

## ORIGINAL ARTICLE

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# Construction and Functional Characterization of a Fully Human Anti-mesothelin Chimeric Antigen Receptor (CAR) Expressing T Cell

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## ABSTRACT

Chimeric antigen receptor (CAR) T cell therapy is considered as an encouraging approach for the treatment of hematological malignancies. However, its efficacy in solid tumors has not been satisfying, mainly in the immunosuppressive network of the tumor microenvironment and paucity of appropriate target antigens. Mesothelin (MSLN) is a tumor-associated antigen (TAA) expressed in numerous types of solid tumors such as gastrointestinal, ovarian, and pancreatic tumors. Owing to high expression in tumor cells and low expression in normal tissues, MSLN-targeted therapies like monoclonal antibodies have been previously developed.

In the present study, a CAR T cell harboring the second-generation of a fully human anti-MSLN-CAR construct containing CD3 $\zeta$  and 4-1BB signaling domains was produced and it was functionally evaluated against an MSLN-expressing cell line.

The findings showed potent, specific proliferation, cytotoxic activity, and interleukin (IL)-2, Tumor necrosis factor-(TNF)  $\alpha$ , and Interferon-(IFN)  $\gamma$  production in an antigen-dependent manner. Cytotoxic activity was shown in effector-to-target ratio from 1:1 to 20:1, but the most adequate efficacy was observed in the ratio of 10:1. Non-specific activity against MSLN negative cell line was not observed.

Our data demonstrated that primary human T cells expressing fully human MSLN-CAR construct are effective against MSLN-expressing cell lines in vitro, suggesting this MSLN-CAR construct as a potential therapeutic tool in a clinical setting.

**Keywords:** Adoptive immunotherapy; Chimeric antigen receptor; Mesothelin

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## INTRODUCTION

Effective adoptive T cell therapy (ATC) for solid tumors because of complicated microenvironment has

been encountered with some challenges including identifying proper target antigens, large-scale production of powerful T cells, and overcoming hostile tumor microenvironment.<sup>1</sup> Although several methods have been developed to make ATC more effective against solid tumors,<sup>2</sup> establishing an acceptable immunotherapy approach for the treatment of solid tumors is still demanded. Genetic manipulation of T cells to express chimeric antigen receptors (CAR) is a promising approach for overcoming such barriers in the immunotherapy of solid tumors.<sup>3</sup> In this strategy, the T cell will be genetically engineered to take advantage of the specificity of the monoclonal antibodies for antigen recognition and the ability of T cells to suppress tumor cells. CAR construct is composed of an extracellular single-chain fragment variable (scFv) for the recognition of target antigen followed by intracellular domains, including activation domain (CD3 $\zeta$ ) and co-stimulatory domains (CD28 or 4-1BB).<sup>4</sup> The first food and drug administration (FDA)-approved CAR T cell was introduced against CD19-a pan-B cell molecule in August 2017. Limited expression of CD19 on B cells makes it a good candidate for CAR T cell therapy in B cell malignancies. However, the selection of proper target antigens is one of the major challenges in the immunotherapy of solid tumors. MSLN, a glycosylphosphatidylinositol (GPI)-linked glycoprotein is an attractive target for the immunotherapy of several solid tumors mainly due to; the high expression in many types of cancers (ovarian, stomach, pancreas, lung and mesothelioma), low expression in normal tissues/cells, and effective roles in metastasis, proliferation, and chemoresistance of tumor cells.<sup>4</sup> Several mesothelin-based immunotherapy modalities such as MSLN-targeting Immunotoxins,<sup>5</sup> chimeric antibodies, anti-MSLN antibody-drug conjugates<sup>6</sup>, and anti-MSLN vaccine, have been previously developed.<sup>7</sup> Nevertheless, concerning to the short half-life of drugs and monoclonal antibodies as well as the issues related to their solubility and delivery,<sup>8-10</sup> many studies have focused on MSLN targeting CAR T cells. Further studies are required to characterize and address these challenges.<sup>11-13</sup>

It is worth mentioning that the origin of the scFv fragment in CAR construct plays a substantial role in determining the in vivo persistence of CAR T cells. Murine originated complementarity-determining regions (CDRs) in humanized monoclonal antibodies (mAbs) could stimulate immune reactions, resulting in

attenuated in vivo activity of CAR T cells.<sup>14,15</sup>

In the present study, we constructed a second-generation fully human anti-MSLN-CAR construct, which its function was subsequently scrutinized. Our data indicated that anti-MSLN-CAR T cells displayed specific proliferation, cytokine secretion, and killing activity against MSLN-expressing cell lines, highlighting their potential for the treatment of different types of MSLN-expressing solid tumors.

## MATERIALS AND METHODS

### CAR Construction

The fully human MSLN-CAR gene is composed of a Kozak consensus ribosome-binding sequence, a human CD8 signal peptide (SP), a fully human scFv, a CD8 $\alpha$  hinge domain, the transmembrane (TM) region of the human 4-1BB molecule, and an intracellular signaling domain containing both 4-1BB and CD3 $\zeta$  domains. The MSLN-CAR transgene was cloned into the pCDH-CMV-MCS-EF1 $\alpha$ -GreenPuro lentivector backbone to generate the second-generation CAR construct.

### Viral Vector Production

The HEK293T cells, as packaging cell, were seeded for 24 hours in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% FBS (Gibco Laboratories, Grand Island, NY), and 1% L-glutamine, then being transfected by the plasmid of lentivector containing CAR sequence and the packaging plasmids including pMDLg/pRRE, pMD.G, and pRSV/Revvia the standard calcium phosphate DNA precipitation technique<sup>13</sup> in 15 cm tissue culture plate pre-treated with Poly-L-Lysin solution (Sigma, St. Louis, MOCon 0.01%). After 6 hours, the media was refreshed and afterward, virus-containing media (VCM) was harvested three times after transfection. After being filtered through a 0.45  $\mu$ m membrane filter to eliminate cell debris, harvested VCM was concentrated using ultracentrifugation (4°C, 2 h, 26, 000 rpm), (Beckman Coulter Optima LE-80K Ultracentrifuge, Indianapolis, IN). The lentiviral particles were suspended in complete DMEM containing 10% FBS and then, they were stored in single-use aliquots at -80°C. Concentrated VCM titers were determined by a limiting dilution method on peripheral human T cells activated by anti-CD3/CD28-coated magnetic beads using flow cytometric analysis for MSLN-CAR.<sup>16</sup>

### Human Primary T Cells Transduction and CAR T Cells Generation

Whole blood from healthy donors was provided by the Iranian Blood Transfusion Organization (IBTO) and handled with all safety and ethical considerations. The protocol of this research was approved by the Ethics Committee of Tehran University of Medical Sciences (Tehran, Iran) (approval number: IR.TUMS.MEDICINE.REC.1397.775). Peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll-Paque gradient centrifugation method. T cells were negatively isolated by applying immunomagnetic beads (pan T cell isolation kit II, human, MiltenyiBiotec Inc., Germany) and their purity was evaluated by APC conjugated anti-human CD3 (BioLegend, San Diego, CA) using a flow cytometric measurement. To activate isolated T cells, cells were treated by anti-CD3 and anti-CD28 antibodies-coated magnetic beads (Life Technologies, Grand Island, NY). The resultant T cells were pretreated with polybrene (8 µg/mL, Santa Cruz Biotechnology, Santa Cruz, CA), transduced using concentrated lentiviruses (at a multiplicity of infection [MOI] of ~7), cultured in RPMI 1640 (Gibco Laboratories, Grand Island, NY) supplemented with 100 IU/ml of human interleukin (IL)-2 (MiltenyiBiotec, Germany), 10-mmol/L HEPES, 10% heat-inactivated FBS, 1% L-glutamine and 1% penicillin/streptomycin (PANBiotech, Aidenbach, Germany), and immediately spinoculated (1 h, 32°C, 2,100 rpm) in 12-well costar tissue culture plates (Corning Costar; Cambridge, MA). After 4 days, magnetic beads were removed and expression of MSLN-CAR was investigated by two-step staining with goat anti-human IgG F(ab')<sub>2</sub> Biotin (BioRad, Hercules, CA, USA) and Streptavidin APC (BioRad, Hercules, CA).<sup>16</sup>

### Cell Lines

Hela, SKOV3, and OVCAR3 cell lines as MSLN-expressing cell lines, the NALM-6 as MSLN-negative cell line, and HEK293T cell line were purchased from the Iranian Biological Resource Center, Iran. Before conducting experiments, MSLN expression levels were measured for NALM-6, Hela, SKOV3, and OVCAR3 cells by flow cytometry using APC-conjugated anti-human MSLN antibody (BioRad, Hercules, CA). Hela and NALM-6 cells were cultured in RPMI 1640 containing 25 mmol/L HEPES, 10% FBS, 1% penicillin/streptomycin, and 1% L-glutamine, then

being incubated at 37°C in 5% CO<sub>2</sub>. HEK293T, OVCAR3, and SKOV3 cells were cultured in DMEM medium supplemented with, 10% FBS, 1% penicillin/streptomycin, and 1% L-glutamine, consequently being incubated at 37°C in 5% CO<sub>2</sub>.

### In vitro Cytotoxicity Assay

In vitro cytotoxicity assay was done by the method described by Mirzaei et al with minor modifications.<sup>16</sup> Briefly, Hela, OVCAR3, and NALM-6 cells were labeled with PKH26 red fluorescent cell linker (Sigma Chemical Co., St. Louis, MO, USA) for 5 min at 37°C, to distinguish between the target cells and effector cells. Labeled Hela, OVCAR3 and NALM-6 cells were co-incubated at 1:1, 1:5, 1:10, and 1:20 ratios with MSLN-CAR T, Un-T (un-transduced T cell) and Mock T cell (empty vector transduced T cell) for 18 hours in 48-well U-bottomed plates in complete RPMI 1640 in a final volume of 600 µL/well. 7AAD (Miltenyi Biotech, Germany) was used as a viability dye for flow cytometry. FlowJo (v7.6.1) software was used for analyzing of data. Flow cytometry analysis was performed with forwarding (FSC) and side scatter (SSC) gating to determine viable cells, PKH26 and 7AAD double-stained cells were considered as dead target cells. To calculate cytotoxicity, the percentage of spontaneous lysis of target cells (PKH26 positive/7AAD positive) was subtracted from the percentage of lysed target cells treated with an effector (CAR) T cells. The FSC threshold was kept low to avoid missing dead cells from the analysis.<sup>17</sup>

### Proliferation Assay and Cytokine Measurement of MSLN CAR T Cells Post/Upon Antigen Stimulation

In vitro, antigen-dependent proliferation assay and cytokine measurement were carried out by the method previously described with slight modifications.<sup>16</sup> In Brief, Hela, OVCAR3 and NALM-6 cells were treated with 25 µg/mL of mitomycin C (Sigma Chemical Co., St. Louis, MO, USA) for 1 hour at 37°C, then being washed with PBS.<sup>18</sup> CAR T, Un-T, and Mock T cells were labeled by PKH26 dye (Sigma Chemical Co., St. Louis, MO, USA) according to the manufacturer's instructions. The PKH26-labeled T cells (2×10<sup>5</sup>/well) were co-cultured at 1:1 ratio with target cells or alone media (as control of auto-proliferation) in the absence of exogenous IL-2 in 48-well plates at a final volume of 800 µL/well. After 72 hours, to track (CAR) T cell proliferation, cells were stained with anti-CD3-APC

(MiltenyiBiotec, BergischGladbach, Germany) followed by gating CD3<sup>+</sup> lymphocytes, PKH26 dilution in CD3<sup>+</sup> population was considered as the amount of proliferation, analyzed by flow cytometry.<sup>19</sup> To measure the concentration of secreted IFN- $\gamma$ , TNF- $\alpha$ , and IL-2 by effector cells, supernatants were harvested 24 hours after co-culture and stored at -20°C until measurement by the enzyme-linked immunosorbent assay (ELISA).

## Flow Cytometric Analysis

Acquisition and analysis of all samples were performed with a BD FACS (BD Biosciences, San Jose, California) with FlowJov7.6.1 software. All assays were done with technical replicates and repeated six times.

## Statistical Analysis

Statistical analysis was performed using IBM SPSS Statistics 20.0. Analysis of variance (ANOVA) was followed by Tukey's post hoc comparison tests. The independent T-test was used to demonstrate the difference among treatment groups. *p* values less than 0.05 were considered statistically significant.

## RESULTS

### MSLN-CAR Generation Using Lentiviral Gene Transfer

Transfection efficacy was confirmed by immunofluorescence microscopic observation of transfected HEK293T cells with lentivector carrying CAR sequence and the packaging plasmids including pMDLg/pRRE, pMD.G, and pRSV/Rev three days

post-transfection (Figure 1A). MSLN-CAR construct was cloned into the pCDH-CMV-MCS-EF1-Green Puro lentivector backbone. MSLN-CAR construct encodes a fusion protein containing CD8 signal peptide, fully human anti-MSLN scFv, human CD8 $\alpha$  as hinge region, the transmembrane (TM) region of human 4-1BB, the intracellular signaling domains consisting 4-1BB, and CD3 $\zeta$  chains (Figure 1B). Activated human CD3<sup>+</sup> T cells were infected by lentivirus encoding MSLN-CAR and Mock cell at MOI of ~7. The CAR expression was detected by two-step staining with an APC-conjugated goat anti-human IgG F(ab')<sub>2</sub> antibody. The results demonstrated 58.7% CAR expression on MSLN-CAR T cells versus 0.1 and 0.09 %on Un-T and Mock cells respectively (Figure 1C).

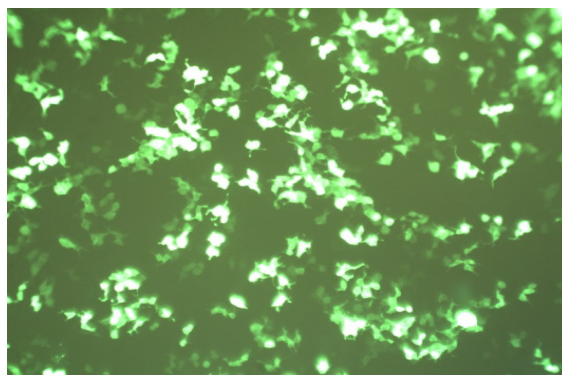
### MSLN Expression on MSLN Expressing Cell Lines

MSLN expression in Hela cell, OVCAR3, and SKOV3 cells were 52.4%, 20.2%, and 7.12%, respectively. Also mesothelin expression was 2.47% in NALM-6 as irrelevant cell line (Figure 2). Hela cell line was selected as a target cell line for functional assays. Likewise, all functional except cytokine measurements were performed on OVCAR3 and NALM-6.

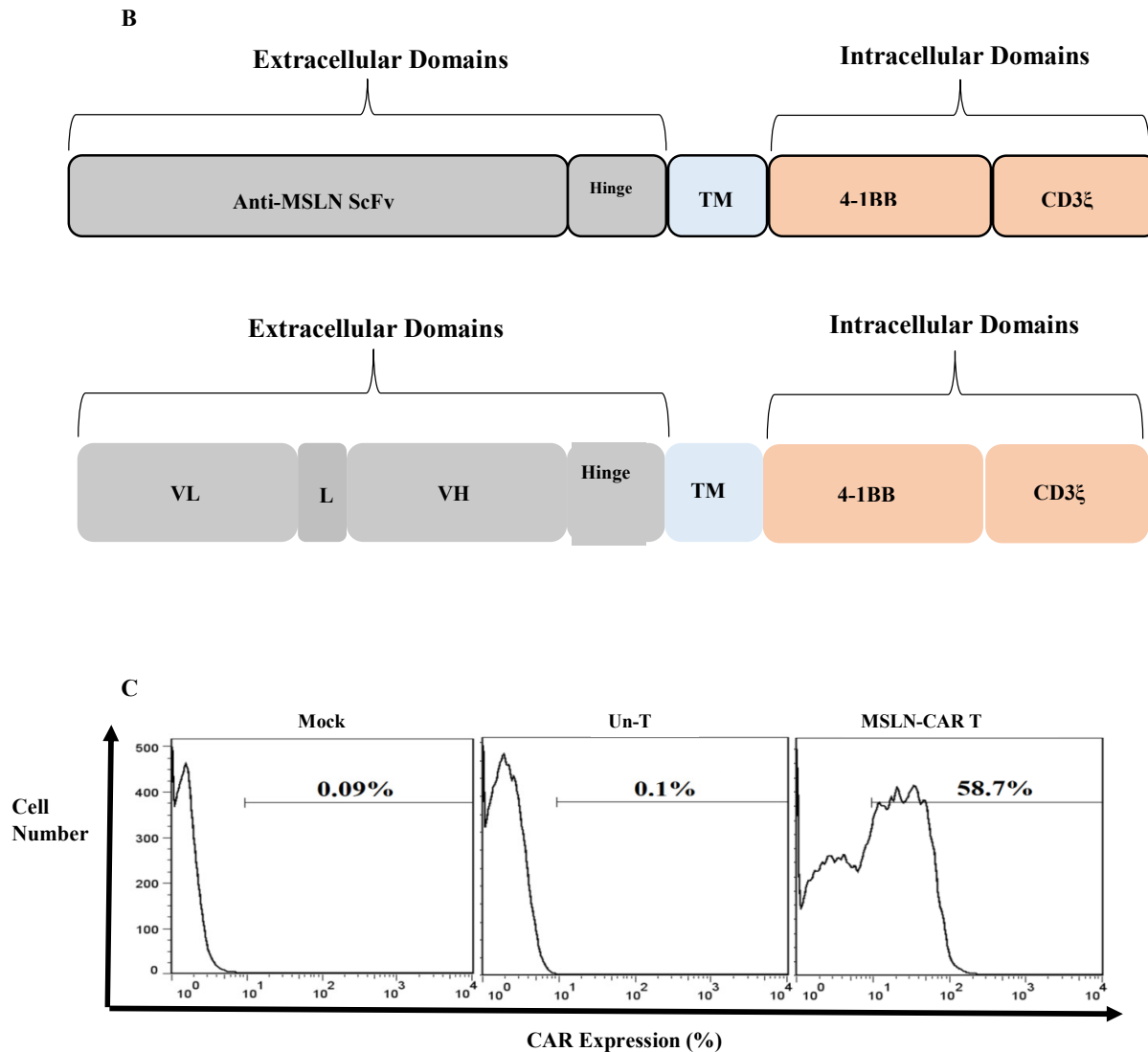
### In vitro Specific Cytotoxicity of MSLN-CAR T Cells

MSLN-CAR T cells were co-cultured with PKH26 pre-labeled Hela, OVCAR3, and NALM-6 cell lines at a different effector to target (E: T) ratios for 18 hours. The lysis percentage was determined by calculating the percentage of double target cells (PKH<sup>+</sup>/7AAD<sup>+</sup>) by flow cytometry. The results disclosed a significant MSLN-CAR T cell cytotoxicity in all ratios compared

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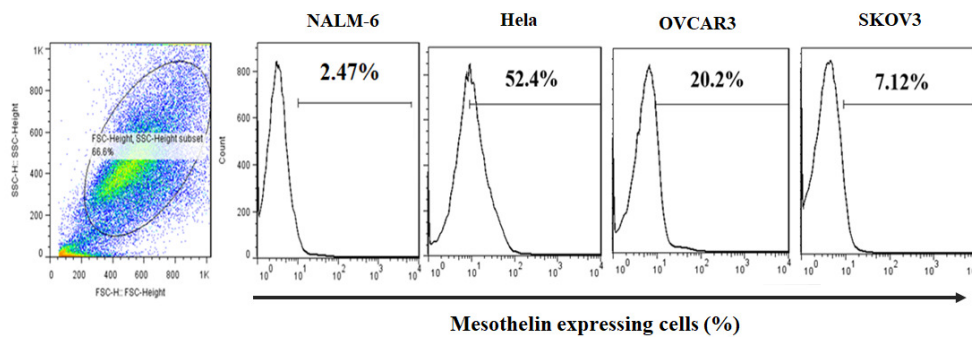


GFP<sup>+</sup> transfected HEK293T cells

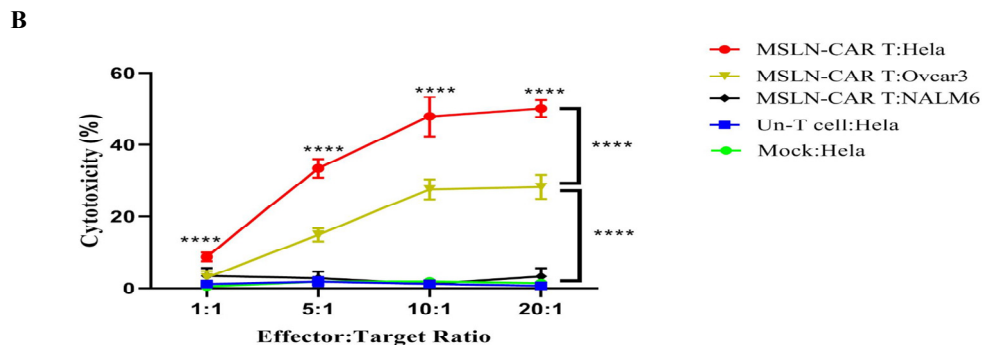
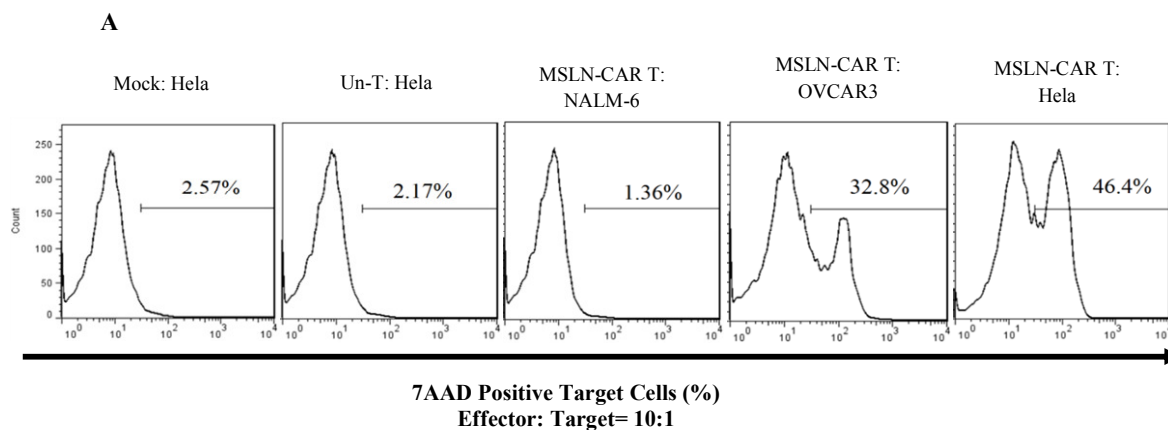


**Figure 1. Design, transduction, and generation of MSLN-CAR T cell.** A) The efficient transfection of HEK293T cells through lentiviral vectors was confirmed using the green fluorescent protein (GFP)-detection by immunofluorescence microscopy. B) Schematic representation of second-generation anti-MSLN CAR construct. CAR construct contains, the single-chain fragment variable (scFv) against MSLN, CD8a hinge, and 4-1BB as transmembrane (TM) region followed by intracellular domains (4-1BB and CD3ξ). C) Flowcytometry histograms represented the percentage of MSLN-CAR positive cells. Transduction was done three days after T cell activation by MSLN-CAR encoding lentiviral vector at an MOI of ~7. CAR expression was measured four days after transduction by two-step staining with a goat anti-human IgG F(ab')<sub>2</sub> Biotin as primary antibody and Streptavidin in APC as the secondary antibody. CAR expression was analyzed in three groups of cells including MSLN-CAR T cells which were transduced by CAR-containing lentivector and un-transduced T cell (Un-T) and empty vector transduced T cell (Mock) as negative controls. CAR expression was assayed using a goat anti-human IgG F(ab')<sub>2</sub> Biotin as primary antibody and Streptavidin APC as the secondary antibody. APC: Allophycocyanin; MOI: multiplicity of infection.

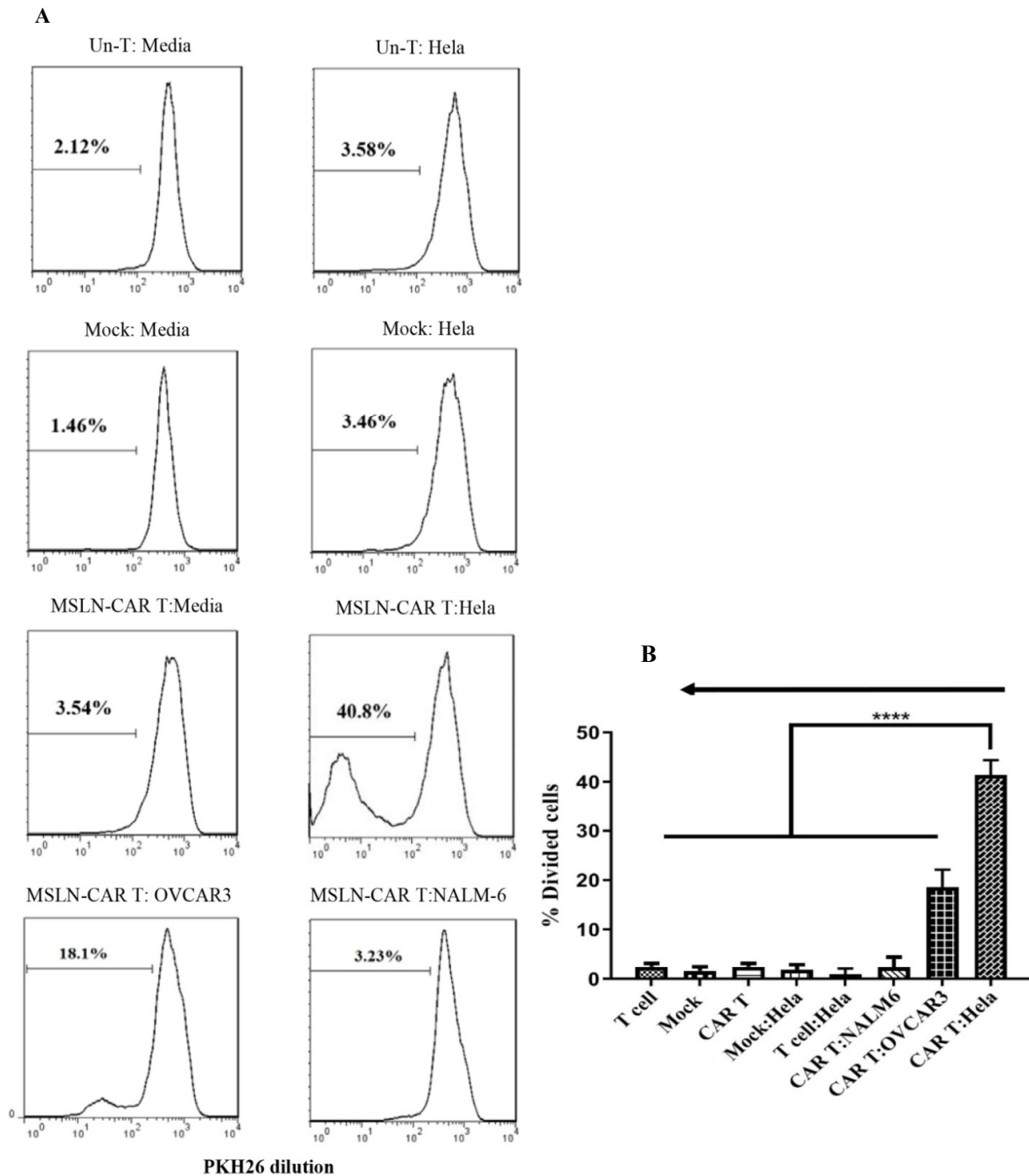
## Construction and Functional Characterization of an Anti-mesothelin CAR T Cell



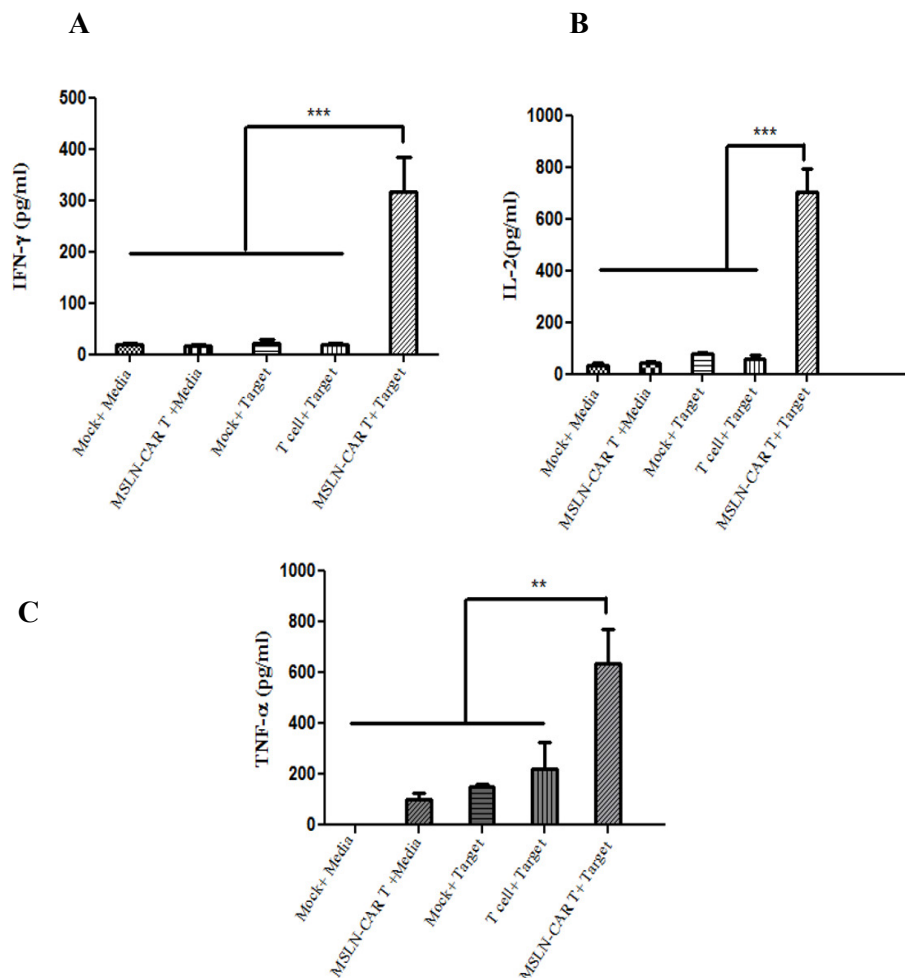
**Figure 2.** MSLN expression in different tumor cell lines. For the detection of the cell surface mesothelin expressions,  $10^5$  HeLa, OVCAR3, Skov3, and NALM-6 cells were stained using APC-conjugated anti-human mesothelin antibody. The flowcytometry histograms showed the percentage of mesothelin positive cells.



**Figure 3.** Strong cytotoxic activity of MSLN-CAR T cells. MSLN-CAR T, Un-T, and Mock cells were co-cultured at 1:1, 5:1, 10:1, and 20:1 ratios with PKH26 labeled HeLa, OVCAR3, and NALM-6 cells for 18 hours and then stained with 7AAD dye. the cytotoxic function of CAR T cells was evaluated by comparison of the percentage of lysed target cells (PKH26<sup>+</sup>/7AAD<sup>+</sup> cells) with the negative control (cultured PKH26 labeled target cells without effector T cells). A) Representative flowcytometry histograms show cytotoxicity of MSLN-CAR T cells against NALM-6, HeLa, and OVCAR3 cells and Mock and Un-T cells against HeLa cells at an effector target ratio 10:1. B) Line plots-display cytotoxicity of MSLN-CAR T cells against HeLa, OVCAR3, and NALM-6, also Un-T and Mock cells against HeLa cells. Statistical analysis was performed by one-way ANOVA following Tukey's post hoc. Data are representative of six independent experiments and presented as mean $\pm$ SD.  $p < 0.05$  was considered statistically significant. 7AAD: 7-Aminoactinomycin D. Un-T: un-transduced T cell.



**Figure 4. Antigen dependent MSLN-CAR T cell proliferation.** Eight days after expansion,  $2 \times 10^5$  MSLN-CAR T cells were co-incubated with mitomycin C-treated Hela, OVCAR3, and NALM-6 cells for 72 hours in the absence of exogenous IL-2. Cell proliferation was measured by flow cytometry. A) Flowcytometry histograms represent PKH26 dilution in MSLN-CAR T, Un-T, and Mock either co-cultured with Hela (in a 1:1 ratio) or cultured in media. B) Bar graphs represent the percentage of proliferated MSLN-CAR T, Mock, and Un-T cells. The results are representative of six independent experiments and exhibited as mean $\pm$ SD.  $p < 0.05$  was considered statistically significant. All experiments were done in duplicate technical replication.



**Figure 5.** Antigen dependent MSLN-CAR T cell cytokine production.  $2 \times 10^5$  MSLN-CAR T, Mock, and Un-T cells were co-incubated with an equal number of Hela cells or without for 24 hours. Afterwards, the concentration of cytokines in the collected supernatant was measured by ELISA. A, B, and C) Bar graphs show IFN- $\gamma$ , IL-2, and TNF- $\alpha$  cytokines level in the supernatant of Mock, Un-T, and MSLN-CAR T cells either co-cultured with Hela cells (at 1:1 ratio) or without, respectively. The results are representative of six independent experiments. The error bars represent SD.

to Un-T and Mock cells ( $p=0.00$ ). However, there was no difference among any of the ratios in Un-T and Mock cells ( $p=0.883$ ) (Figure 3). Different ratios of CAR T cells co-cultured with Hela cells showed a significant difference between 1: 1 ratio with 5:1 ( $p=0.000$ ), 10: 1 ( $p=0.000$ ) and, 20: 1( $p=0.000$ ), similarly, among 5: 1 ratio ( $p=0.002$ ) and 20: 1 ( $p=0.000$ ), a significant difference was observed when compared to Un-T and Mock cells. CAR T cells co-cultured with OVCAR3 cells showed significant

cytotoxicity in all E:T ratios except 1:1 compared to Un-T and Mock cells. There was no significant difference between 10: 1 and 20: 1 ratio of CAR T cells co-cultured with both of Hela ( $p=0.557$ ) and OVCAR3 cells ( $p=0.81$ ). Antigen dependent lysis was also evaluated by co-culturing MSLN-CAR T cells with NALM-6 as an unrelated cell line. Demonstrative histograms and bar graphs display cytotoxicity of MSLN-CAR T cells in comparison with Un-T and Mock cells in different E: T ratios (Figures 3A and 3B).



### MSLN-CAR T Cell Antigen Dependent Proliferation

The proliferation of MSLN-CAR T, Un-T, and Mock T cells stimulated by Hela and OVCAR3 cells was evaluated. To examine the non-specific proliferation of CAR T cells, NALM-6 cells were also used as non-relevant target cells. MSLN-CAR T cells co-cultured with Hela cells displayed a significant proliferation relative to Un-T and Mock cells as well as showing significant proliferation when co-cultured with Hela cells compared to OVCAR3 cells. The negligible proliferation of culturing CAR T cells in the absence of target cells displayed no proliferation by tonic signaling of MSLN-CAR T cells (Figures 4A and 4B).

### MSLN-CAR T Cells Cytokine Production after MSLN Stimulation

In this study, IFN- $\gamma$ , IL-2, and TNF- $\alpha$  production were measured using the ELISA technique in MSLN-CAR T cells. As a Result of MSLN-CAR activation with Hela cells, MSLN-CAR T cells produced significant amounts of IFN- $\gamma$ , IL-2, and TNF- $\alpha$  compared to Mock and Un-T cells stimulated by endogenous TCR and CD28 receptor. Producing cytokines showed an effective antitumor cellular immune response (Figures 5 A, B and C).

## DISCUSSION

Targeted immunotherapy of solid tumors is comprised of two main approaches including monoclonal antibodies and cell-based immunotherapies.<sup>20,21</sup> The most important limitation of targeted immunotherapy of tumors is to find the appropriate tumor antigen with an exclusive expression on cancer cells. MSLN is a tumor antigen that plays several roles in the development and growth of many cancer cells.<sup>22</sup> MSLN has been considered as an ideal target for anti-MSLN antibody therapy of MSLN positive tumors.<sup>23,24</sup> However, because of restricted tissue accessibility and short-term therapeutic effects, desirable results have not been achieved yet.<sup>8</sup> CAR T cell-based immunotherapy has shown remarkable results in the treatment of hematologic malignancies. However, the results of CAR T cell therapy in MSLN-positive solid tumors have not been very promising partly due to the immunosuppressive microenvironment of solid tumors and the development of host immune responses against fully murine or humanized anti-MSLNCAR transgenes.<sup>15,25</sup> Maus et al

have evaluated the antitumor activity and safety of CAR T cell therapy in four patients who received autologous T cells that had been electroporated with mRNA encoding a CAR construct derived from a murine antibody against human MSLN. Their results indicated that murine anti-MSLN CAR could trigger anti-CAR host immune responses.<sup>15</sup>

In the current study, a fully human anti-MSLN CAR comprised of human MSLN-specific scFv fragment coupled to CD3 $\zeta$  and 4-1BB signaling domains were constructed. To improve the survival of CAR T cell, a 4-1BB signaling domain was embedded in the CAR construct; however, it remained to be assessed in future studies.<sup>26</sup>

The primary human T cells expressing MSLN-CAR showed potent antigen-dependent proliferation and cytokine production as well as specific cytolytic activity against MSLN positive cell lines. A significant cytotoxic activity of MSLN-CAR T cells at four ratios of the effector: target cells (from 1:1 to 20:1) was observed after 18 hours. An increasing pattern of cytolytic activity was found from 1:1 to 10:1 at 3 ratios, while there was no significant difference between 10:1 and 20:1 ratios in terms of cytotoxic activity. This finding exemplifies that CAR T cell doses greater than 10:1 are not necessary to achieve the high cytolytic activity, and it could provide more safety with lower doses while maintaining desirable results. This finding is consistent with the cytotoxicity of the murine anti-human MSLN CD8<sup>+</sup> CAR (SS1) T cells after 4 hours co-incubation with MSLN-expressing target cells.<sup>26</sup> However, some studies reported a higher level of cytokine production (e.g. IFN- $\gamma$ ) and cytotoxicity of MSLN-CAR T cells<sup>13,16</sup> as the second-generation CD28-based CAR T cells similar to CAR T cells used in Lanitis et al study which produced a larger amount of cytokines compared to our second-generation 4-1BB based-CAR T cells.<sup>27,28</sup> Moreover, Lanitis et al indicated that IFN- $\gamma$  production depends on the level of mesothelin expression on the tumor cell surface, and this could partly explain the lower IFN- $\gamma$  production of our CAR T cells against Hela and OVCAR3 cells with low mesothelin expression, 52%, and 20% respectively.<sup>13</sup> In the present study, we took advantages of different transmembrane (TM) and signaling domains as compared to other CAR T cell studies including Lanitis et al. It has been previously shown that the type of TM in CAR construct has a major effect on CAR persistence and enhancing antitumor

efficacy of third-generation ICOS based CAR constructs. Recent studies have also shown that the type of TM affects the production of effector cytokines, which may be important in CAR T cell therapy in cancer-related complications such as cytokine release syndrome (CRS).<sup>29-33</sup> Various studies have shown that different types of signaling domains in the structure of the CAR construct substantially affect the antitumor activity of CAR T cells. It has been sufficiently described that second-generation CD28-based CAR T cells produce larger amounts of cytokines relative to second-generation 4-1BB based-CAR T cells.<sup>27,34</sup> Hence, the use of 4-1BB in the CAR construct along with a low expression of MSLN on target cell lines could explain the lower level of IFN- $\gamma$  production in our study. Altogether, these data indicate that our fully human anti-MSLN-CAR was able to deliver cytotoxic, proliferative and cytokine production signals into CAR T cells. While we did not measure Th2 cytokines, it appears that the cytokine production pattern was similar to the Th1 cells generated by anti-CD3 and CD28 stimulatory bead, which is required to an effective antitumor immune response.<sup>35</sup> In agreement with our results, Shuford et al showed that the incorporation of the 4-1BB domain into CAR constructs decreased the production of Th2 cytokines.<sup>36</sup>

These data certify that the type of co-stimulatory domains of CAR constructs affects cytokine secretion and CAR T cell persistence.<sup>16,37,38</sup> Furthermore, Qijun Qian et al showed that targeting the membrane-proximal region of mesothelin in comparison to the membrane-distal region leads to stronger cytotoxicity, higher CD107 expression, and elevated production of cytokines by MSLN-CAR T cells.<sup>39</sup> Moreover, it has been shown that the effectiveness of CAR T cells for gene therapy is dependent on the persistence of T cells following administration to the body.<sup>40</sup> However, host immune responses against the CAR construct might restrict the persistence of the CAR T cells. Therefore, the generation of fully human anti-MSLN CAR not only blunts such as a host immune response but also maximizes the effector function of these cells.

In summary, we produced a second-generation fully human anti-MSLN CAR T cell composed of anti-MSLN scFv and CD3 $\zeta$ -4-1BB transduced by the third-generation lentiviral vector. The results showed the CAR construct elicited strong in vitro antigen-specific proliferation, cytotoxicity, and cytokine production, while they didn't show any tonic signaling. The

application of this fully human MSLN-CAR will not only increase the in vivo persistence of anti-MSLN CAR T cells, there by diminishing the risk of tumor relapse but also enables us to administer multiple doses of CAR T cells into the same patient.

## CONFLICT OF INTEREST

The authors declare no conflicts of interest.

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