6$ PBMCs were thawed and seeded in anti-human CD3 (1 μg/mL) precoated 24-well cell

culture plates (Costar, Cambridge, MA, USA). These cells were further cultured in 2 mL of RPMI1640 medium (Gibco, USA) supplemented with 10% heat-inactivated FBS (Gibco, USA), 1% penicillin/streptomycin (Gibco, USA), 3 μg/mL anti-human CD28 mAb, and 100 IU/mL human recombinant IL-2 (Miltenyi Biotec, Germany). After 3 days of culture, cells were harvested, counted, and checked for viability with trypan blue staining and used for further analysis.

For the activation of T cells with soluble anti-human CD3 and CD28, $1.5x10^6$ PBMCs were thawed and seeded in 24-well cell culture plates. PBMCs were cultured in 1.5 mL of RPMI 1640 medium supplemented with 10% heat-inactivated FBS, 1% penicillin/streptomycin, 0.2 μg/mL anti-human CD3, 0.6 μg/mL anti-human CD28, and 100 IU/mL human recombinant IL-2 (Miltenyi Biotec, Germany). On the third day, 0.5 mL complete RPMI 1640 media containing 100 IU/mL of IL-2 was added to each well. After four days, cells were harvested, counted, and checked for viability with trypan blue staining and used for further analysis.

Flow cytometry

Immunophenotyping was performed on T cells activated with coated and soluble activation protocols on days 3 and 4 postactivation, respectively. Briefly, cells were washed with the washing buffer (PBS + 2% FBS). The cells were then counted, and 10^5 cells were labeled with fluorochrome-conjugated antibodies according to the manufacturer's instructions. After 30 minutes of incubation with antibodies at 4°C, cells were washed and resuspended in the washing buffer. Positively stained cells were differentiated from the background using unstained controls. Flow cytometry was performed using a BD FACSCalibur system.

Statistical Analysis

Flow cytometry data were analyzed using FlowJo software (Tree Star Inc. version 10.6). Statistical analysis was performed using the SPSS software (version 26.0). Statistical significance was determined using two-way analysis of variance. In all tests, $p<0.05$ were considered statistically significant. The graphs were illustrated using GraphPad Prism software (version 8.3.0).

RESULTS

T Cells Were Enriched with Low Concentrations of Soluble Anti-CD3/28 mAbs

PBMCs were thawed and activated with soluble anti-CD3 and anti-CD28 mAbs (0.2 μg/mL and 0.6 μg/mL, respectively). Thawed PBMCs were also activated with a conventional T-cell activation protocol in which anti-CD3 was coated (1 μg/mL), and anti-CD28 was added to the culture at a concentration of 3 μg/mL. To investigate the potential difference between the two protocols, we counted the number of T cells postactivation. Our results demonstrated a significant difference in T-cell expansion between the two protocols (Figure 1A). As expected, T cells activated with immobilized mAbs exhibited a higher expansion rate. The frequency of the $CD3^+$ population was greater than 90% in both protocols (Figure 1B). Interestingly, the fraction of CD8+ T cells was significantly higher in T cells activated with soluble mAbs (Figure 1C). However, no significant difference was observed in terms of $CD4^+$ T-cell abundance and CD4/CD8 ratio between the two protocols (Figures 1D and 1E).

T Cells Undergo Full Activation Even with Low Concentrations of Soluble Anti-CD3/28 mAbs

Next, we investigated whether the stimulated T cells from the modified protocol (soluble anti-CD3/CD28 mAbs) were equally activated as T cells stimulated with plate-coated activation protocol. As there are several ways of measuring T-cell activation, including evaluation of T-cell proliferation, cytokine production, CD69 (as an early activation marker), and CD25 (as a late activation marker) expression, we measured CD25 and CD69 expression on T-cell postactivation in both protocols. Our results exhibited no statistical significance in the expression of early and late activation markers between the two protocols (Figures 2A and 2B). Thus, both protocols could similarly activate T cells.

Stimulation of T Cells with Lower Concentrations of Soluble Anti-CD3/28 mAbs Promoted their Differentiation Towards Memory Phenotype

As T cells stimulated with immobilized anti-CD3 and higher concentrations of mAbs have higher expansion rates, we hypothesized that conventional T cell activation protocol could induce a higher rate of T cell differentiation. To investigate our hypothesis, we compared the frequency of naïve T cells, central

memory T cells, effector memory T cells, and terminally differentiated T cells within the $CD3^+$ T cell population postactivation between the two protocols. Our findings suggested that stimulation of PBMCs with lower concentrations of soluble anti-CD3/28 mAbs resulted in significant enrichment of naïve, central memory, and effector memory T cells compared to plate-coated PBMC activation protocol (Figures 3A-C). We also observed a nonsignificant decrease in the frequency of terminally differentiated T cells in the PBMCs stimulated with soluble/low concentrations of anti-CD3/28 mAbs (Figure 3D).

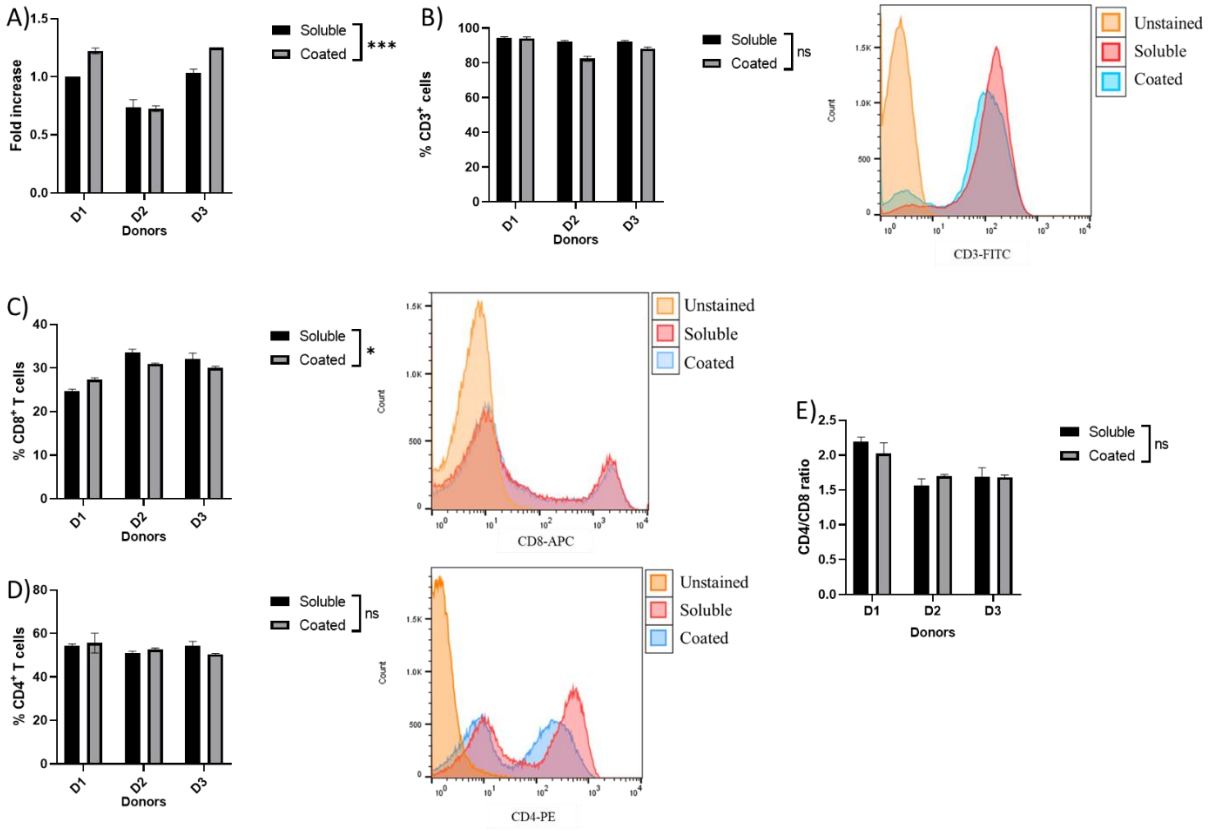

Figure 1. T-cell enrichment with low concentrations of soluble anti-CD3/28 mAbs. Fold expansion of T-cell postactivation with soluble and immobilized mAbs (A). Expression of CD3 (B), CD8 (C), CD4 (D), and the CD4/CD8 ratio (E) postactivation with soluble and immobilized mAbs. The bar graphs represent 3 independent donors. Histograms represent overlaid results of 1 donor. All data were analyzed using two-way analysis of variance. *p<0.05; ***p<0.001. Data are represented as mean±SD. The experiments were repeated 3 independent times.

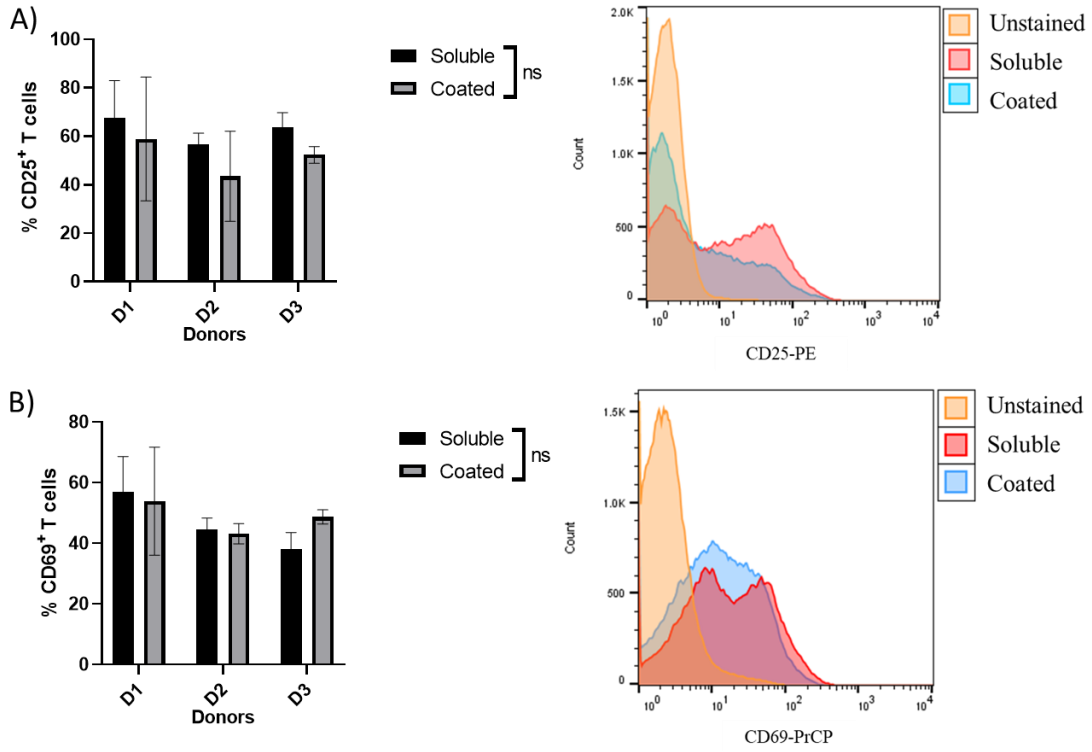

Figure 2. The activation of T cells with low concentrations of soluble anti-CD3/28 mAbs. Expression of CD25 (A) and CD69 (B) on T cells postactivation. All data were analyzed using two-way analysis of variance. Bar graphs represent 3 independent donors. Histograms display the overlaid results of 1 donor. ns: not significant. Data are represented as mean±SD. The experiments were repeated 3 independent times.

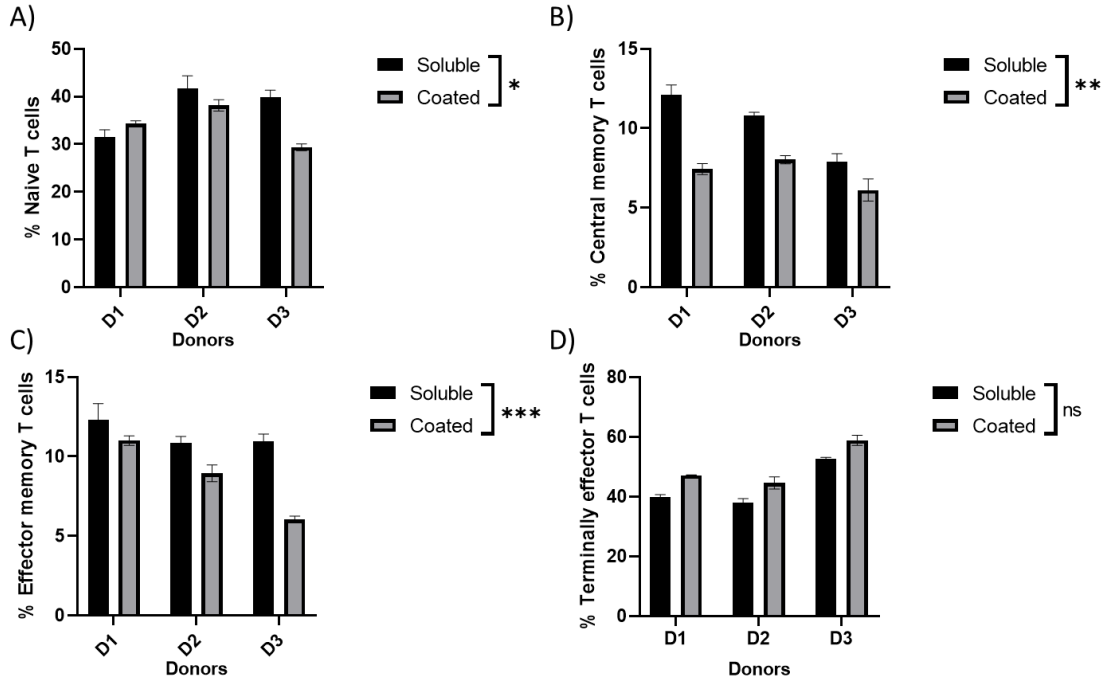

Figure 3. Immunophenotyping of T cell subsets postactivation for the two protocols. Frequency of naïve T cells ($CCR7^+CD45RO^-$) (A), central memory T cells ($CCR7^+CD45RO^+$) (B), effector memory T cells ($CCR7^-CD45RO^+$) (C), and terminally differentiated effector T cells ($CCR7^-CD45RO^+$) postactivation with soluble and immobilized mAbs (D). The bar graphs represent 3 independent donors. All data were analyzed using two-way analysis of variance. *p<0.05; **p<0.01; ***p<0.001. Data are represented as mean±SD. The experiments were repeated 3 independent times.

DISCUSSION

The intrinsic qualities of engineered T cells, such as persistence and proliferation capacity, have been proven to be critical determinants of clinical outcomes following adoptive T cell therapies.[11] Different T-cell subsets exhibit diverse inherent properties. For instance, memory T cells offer a higher rate of antitumor response, whereas terminally differentiated T cells often show weaker antitumor activity.[6,12] The number of T cells and the differentiation status of adoptively transferred T cells are two important factors that appear to be correlated with the response to treatment in patients with metastatic cancers and solid tumors.[13] This notion is generally supported by the fact that a large number of effector T cells are required for tackling the outgrowth of tumor cells in a tumor bulk.[14] As the tumor-immune interface is like a battleground, a sufficient number of tumor-specific T cells or continuous replenishment of tumor-specific T cells by tumor-specific memory T cells can give T cells an upper hand in fighting against tumor cells.[4,5,14] Unfortunately, the immunobiological coupling of expansion and effector differentiation of T cells offers a substantial therapeutic roadblock against effective T-cell–based cancer therapies. Therefore, scientists in the field of cell-based cancer immunotherapy have long been struggling how to expand T cells ex vivo without triggering cellular differentiation and replicative senescence.[14] Recently, genetic modification of cytokine signaling and epigenetic modification of T cells have led to a higher expansion rate of engineered T cells while retaining their central memory and/or stem cell memory phenotype.[15,16] In this study, we demonstrated that low concentrations of anti-CD3/28 mAbs could decrease the rate of T-cell differentiation to the terminal effector stage and promote the enrichment of less differentiated T cells.

In another study, soluble mAbs triggered short-term activation, while surface-bound mAbs led to cross-linking and clustering of an adequate number of TCR and induced extensive intercellular signaling with long-term effects.[17] Mechanistically, blockade of

phosphatidylinositol 3-kinase δ (PI3Kδ), as a critical protein involved in downstream signaling pathways of T cell activation, has been shown to inhibit the terminal differentiation of T cells during anti-CD3/28 ex vivo expansion.[18] Therefore, a low concentration of soluble anti-CD3/28 may regulate intercellular signaling pathways and decrease the rate of terminal differentiation. Moreover, high concentrations of stimulators have been reported to induce exhaustion-related markers.[19] Thus, it can be concluded that higher concentrations of anti-CD3/28 mAbs lead to intensive downstream signaling and promote terminal differentiation and probably T-cell exhaustion. However, the expression of exhaustion markers should be evaluated in enriched T cells based on two protocols in future studies. Since our modified protocol utilizes lower anti-CD3/28 mAbs, this protocol could be more cost-effective for generating engineered T cells than conventional methods.

Finally, we suggest that future studies explore other memory T cell subsets, such as T memory stem cells. In addition, the effects of different clones of anti-CD3/CD28 antibodies (from other suppliers) on the proliferation and differentiation of T cells should be assessed. The functional characterization of activated T cells can further validate the developed protocol.

STATEMENT OF ETHICS

The participants gave written informed consent for the study protocol approved by the Institutional Review Board of Tehran University of Medical Sciences (IR.TUMS.MEDICINE.REC.1399.715).

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CONFLICT OF INTEREST

The authors declare no conflicts of interest.

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REFERENCES

1. Emens LA, Ascierto PA, Darcy PK, Demaria S, Eggermont AMM, Redmond WL, et al. Cancer immunotherapy: Opportunities and challenges in the rapidly evolving clinical landscape. Eur J Cancer. 2017;81:116-29.
2. Kirtane K, Elmariah H, Chung CH, Abate-Daga D. Adoptive cellular therapy in solid tumor malignancies: review of the literature and challenges ahead. Journal for immunotherapy of cancer. 2021;9(7).
3. Gattinoni L, Speiser DE, Lichterfeld M, Bonini C. T memory stem cells in health and disease. Nat Med. 2017;23(1):18-27.
4. Klebanoff CA, Gattinoni L, Torabi-Parizi P, Kerstann K, Cardones AR, Finkelstein SE, et al. Central memory self/tumor-reactive CD8+ T cells confer superior antitumor immunity compared with effector memory T cells. Proc Natl Acad Sci U S A. 2005;102(27):9571-6.
5. Li Y, Wu D, Yang X, Zhou S. Immunotherapeutic Potential of T Memory Stem Cells. Front Oncol. 2021;11:723888.
6. Ghassemi S, Nunez-Cruz S, O'Connor RS, Fraietta JA, Patel PR, Scholler J, et al. Reducing Ex Vivo Culture Improves the Antileukemic Activity of Chimeric Antigen Receptor (CAR) T Cells. Cancer Immunol Res. 2018;6(9):1100-9.
7. Ghassemi S, Durgin JS, Nunez-Cruz S, Patel J, Leferovich J, Pinzone M, et al. Rapid manufacturing of non-activated potent CAR T cells. Nat Biomed Eng. 2022;6(2):118-28.
8. Li Y, Kurlander RJ. Comparison of anti-CD3 and anti-CD28-coated beads with soluble anti-CD3 for expanding human T cells: differing impact on CD8 T cell phenotype and responsiveness to restimulation. J Transl Med. 2010;8:104.
9. Rostamian H, Khakpoor-Koosheh M, Jafarzadeh L, Masoumi E, Fallah-Mehrjardi K, Tavassolifar MJ, et al. Restricting tumor lactic acid metabolism using dichloroacetate improves T cell functions. BMC Cancer. 2022;22(1):39.
10. Jafarzadeh L, Masoumi E, Mirzaei HR, Alishah K, Fallah-Mehrjardi K, Khakpoor-Koosheh M, et al. Targeted knockdown of Tim3 by short hairpin RNAs improves the function of anti-mesothelin CAR T cells. Mol Immunol. 2021;139:1-9.
11. Jafarzadeh L, Masoumi E, Fallah-Mehrjardi K, Mirzaei HR, Hadjati J. Prolonged Persistence of Chimeric Antigen Receptor (CAR) T Cell in Adoptive Cancer

Immunotherapy: Challenges and Ways Forward. Front Immunol. 2020;11:702.
12. Liu Q, Sun Z, Chen L. Memory T cells: strategies for optimizing tumor immunotherapy. Protein Cell. 2020;11(8):549-64.
13. Klebanoff CA, Gattinoni L, Palmer DC, Muranski P, Ji Y, Hinrichs CS, et al. Determinants of successful CD8+ T-cell adoptive immunotherapy for large established tumors in mice. Clin Cancer Res. 2011;17(16):5343-52.
14. Crompton JG, Sukumar M, Restifo NP. Uncoupling T-cell expansion from effector differentiation in cell-based immunotherapy. Immunol Rev. 2014;257(1):264-76.
15. Akbari B, Ghahri-Saremi N, Soltantoyeh T, Hadjati J, Ghassemi S, Mirzaei HR. Epigenetic strategies to boost CAR T cell therapy. Mol Ther. 2021;29(9):2640-59.
16. Ghahri-Saremi N, Akbari B, Soltantoyeh T, Hadjati J, Ghassemi S, Mirzaei HR. Genetic Modification of Cytokine Signaling to Enhance Efficacy of CAR T Cell Therapy in Solid Tumors. Front Immunol. 2021;12:738456.
17. Poltorak MP, Graef P, Tschulik C, Wagner M, Cletiu V, Dreher S, et al. Expamers: a new technology to control T cell activation. Sci Rep. 2020;10(1):17832.
18. Petersen CT, Hassan M, Morris AB, Jeffery J, Lee K, Jagirdar N, et al. Improving T-cell expansion and function for adoptive T-cell therapy using ex vivo treatment with PI3Kdelta inhibitors and VIP antagonists. Blood Adv. 2018;2(3):210-23.
19. Kouro T, Himuro H, Sasada T. Exhaustion of CAR T cells: potential causes and solutions. J Transl Med. 2022;20(1):239.