# Targeted knockdown of Tim3 by short hairpin RNAs improves the function of anti-mesothelin CAR T cells

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**Research**

**Keywords:** Mesothelin, Chimeric antigen receptor, Tim3, Targeted knockdown, Tumor microenvironment.

**Posted Date:** July 14th, 2020

**DOI:** https://doi.org/10.21203/rs.3.rs-41304/v1



**Version of Record:** A version of this preprint was published at Molecular Immunology on November 1st, 2021. See the published version at https://doi.org/10.1016/j.molimm.2021.06.007.

# Abstract

**Background:** T-cell immunoglobulin mucin 3 (Tim3) is an immune checkpoint receptor that plays a central role in chimeric antigen receptor (CAR) T cell exhaustion within the tumor microenvironment. This study was aimed to evaluate the effects of targeted-knockdown of Tim3 on the antitumor function of anti-mesothelin (MSLN)-CAR T cells.

**Methods:** To knockdown *Tim3* expression, three different shRNA sequences specific to different segments of the human *Tim3* gene were designed and co-inserted with an *anti-MSLN-CAR* transgene into lentiviral vectors. To investigate the efficacy of Tim3 targeting in T cells, expression of Tim3 was assessed before and after antigen stimulation. Afterwards, cytotoxic effects, proliferative response and cytokine production of MSLN-CAR T cells and Tim3-targeted MSLN-CAR T cells were analyzed.

**Results:** Our results showed that activation of T cells and MSLN-CAR T cells led to up-regulation of Tim3. Tim3 knockdown significantly decreased its expression in different groups of MSLN-CAR T cells. Tim3 knockdown significantly improved cytotoxic function, cytokine production and proliferation capacity of MSLN-CAR T cells.

**Conclusions:** Our findings indicate that targeted knockdown of Tim3 allows tumor-infiltrating CAR T cells that would otherwise be inactivated to continue to expand and carry out effector functions, thereby altering the tumor microenvironment from immunosuppressive to immunosupportive via mitigated Tim3 signaling.


# Background

Over the past two decades, adoptive T cell immunotherapy (ACT) for cancer has received considerable attention by the scientific community. Better understanding of the function of immune system as well as identification of new tumor antigens has led to the development of novel ACT approaches. Among these approaches, genetic modification of polyclonal T cells to express chimeric antigen receptors (CAR) against tumor antigens has been very successful and it might have the potential to become a cornerstone of cancer treatment (1).

Various tumor antigens have been targeted in CAR T cell studies. For instance, CD19 has been extensively used as a target tumor antigen in hematological malignancies (2–5). However, the paucity of tumor-specific antigens, that can be targeted safely and effectively, has been a major challenge in developing CAR T cells for solid tumors(6–8). Mesothelin is a tumor-associated antigen (TAA) which is overexpressed in several cancers including mesothelioma, lung, ovary and pancreatic cancers(9). Recent clinical trials have demonstrated that treatment of pancreatic adenocarcinoma or mesothelioma with active immunization or immunoconjugates targeting mesothelin results in favorable clinical responses without toxicity (10–12). These results and preclinical CAR T cell therapy models have introduced mesothelin as an ideal target of CAR T cell therapy in multiple solid tumors.

Although CAR-expressing T cells have made great strides in the treatment of patients with advanced hematological malignancies, their efficacy in solid tumors has been limited in part due to the presence of immunosuppressive cells and molecules within tumor microenvironment (TME) (6–8). The latter includes immunosuppressive cytokines (e.g. TGF-β) and immune checkpoint molecules such as PD-1, CTLA-4 and Tim3. Among the inhibitory molecules, Tim3 is preferentially upregulated in exhausted tumor-infiltrating lymphocytes (13, 14). Upon engagement with its cognate ligands, Tim3 plays a crucial role in tumor-induced immunosuppression likely through induction of intracellular calcium flux and T cell death (15). Various studies have demonstrated that Tim-3 blockade reverses immunosuppression through reducing regulatory T cells and increased production of IFN-γ by T cells (16, 17). In the current study, in order to diminish the negative effects of Tim signaling on antitumor function of CAR T cells, fully human second-generation anti-MSLN-CAR T cells were generated that co-expressed short hairpin RNA (shRNA) sequences against Tim-3 (Tim3.sh.MSLN-CAR T cell). Tim3 expression was evaluated in these cells, and the cells were analyzed for their proliferative response, cytokine production and antitumor cytotoxic effects.

## Materials And Methods

# Cell lines

HeLa (human cervical cancer cell line), HEK293T cell (human embryonic kidney T 293), Skov3 (human ovarian cancer cell line), Ovcar3 (human ovarian cancer cell line) and NALM-6 (pre-B cell leukemia cell line) cells were purchased from the Iranian Biological Resource Center (IBRC), Iran., Skov3, Ovcar3 cell lines were used as mesothelin-expressing cell lines and NALM-6 was used as a mesothelin negative control. Before the experiments, expression of mesothelin in these cells was analyzed by flow cytometry using PE-conjugated anti-human mesothelin antibody (R&D Systems, Minneapolis, MN, USA). HEK293T cells were used as the packaging cell line for the production of lentiviral particles. Mycoplasma contamination of all cell lines was routinely examined by polymerase chain reaction (PCR). KEK293T, and Skov3 cells were cultured in DMEM (Gibco Laboratories, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS) (Sigma Chemical Co., St. Louis, MO, USA) and 100 µg/ml penicillin-streptomycin (PAN Biotech, Aidenbach, Germany), and incubated at 37 °C in 5% CO2, 95% humidity atmosphere. Ovcar3 was cultured in RPMI-1640 (Gibco Laboratories, Grand Island, NY, USA) supplemented with 10% FBS and 100 µg/ml penicillin-streptomycin, and 2 Mm L-glutamine (Gibco Laboratories, Grand Island, NY, USA) incubated at 37 °C in 5% CO2, 95% humidity atmosphere.

# Genetic Modification Of T Cells

To generate a fully human anti-MSLN CAR construct, a Kozak consensus ribosome-binding sequence, a human CD8a signal peptide (SP) and a fully human anti-MSLN scFv were linked to the CD8a hinge, 4-1BB transmembrane region (TM) and intracellular domains containing the 4-1BB and CD3ζ domains. Mesothelin-specific scFv fragment originated from P4-scFv (18). Other fragments of CAR construct have

been described previously(3). The CAR expression cassette was under the control of CMV promoter. To knockdown Tim3 expression, three shRNA-encoding sequences against three different regions of Tim3 gene were designed and inserted after the CD3ξ domain sequence in MSLN-CAR constructs (hereafter referred to Tim3.sh1.MSLN-CAR, Tim3.sh2.MSLN-CAR and Tim3.sh3.MSLN-CAR). The expression of shRNA-encoding sequences was under the control of a CMV promoter. ShRNA sequences are shown in Table 1. MSLN-CAR gene cassettes and different shRNA-coding sequences containing 5'-Flank-Sense-Loop-Antisense-3'Flank segments were cloned into a pCDH-CMV-MCS-EF1α-cGFP-2A-Puro lentivector backbone for production of third-generation lentiviral particles.

Table 1
shRNA sequences against different regions of Tim3 transcript.

| Name | Sense sequence | Anti-sense sequence | Target gene |
|---|---|---|---|
| shRNA1 (sh1) | CCATAGAGAATGTGACTCTAGC | GCTAGAGTCACATTCTCTATGG | Tim3 (HAVCR2), NM_032782.5 |
| shRNA2 (sh2) | TCGCTCAGAAGAAAACATCTAT | ATAGATGTTTTCTTCTGAGCGA | |
| shRNA3 (sh3) | GCACTGAACTTAAACAGGCAT | CATGCCTGTTTAAGTTCAGTGC | |

# Production Of Third-generation Lentiviral Particles

To generate three different Tim3-targeted MSLN-CAR T cells, HEK293T cells were transfected with plasmids encoding three different shRNAs against Tim3 (Tim3.sh1, sh2 and sh3.MSLN-CAR). Two other plasmids, including MSLN-CAR and empty vector (CAR null) were also used for production of MSLN-CAR T cells and Mock-T cells. Different MSLN-CAR plasmids and packaging plasmids (pMDLg/pRRE, pMD.G and pRSV-Rev) were co-transfected into HEK293T cells using a standard calcium phosphate precipitation method. Virus-containing media (VCM) was then harvested 24, 36 and 48 hours post-transfection and pooled. To discard cell debris, VCM was filtered through 0.45 µm pore size membrane filters and concentrated by ultracentrifugation at 26,000 rpm for 2 h at 4 °C (Optima LE-80 K Ultracentrifuge, Beckman Coulter, Indianapolis, IN). The viral particles were resuspended in complete DMEM media and stored at -80 °C in single-use aliquots. Titers of concentrated VCMs were determined by a limiting dilution method using flow cytometry on primary human T cells activated with anti-CD3 and anti-CD28 antibodies.

# Primary human T cell purification, activation and lentiviral gene transduction

Buffy coat from healthy donors was provided by the Iranian Blood Transfusion Organization (IBTO) (consented under Institutional Review Board approved research protocols at the Tehran University of

Medical Sciences). Peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll-Paque gradient centrifugation. T lymphocytes were then enriched and activated *in vitro* by culturing PBMCs at $1 \times 10^6$ cells/well in 6-well cell culture plates (Costar, Cambridge, MA, USA) coated with 1 µg/ml anti-CD3 mAb (Miltenyi Biotec, Bergisch Gladbach, Germany), 3 µg/ml anti-CD28 soluble mAb (Miltenyi Biotec, Bergisch Gladbach, Germany), and 100 IU/ml human recombinant IL-2 (Miltenyi Biotec, Bergisch Gladbach, Germany). After three days, more than 95% of the cells in the culture were $CD3^+$ T lymphocytes, as confirmed by flow cytometry. Activated T cells were then transduced by concentrated lentiviral supernatants at multiplicity of infection (MOI) of 7 in the presence of polybrene (8 µg/ml, Santa Cruz Biotechnology, Santa Cruz, CA, USA) in a 12-well cell culture plate. The plate was then spinoculated at 2100 rpm at 32 °C for 60 min, followed by incubation at 37 °C. Next 6–7 hours, 2 ml of fresh complete RPMI-1640 media supplemented with IL-2 (100 IU/ml) (Miltenyi Biotec, Bergisch Gladbach, Germany) was added to each well. After 4 days, cell surface expression of anti-MSLN CAR was measured by flow cytometry using biotinylated goat anti-human IgG F(ab')2 (BioRad, Hercules, CA, USA) and APC-conjugated Streptavidin (BioRad, Hercules, CA, USA).

# Tim-3 Knockdown Experiments

Percentage of Tim3 positive cells and mean fluorescence intensity (MFI) of Tim3 were evaluated by flow cytometry using human Tim3 (CD366) APC-conjugated antibody (BioLegend, San Diego, CA, USA) in T cells before activation, activated/un-transduced T cells (Un-T), and activated/transduced T cells (i.e. Mock-transduced and CAR T cells). Percentage of Tim3 positive Mock T cells (transduced with empty vector) and CAR T cells (with and without shRNA) was also determined following co-incubation with MSLN-positive target cells. To analyze efficacy of Tim3-knockdown, the MFI/percentage of Tim3 positive cells in MSLN-CAR T cells, Tim3.sh1, sh2, and sh3.MSLN-CAR T cells as well as Tim3.sh3.sh2.MSLNCAR T cells (i.e. T cells that co-transduced with two viruses carrying Tim3.sh2.MSLN.CAR and Tim3.sh3.MSLN.CAR) was assessed by flow cytometry. In addition, MFI/percentage of Tim-3 positive cells in Tim3.sh1.sh2.MSLNCAR T cells, Tim3.sh1.sh3.MSLNCAR T cells and Tim3.sh1.sh2.sh3.MSLN CAR T cells (i.e. T cells that co-transduced with three viruses carrying Tim3.sh1.MSLN.CAR, Tim3.sh2.MSLN.CAR and Tim3.sh3.MSLN.CAR) was also determined by flow cytometry.

# In vitro Cytotoxicity assay

At first, target cells (Hela cell line) were labeled with lipophilic dye PKH-26 (Sigma Chemical Co., St. Louis, MO, USA) according to manufacturer's instructions. $3 \times 10^4$ labeled-target cells were then co-cultured at 1:1, 1:5, 1:10, and 1:20 ratios with effector cells (CAR T, Un-T, and Mock T cells) in a 48-well culture plate, maintained for 18 hr at 37°C in 5% CO2. Next, PKH-26-labeled target cells were stained with 7-AAD (Miltenyi Biotec, Bergisch Gladbach, Germany) as a viability dye. Flow cytometry analysis was done with the use of forward and side scatter gating to select viable cells, whereas PKH-26 and 7-AAD labeling was used to distinguish effector cells from dead target cells. Double positive ($PKH26^+$ /$7AAD^+$) target cells

were considered as dead cells. To calculate the percentage of double-positive dead target cells, the percentage of target cells autolysis was subtracted from the percentage of target cells co-cultured with effector cells. FlowJo (v7.6.1) software was used to analyze the cytometric data.

# Measurement of MSLN-dependent CAR T cell proliferation and cytokine production

Target cells were treated with 25 µg/ml of mitomycin C (Sigma Chemical Co., St. Louis, MO, USA) for 30 min at 37 °C followed by extensive washing with PBS. To track cell proliferation, CAR T, Un-T, and Mock T cells were labeled with lipophilic dye PKH26 (Sigma Chemical Co., St. Louis, MO, USA). The PHK26-labeled cells ($2 \times 10^5$/well) were co-incubated at a 1:1 ratio with mitomycin C-treated cells or cultured without target cells as a control for spontaneous proliferation (in the absence of exogenous IL-2) in 48-well plates in a final volume of 800 µl/well for 72 hr. For cytokine (IFN-γ, TNF-α and IL-2) quantification, supernatant was harvested 24 hr. after plating and kept at -80 °C until subsequent measurement by the enzyme-linked immunosorbent assay (ELISA). After 72 hr., labeled cells were stained with anti-CD3-APC (BioLegend, San Diego, CA, USA) to differentiate T cells from target cells. PKH-26 dilution of CD3-gated lymphocytes was used as a measure of proliferation.

# Flow Cytometric Analyses

Data acquisition was performed on a flow cytometer (BD FACSCalibur, BD Biosciences, San Jose, California). Analysis of all samples was done using FlowJo software (v7.6.1). All experiments were done in triplicate and repeated at least three times.

# Statistical analysis

Analysis of variance (ANOVA) followed by Tukey’s post-hoc was used to reveal any significant differences among treatment groups. P values below 0.05 were considered significant. Statistical analyses were performed with Prism 8 software (GraphPad Software, Inc., San Diego, USA).

## Results

# Efficient generation of MSLN-CAR T cells and Tim3-targeted MSLN-CAR T cells using lentiviral gene transfer

To examine the effect of Tim3 targeting on anti-MSLN-CAR expression, primary T cells were exposed to third-generation lentiviral vectors at an MOI of ~ 7 to generate four types of anti-MSLN CAR T cells; a fully human second-generation anti-MSLN CAR T cell as well as three groups of CAR T cells encoding three different anti-Tim3 shRNAs [referred to as Tim3.sh1, sh2, and sh3.MSLN-CAR T cells (Fig. 1a)]. To

evaluate the effect of single and combinatorial targeting of Tim3 on CAR expression, primary T cells were transduced with one, two or three lentiviral vectors to generate different MSLN-CAR T cells (Fig. 1a). Un-transduced T cells (Un-T) and Mock T cells were used as control. Flow cytometric analysis revealed that approximately 50 percent of cells in each type of CAR T cells were CAR positive (Fig. 1b).

# Efficient Targeting Of Tim3 In Msln-car T Cells

To explore the efficiency of Tim3 targeting by shRNAs, cell surface expression of Tim3 was evaluated in 5 different donors. Flow cytometric analysis revealed that Tim3-targeted shRNAs alone (i.e. sh1, sh2 and sh3.MSLN-CAR T cells) or in various combinations (i.e. Tim3.sh1.sh2, sh1.sh3, sh2.sh3 and sh1.sh2.sh3.MSLN-CAR T cells) could significantly knockdown Tim3 expression compared with MSLN-CAR T cells (Fig. 2a). The lowest and highest percentages of Tim3 knockdown were observed in the sh1.MSLN-CAR T cells and sh2.sh3.MSLN-CAR T cells, respectively. The percentages of Tim3 positive T cells, activated Un-T cells, and six types of MSLN-CAR T cell from 5 donors are shown in Fig. 2a and 2b. In line with these findings, analysis of MFI also showed that cell surface expression of Tim3 expression was significantly diminished in all Tim3.sh.MSLN-CAR T cells (except Tim3.sh1 and sh1.sh3.MSLN-CAR T cells) compared with MSLN-CAR T cells (Fig. 2c). Moreover, the expression levels of Tim-3 in Un-T cells and Mock T cells before and after activation with anti-CD3/CD28 antibodies were measured by flow cytometry (Fig. 2a). The results showed that anti-CD3/CD28 activation significantly augmented Tim3 expression in all donors. Altogether, our data revealed that expression levels of Tim3 were significantly increased in Un-T and MSLN-CAR T cells after stimulation with anti-CD3/CD28 antibodies or target cells. MSLN-CAR T cells and Mock T cells up-regulated Tim3 expression, even before their exposure to target cells (Fig. 2a.). Gene expression levels of Tim3 were not significantly affected in Tim3.sh2 and sh3.MSLN-CAR T cells as well as Tim3.sh1.sh2, sh1.sh3, sh2.sh3 and sh1.sh2.sh3.MSLN-CAR T cell groups before and after exposure to target cells. Considering that the weakest and strongest Tim3 knockdown was respectively seen in Tim3.sh1 and Tim3.sh2.sh3.MSLN-CAR T cells (Fig. 2d), these CAR T cells were chosen for further experimentation.

# Tim3 knockdown augments antigen-dependent cytotoxic function of MSLN-CAR T cells

Prior to testing the functionality of Tim3-targeted MSLN-CAR T cells, MSLN expression levels were examined in three MSLN-positive cell lines; Hela cells, Ovcar3 and Skov3. Among these cell lines, HeLa cells showed the highest expression levels for MSLN (approx. 52%) (data not shown). Therefore, this cell line was selected as the target cell for all functional experiments described below.

To investigate the effects of Tim3 knockdown on the cytotoxic function of MSLN-CAR T cells, a PKH26/7AAD-based cytotoxic assay was used. All cytotoxic assays were performed at an effector to target ratios of 1:1, 5:1, 10:1, and 20:1 (Figs. 3 and 3b). Unlike Mock T cells and Un-T cells, MSLN-CAR T

cells and two Tim3-targeted MSLN-CAR T cells showed significant cytotoxic activity against target cells (Fig. 3c). Our data showed the highest level of cytotoxicity in sh2.sh3.MSLN-CAR T cells at target to effector ratio of 20:1. MSLN-CAR T cells and Tim3-targeted MSLN-CAR T cells were not significantly different in their cytotoxic function at effector to target ratios of 1:1, 5:1 and 10:1 (Fig. 3c). Finally, our results revealed that Tim3-knockdown had no negative impact on the cytotoxic function of Tim3-targeted MSLN-CAR T cells. We did not observe any significant cytotoxicity against MSLN-negative NALM-6 cells (data not shown).

# Tim3 Knockdown Enhances Antigen-dependent Proliferation Of Msln-car T Cells

To explore the effects of Tim3 knockdown on the proliferative response of MSLN-CAR T cells, MSLN-dependent proliferation of these cells was evaluated following co-culture with target cells. To do this experiment, PKH26-labelled T cell/MSLN-CAR T cells were co-incubated with mitomycin C-treated target cells for 72hr at a 1:1 ratio. Our data revealed that both MSLN-CAR T cells and Tim3-targeted MSLN-CAR T cells had higher proliferative capacity compared with Mock T cells and Un-T cells (Fig. 4a). Consistent with the results of cytotoxicity assays, Tim3.sh2.sh3.MSLN-CART cells showed significantly higher proliferative responses compared with Tim3.sh1.MSLN-CAR T cells and MSLN-CAR T cells (Fig. 4b). However, none of the T cell groups showed any significant proliferative response when they were cultured in the absence of target cells (Fig. 4b) or in the presence of MSLN-negative NALM-6 cells (data not shown).

# Tim3 Knockdown Improves Antigen-dependent-cytokine Production Of Msln-car T Cells

We next analyzed cytokine production by different groups of MSLN-CAR T cells. To do this, supernatants of Mock T cells, MSLN-CAR T cells and Tim3-targeted MSLN-CAR T cells in the presence and absence of target cells (1:1 ratio) were harvested. Concentrations of cytokines TNF-α, IL-2 and IFN-γ were measured by ELISA. Our results showed that culturing of T and/or CAR T cells in the absence of target cells led to the minimal cytokine production (Fig. 5a-c). However, co-culturing all types of MSLN-CAR T cells with target cells did significantly induce TNF-α, IL-2 and IFN-γ production (Fig. 5a-c). Nonetheless, minimal levels of TNF-α, IL-2 and IFN-γ were detected in cultures of Mock T cells in the presence or absence of target cells. TNF-α, IL-2 and IFN-γ levels were significantly higher in Tim3.sh2.sh3.MSLN-CAR T cells compared with Mock T cells, MSLN-CAR T cells and Tim3.sh1.MSLN-CAR T cells (Fig. 5a-c).

## Discussion

Negative immune regulator Tim3 is highly expressed on exhausted or impaired T cells in chronic viral infections and tumor-bearing hosts (14, 19–21). Consistent with its role as an inhibitory molecule, it has

been shown that the blockade of Tim3-Tim3L signaling with blocking antibodies or by genetic targeting not only improves the secretion of IFN-γ by activated T cells but can also restore the function of exhausted T cells. This underscores the importance of targeting Tim3 signaling for improving immunity against chronic viral infections and cancers (22–24). So far, several clinical trials using Tim3 blocking monoclonal antibodies have been started aiming to evaluate the therapeutic efficacy, safety, tolerability and dose-limiting toxicities of Tim3 blockers (ClinicalTrials.gov, NCT03652077and NCT03489343). In the current study, the effects of Tim3 gene knockdown on MSLN-specific CAR T cells were investigated. Compared with co-administration of CAR T cells with anti-Tim3 monoclonal antibodies, genetic targeting of Tim3 in CAR T cells may not only overcome the need for repeated administration of monoclonal antibodies but also minimizes the risk of systemic toxicity (25). Moreover, stable expression of a shRNA sequence leads to continuous repression of Tim3 expression after infiltration of CAR T cells into the tumor microenvironment, while the concentrations of a systemically-administered anti-Tim3 antibodies might not reach sufficient levels inside tumors. It has also been shown that the extracellular domain of Tim3 contains multiple ligand-binding sites. Anti-Tim3 monoclonal antibodies might not completely block all of these binding sites, making genetic targeting of Tim3 a superior approach for hindering its activity (26). In the current study, Tim3-targeting in MSLN-CAR T cells abrogated the inhibitory function of Tim3. ShRNA-mediated stable knockdown of Tim3 could significantly augment proliferation, cytokine production and cytotoxic functions of MSLN-CAR T cells.

Various studies have shown that Tim3 signaling confers its inhibitory effects on T cells through mechanisms including eliciting intracellular calcium flux, inducing apoptosis and reducing IFN-γ production (13, 15, 27). Although the downstream signaling events remain poorly characterized, different studies have shown that ligand-dependent Tim3 signaling leads to repression of TCR signaling and thereby suppression of T cell proliferation and survival (28–30).

It is not well known whether ex vivo manipulation of T cells for cancer immunotherapy has any negative effects on their function through up-regulation of immunoinhibitory receptors such as Tim3. Our study revealed that the activation of T cells with anti CD3/CD28 antibodies (as the first stimulation) can significantly increase the expression levels of Tim3. Primary activation of T cells with anti-CD3/CD28 antibodies is essential for efficient transduction of CAR transgenes(31). More importantly, Tim3 overexpression was also detected in T cells after transduction with CAR-carrying lentiviral vectors (i.e. MSLN-CAR T cells) and also after their co-culture with MSLN-positive target cells. Moreover, these findings are in agreement with previous reports indicating that Tim3 was up-regulated on T and CAR T cells after co-incubation with target cells or administration to tumor-bearing mice (32–35). Considering the inhibitory effects of Tim3, it is clear that overexpression of Tim3, even prior to their exposure to tumor cells, might have a negative impact on (CAR) T-intrinsic qualities such as longevity and functionality which play key roles in determining the efficacy of immunotherapy.

In the present study, we also showed significant improvement in MSLN-CAR T cells cytotoxic function following shRNA-mediated blockade of Tim3. This finding is consistent with Kenderian et al study which has demonstrated that combination therapy with suboptimal doses of anti-CD123 CAR T cells plus anti-

Tim3 antibody could significantly improve complete response (CR) rate (CR = 100%) in a relapsed acute myeloid leukemia (AML) xenograft model compared with suboptimal doses of anti-CD123 CAR T cells alone (CR = 45%) or suboptimal dose of anti-CD123 CAR T cells plus anti-PD-1 antibody (CR = 80%)(35). In line with the above study, Koyoma et al. have shown that Tim3 is up-regulated in patients who were treated with anti-PD-1. Their data also shows that anti-Tim3 therapy could confer survival advantage following treatment failure with anti-PD-1 (36). Altogether, these data indicate that therapeutic regimens with Tim3-knockdown MSLN-CAR T cells might either prevent tumor relapse or have superior antitumor activity in relapsed MSLN-positive tumors. Moreover, various studies have reported that concomitant blockade of Tim-3 and PD-1 could increase cytotoxic activity of T cells (37, 38). In this regard, it seems that dual or multiple targeting of inhibitory checkpoint receptors (either by gene-editing techniques or shRNAs) might further improve the therapeutic outcome T cell-based cancer immunotherapies.

To analyze the effects of Tim3 downregulation on cell proliferation, we investigated proliferation rates of Tim3-knocked down MSLN-CAR T cells. Our data revealed that genetic targeting of Tim3 could significantly improve the proliferative response of Tim3.sh2.sh3.MSLN-CAR T cells in an antigen-dependent manner. This finding is in accordance with previous reports where it has been shown that, unlike Tim3 negative T cells, Tim3 positive T cells had a lower cytokine production and proliferation rate which could be reversed by Tim3 targeting (14, 16, 39–41).

In this study, cytokine analysis revealed that stable knockdown of Tim3 could significantly improve production of cytokines TNF-α, IFN-γ and IL-2 in an antigen-dependent manner. The pattern of produced cytokines is an indicative of T cell polarization towards a favorable antitumor phenotype and highlights the key role of these cytokines in T cell cytotoxic function and proliferation (42–44).

Another facet of Tim3 signaling which is important in cancer immunotherapy is the role of Tim3 signaling in the differentiation and regulation of Tregs, the cells that are thought to be an obstacle in antitumor immunity. Tim3 is thought to exert these effects through influencing the expression of CD80/CD86 or TGF-β and expression of CTLA4 and TIGIT inhibitory molecules (17, 45). We did not analyze Treg markers in this study, but it is likely that improved function of Tim3-targeted MSLN-CAR T cells might have been related to repressed Treg-promoting signals.

Considering different shRNA sequences and their effectiveness, current study could not detect any cellular toxicity (in terms of potency and relative efficacy) in shRNA-targeted MSLN-CAR T cells. Hence, it can be speculated that our strategy for designing and screening of shRNA sequences, before and during knockdown experiments, could empirically identify a combinatorial approach (i.e. co-transduction of two shRNA2 and shRNA3) as the most effective strategy for targeting Tim3 in the MSLN-CAR T cells.

# Conclusions

The results of the current study illustrate that genetic targeting of Tim3 could improve the function of MSLN-CAR T cells. Overexpression of Tim3 in T cells during different phases of CAR T cell production (before and after CAR transduction) highlights the importance of revisiting the existing CAR T cell

production protocols, perhaps by development of novel protocols which do not require initial activation of cells. Dual or multiple targeting of inhibitory checkpoint receptors either pharmacologically or genetically might further improve the therapeutic outcome of CAR T cell-based cancer immunotherapies. Altogether, genetic targeting of Tim3 might be worth considering in clinical studies of CAR T cells.

# Abbreviations

ShRNA: Short hairpin RNA; CAR T cell: Chimeric antigen receptor T cell; Tim3: ; T cell immunoglobulin and mucin domain containing-3 receptor; ACT: Adoptive cell therapy; TAA: Tumor associated antigen; TME: Tumor microenvironment; SP: Signal peptide; ScFv: Single-chain variable fragment; TM: Transmembrane; VCM: Virus-containing media; PBMC: Peripheral blood mononuclear cell; MOI: Multiplicity of infection; MFI: Mean fluorescence intensity; ZAP70: Zeta associated protein70; LAT: Linker for activation of T cells; PLCγ1: Phospholipase Cγ1; AML: Acute lymphoblastic leukemia; CR: Complete response

# Declarations

**Acknowledgements:** We would like to thank Bita Ansaripour for her technical assistance.

**Authors' contributions:** Conception and design of studies: JH, FN. Acquisition, analysis and interpretation:, LJ, EM, HRM, KHA. Drafting article: LJ. Critical review and discussion: JH, FN, LJ, HRM.

**Funding:** This study was supported by grants from the National Institute for Medical Research Development (NIMAD) of Iran (grant no. 942554) and Tehran University of Medical Sciences (grant no. 36174).

### Availability of data and materials

Not applicable.

### Ethics approval and consent to participate

This study was approved by the ethical committees of the Tehran University of Medical Sciences. Buffy coats or fresh whole blood from healthy donors were purchased from the Iranian Blood Transfusion Organization (IBTO) and handled with necessary safety procedures and ethical requirements.

### Consent for publication

Not applicable.

### Competing interests

The authors declare that they have no competing interests.

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# Figures

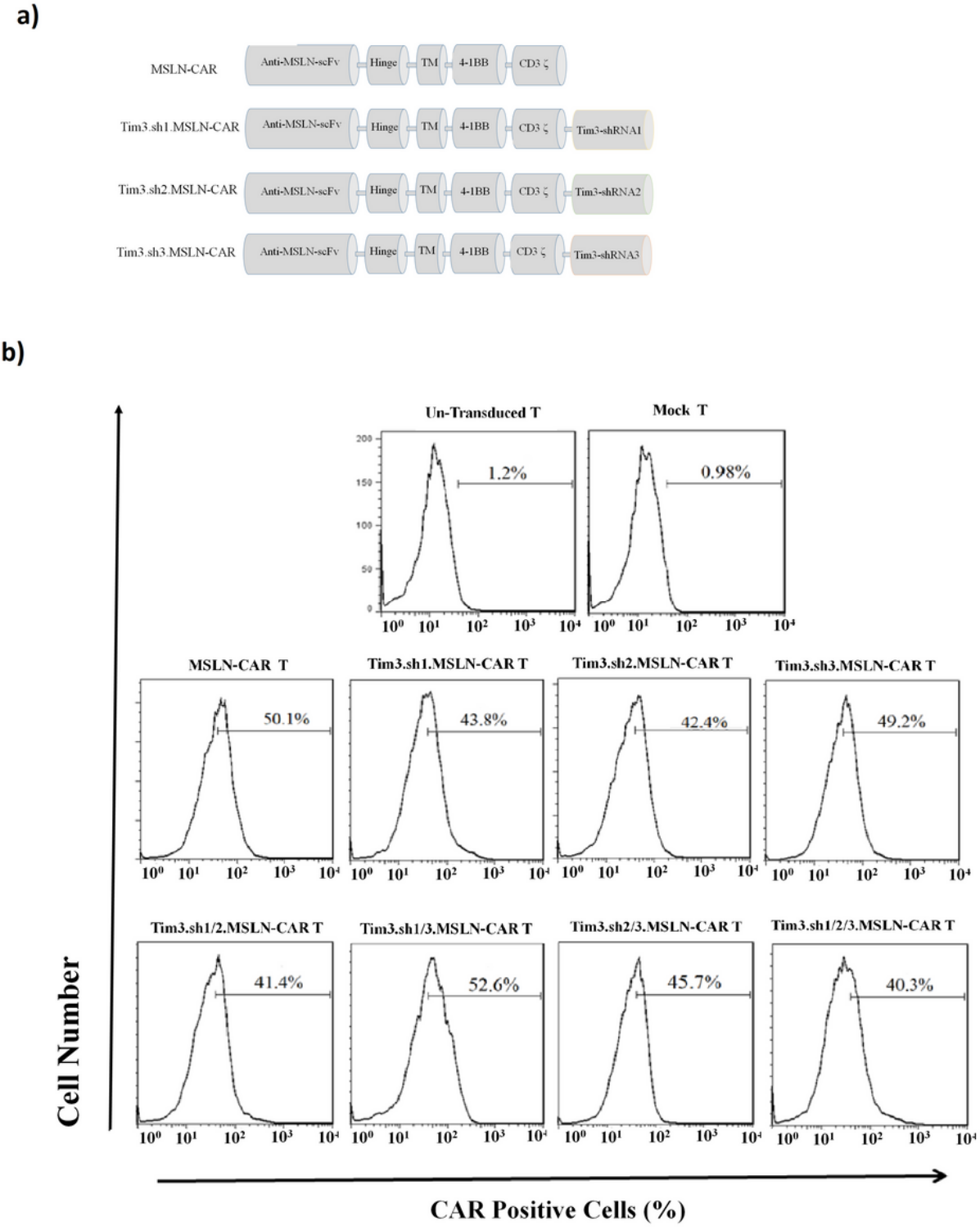
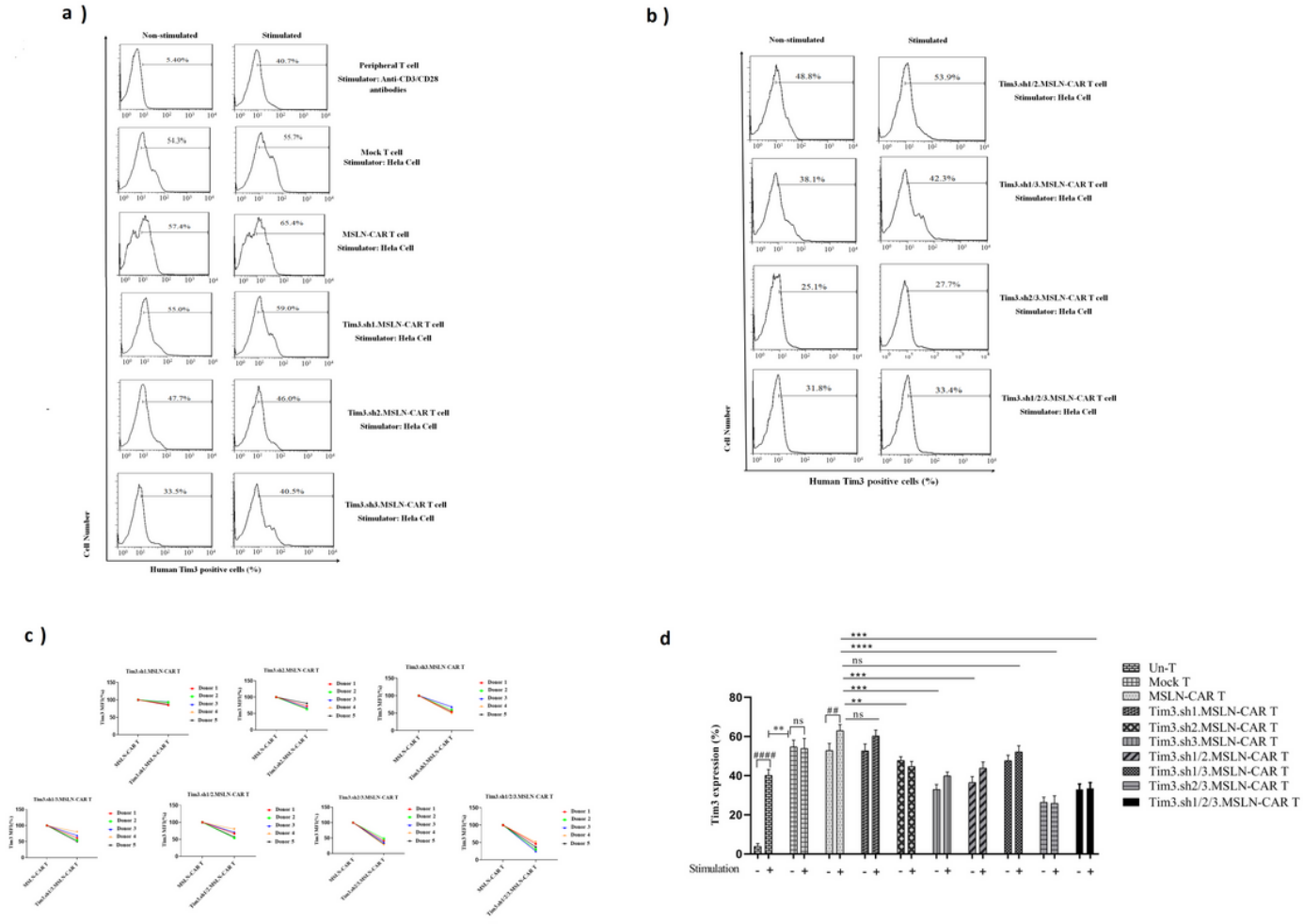



Figure 1

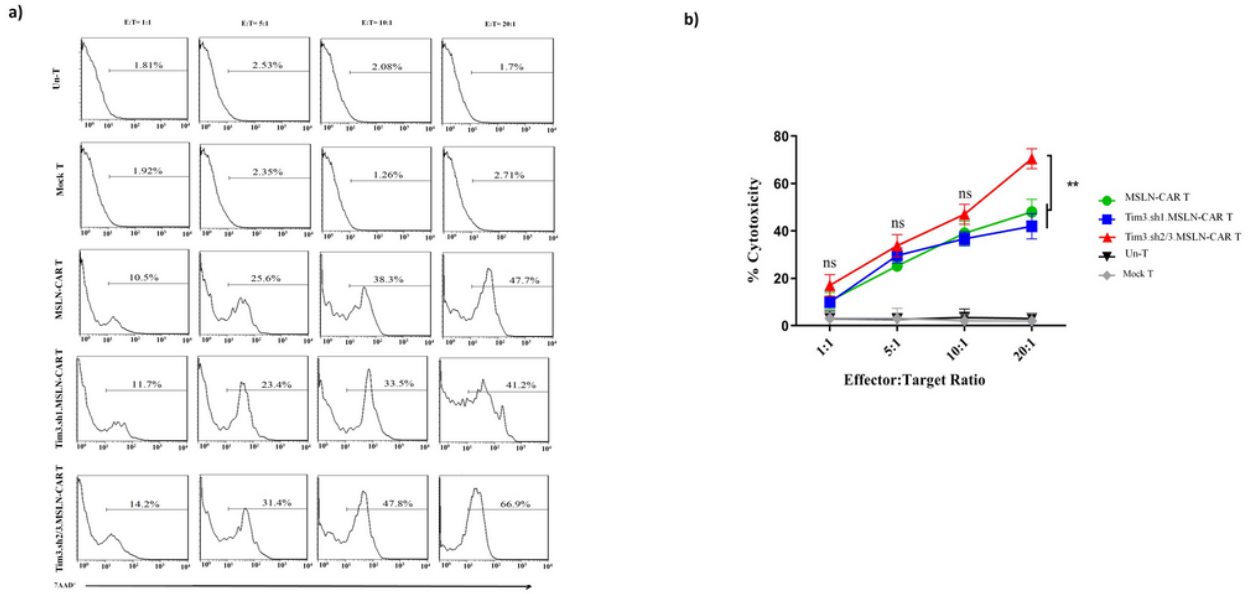
Lentiviral vector construction and generation of MSLN-CAR T cells and Tim3 targeted MSLN-CAR T cells. a) Schematic representation of second generation anti-MSLN CAR construct and three lentiviral MSLN-CAR constructs co-encoding three shRNAs targeting different segments of Tim3 transcript. MSLN-CAR is composed of anti-MSLN scFv, human CD8α hinge, human 4-1BB transmembrane (TM) region and intracellular domains containing the human 4-1BB and CD3ζ domains. Three Tim3-shRNA sequences were incorporated downstream of CD3ζ sequence. b) Histogram plots show the percentage of CAR positive cells (from one donor) 4 days after transduction with the designated MSLN-CAR-expressing viruses at an MOI of ~ 7. MSLN-CAR T cells: fully human anti-mesothelin CAR T cells; Tim3.sh.MSLN-CAR T cells: ShRNA-mediated knockdown of Tim3 in anti-mesothelin-CAR T cells; Mock T: empty vector transduced T cell; Un-T: untransduced T cell; MOI: multiplicity of infection




## Figure 2

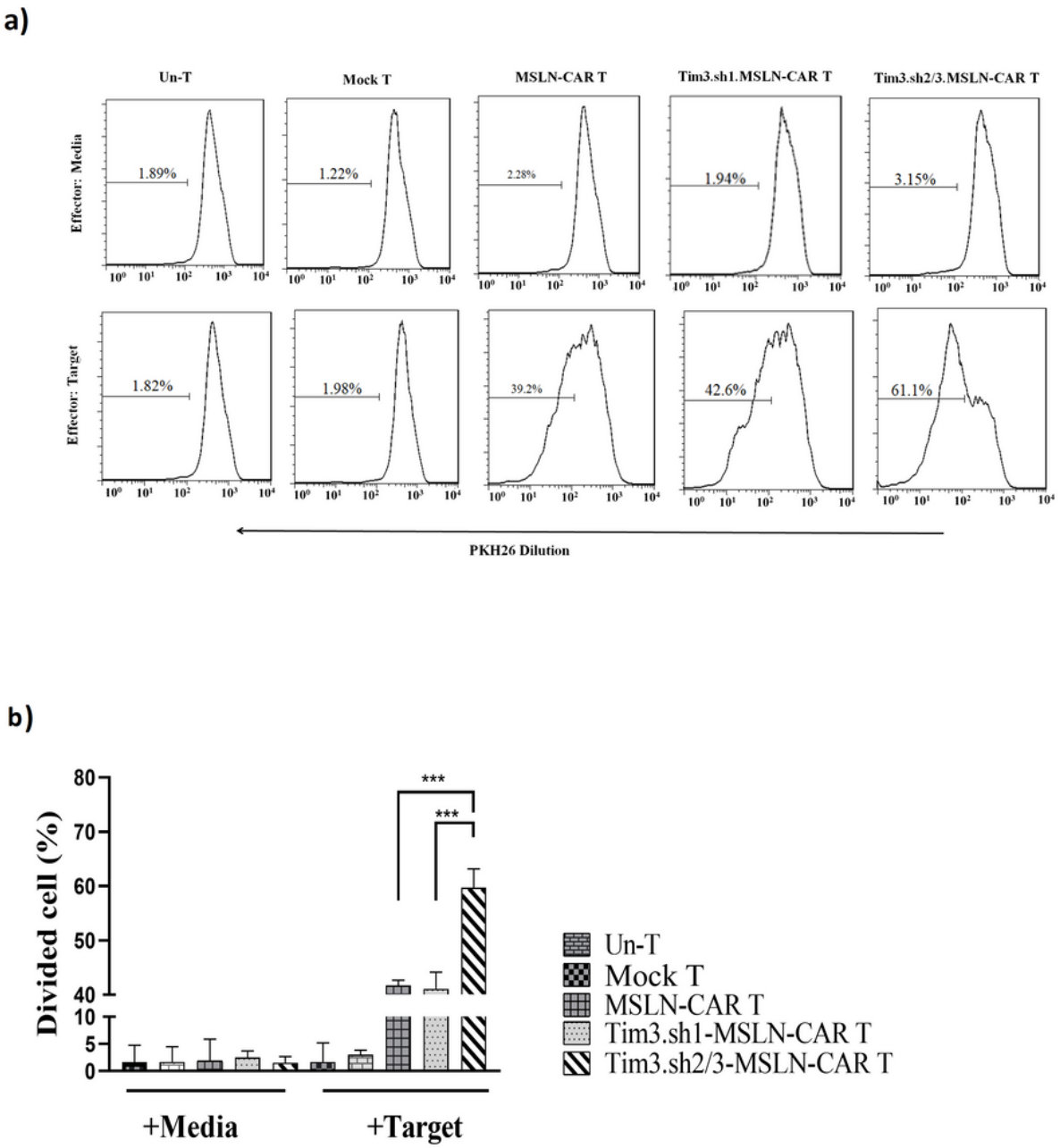
Efficient targeting of Tim3 by shRNAs expressed by MSLN-CAR T cells. a,b) Human fresh T cells, Mock T cells and 8 different groups of CAR T cells from 5 healthy donors were analyzed to determine the percentage of Tim3-expressing cells. All T cells and MSLN-CAR T cells were assessed before and after stimulation with designated stimulators. Flow cytometry histograms show the percentage of Tim3-expressing T cells, Mock T cells and MSLN-CAR T cells from one donor. c) Mean fluorescence intensity (MFI) of Tim3 expression in Tim3.sh2 and sh3.MSLN-CAR T cells as well as in sh1.sh2, sh1.sh3, sh2.sh3

and sh1.sh2.sh3.MSLN-CAR T cells from 5 donors (MFI for MSLN-CAR T cells was set as 100%). d) Bar graph shows the average number of Tim3 positive T cells, Mock T cells and MSLN-CAR T cells from all 5 donors in the presence or absence of stimulator. # Intragroup comparison of Tim3 positive cells before and after stimulation. * Intergroup comparison of the percentage of Tim3–expressing cells in MSLN-CAR T cell groups with Tim3.sh2 and sh3.MSLN-CAR T cells as well as with sh1.sh2, sh1.sh3, sh2.sh3 and sh1.sh2.sh3.MSLN-CAR T cells. The data are shown as mean ± SD. Mean comparisons were performed using one-way ANOVA followed by Tukey's post hoc test. $P < 0.05$ was considered statistically significant. The results are representative of five independent experiments, each performed in duplicate. MSLN-CAR T cells: fully human anti-mesothelin CAR T cells, Tim3.sh.MSLN-CARs: ShRNA-mediated knockdown of Tim3 in anti-mesothelin-CAR T cells; Mock T: empty vector transduced T cell; Un-T: untransduced T cell; MFI: mean fluorescence intensity. (*$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$, ****$P < 0.0001$)




## Figure 3

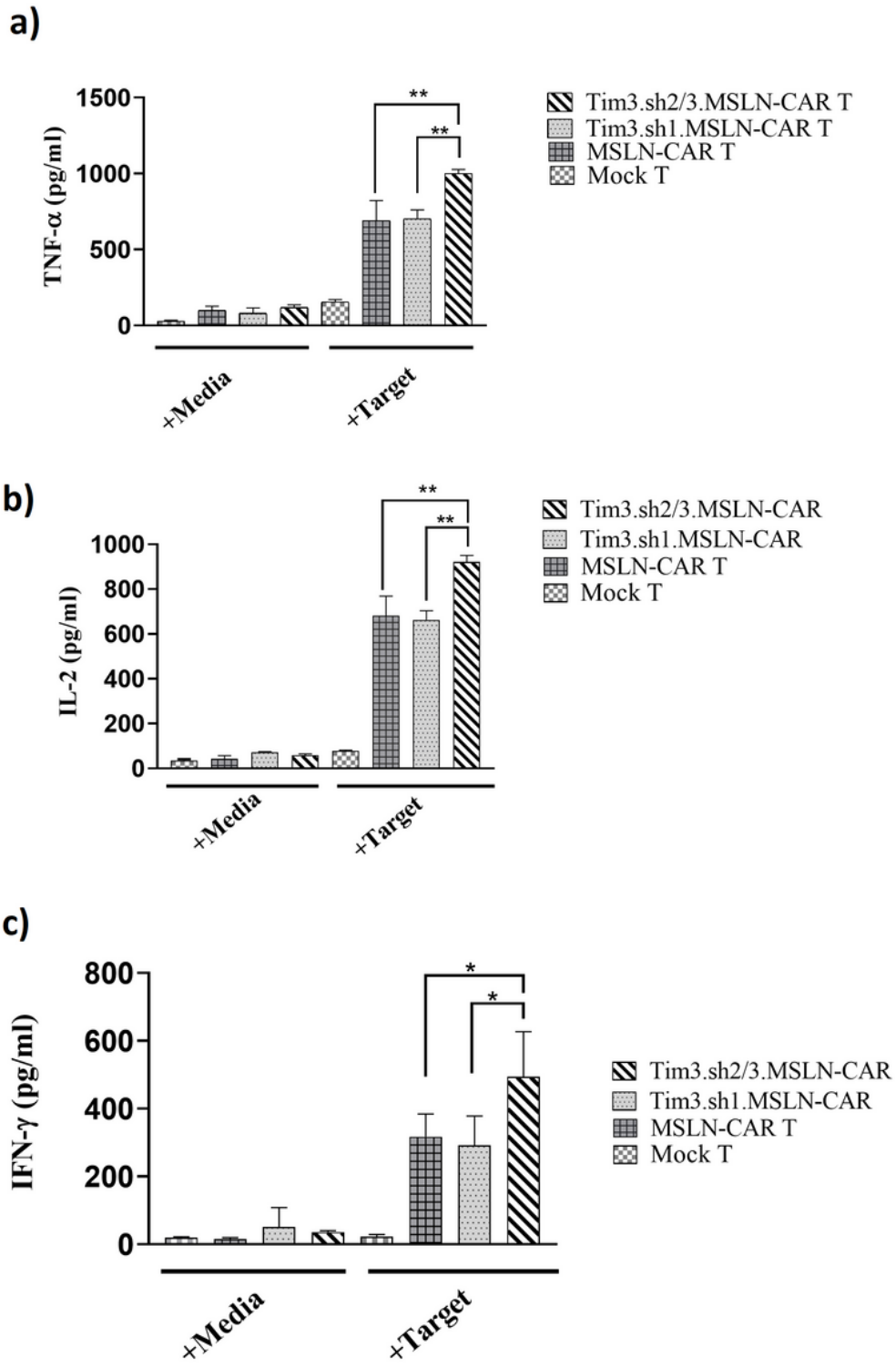
Tim3 knockdown augments antigen-dependent cytotoxic function of MSLN-CAR T cells. a) Representative flow cytometry histograms show the cytotoxic function of MSLN-CAR T cells, Tim3.sh1 and sh2/sh3.MSLN-CAR T cells as well as Un-T cells and Mock T cells (all from one donor). 5 × 104 effector cells were co-incubated with PKH26-labeled HeLa target cells at 4 ratios (effector: target) including 1:1, 5:1, 10:1 and 20:1 for 18 hr and then stained with 7AAD dye to detect dead cells. b) Line plots display the average percent of dead target (double positive; PKH26+ /7AAD+) cells. Data are shown as mean ± SD. Mean comparisons were performed using one-way ANOVA followed by Tukey's post hoc test. $P < 0.05$ was considered statistically significant. The results are representative of three independent experiments, each performed in duplicate. MSLN-CAR: fully human anti-MSLN-CAR; Tim3.sh.MSLN.CARs: Tim3.knocked down anti-MSLN-CAR; Mock T: empty vector transduced T cell; Un-T: untransduced T cell; SD: standard deviation. (**$P < 0.01$).




## Figure 4

Tim3 knockdown enhances antigen-dependent proliferation of MSLN-CAR T cells. a) Representative flow cytometry histograms show the proliferation potency of MSLN-CAR T cells, Tim-3.sh1 and sh2/sh3.MSLN-CAR T cells as well as Un-T cells and Mock T cells (all from one donor) as effector cells. 2 × 105 effector cells were co-incubated with mitomycin C-treated Hela cells as the mesothelin-expressing target cell at a 1:1 ratio for 72 hr in the absence of exogenous IL-2. Anti-CD3 staining was used to

distinguish T cells from target cells. PKH dilution was used as a measure of cell proliferation. a) Histograms display the percentage of proliferated effector cells from one donor. b) Bar graphs show the average percent of divided effector cells in different conditions. The data are shown as mean ± SD. Mean comparisons were performed using one-way ANOVA followed by Tukey's post hoc test. $P < 0.05$ was considered statistically significant. The results are representative of three independent experiments, each performed in duplicate. MSLN-CAR: fully human anti-MSLN CAR; Tim3.sh.MSLN-CARs: Tim3-knocked down anti-MSLN-CAR; Mock T: empty vector transduced T cell; Un-T: untransduced T cell; SD: standard deviation. (***$P < 0.001$)




## Figure 5

Tim3 knockdown improves antigen-dependent-cytokine production of MSLN-CAR T cells. Mock, MSLN-CAR, Tim-3.sh1 and sh2/sh3.MSLN-CAR T as well as Un-T cells were co-incubated with Hela cells in a 1:1 ratio or incubated in media. The supernatants were harvested after 24 hr. and TNF-α (a), IL-2 (b) and IFN-γ (c) concentrations were measured by ELISA. The data are shown as mean ± SD. Mean comparisons were performed using one-way ANOVA followed by Tukey's post hoc test. $P < 0.05$ was considered statistically

significant. The results are representative of three independent experiments, each performed in duplicate. IL: interleukin; MSLN-CAR: fully human anti-MSLN-CAR; Tim3.sh.MSLN-CARs: Tim3-knocked down anti-MSLN-CAR; Mock T: empty vector transduced T. SD: standard deviation. (*P < 0.05, **P < 0.01).