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Research Article

Augmented Tumor Suppression by CAR T Cells with Co-Expressed PD-L1 Antibodies

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ABSTRACT

Chimeric antigen receptor T (CAR-T) cell therapy against solid tumors has shown limited success in clinical trials. Solid tumors with highly immunosuppressive tumor microenvironment limit the anti-tumor efficacy of CAR-T cell therapy. Herein we generated a novel vector that co-express human mesothelin (MSLN)-targeted CAR and secretory human anti-programmed death ligand 1 (PD-L1) antibodies. The anti-PD-L1 antibodies secreted from the transduced T cells were able to block the interaction of PD-1/PD-L1 to overcome PD-L1-mediated immune suppression. The CAR-T cells targeting mesothelin and secreting PD-L1 antibodies enhanced cytolytic activity against MSLN+PD-L1+ tumor cells and increased the production of cytokines IL-2, TNF α , and IFN γ . The CAR-T cells targeting MSLN and secreting PD-L1 antibodies had enhanced anti-tumor efficacy when compared with the MSLN-targeted CAR-T cells in xenograft mouse models of mesothelioma, pancreatic and ovarian cancers. The results of this study demonstrated that the co-expression of PD-L1 antibody enhances the anti-tumor efficacy of CAR-T cell therapy against solid tumors.

Keywords: Chimeric antigen receptor T; PD-L1 antibodies; Anti-tumor efficacy

INTRODUCTION

Chimeric antigen receptor T cell (CAR-T) therapy has shown significant efficacy in treating patients with refractory B-cell leukemia and lymphoma [1,2]. However, CAR-T therapy has yielded less favorable results in treating patients with solid tumors such as pancreatic, ovarian or mesothelioma cancers [3-5]. One of the major obstacles in treating solid tumors with CAR-T is the scarcity of antigen that is uniformly expressed in tumor cells such as CD19 in B cell hematologic malignancies [6,7]. Therefore, designing a CAR against an antigen that is highly expressed in cancer cells but either has negligible or reduced expression in normal tissues to avoid off-tumor toxicity is extremely critical. Several CAR-T clinical trials in treating solid tumors demonstrated poor efficacy and severe toxicities including death because of the low expression of antigen in the normal cells [7-13]. Moreover, the immunosuppressive tumor microenvironment of solid tumors inhibits the T cell infiltration and proliferation limiting the potential of CAR-T cell immunotherapy [14,15].

Mesothelin (MSLN) has emerged as one of the promising targets for CAR-T cell therapy for solid tumors because of its overexpression in cancer cells including pancreatic, ovarian and mesotheliomas but with a basal expression in normal tissues [16-18]. Mesothelin is encoded as a 71-kDa cell surface glycoprotein that is cleaved by a furin protease into an N-terminal 31 kDa soluble fragment megakaryocyte potentiating

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factor (MPF) and C-terminal 40 kDa membrane-bound mesothelin [16,18]. Although mesothelin is expressed in lung, heart, spleen, liver, kidney, and testis of adult tissues, the biological function of mesothelin is not known [19]. Mice harboring a null mutation in the mesothelin gene had a phenotype similar to the wild-type suggesting mesothelin was not required for growth and reproduction [19]. However, mesothelin is believed to involve in cell adhesion in tumor progression since it binds to the CA125/MuC16 facilitating the metastatic proliferation of ovarian cancer onto peritoneal mesothelial cells [20,21].

Programmed death 1 (PD-1) is expressed on B and T cells, macrophages and some of dendritic cells and upon activation regulates the tolerance and autoimmunity [22]. PD-1-/- mice developed lupus-like autoimmune disease confirming the role of PD-1 in maintaining tolerance and autoimmunity [23,24]. PD-L1, a ligand of PD-1, is expressed constitutively on cancer cells and delivers pro-survival signals that favor tumor progression [25]. PD-1 is capable of transmitting inhibitory signals when engaged with PD-L1 expressed on tumor cells and thus inhibiting tumor-specific T-cell proliferation and cytokine production [26]. Therefore, eliminating either PD-1 or PD-L1 and blocking their interaction can result in the breakdown of tumor immune suppression.

In this study, we generated a novel vector that co-expresses human MSLN-targeted CAR with secretory human anti-PD-L1 antibodies to overcome tumor-mediated immunosuppression. We investigated its anti-tumor potential in pancreatic, ovarian and mesothelioma xenograft mouse models. The MSLN-targeted CAR-T cells secreting the anti-PD-L1 antibodies efficiently killed mesothelin-expressing cells *in vitro* as well as showed potent anti-tumor activity in a xenograft mouse model.

MATERIALS AND METHODS

Cell culture

Human pancreatic adenocarcinoma cell line PANC1, human lung squamous cell carcinoma/mesothelioma cell line H226 and human ovarian adenocarcinoma cell line OVCAR3 were purchased from American Type Culture Collection (ATCC, USA). PANC1 and 293T (lentiviral packaging cell line) were maintained in DMEM (Corning, USA) while H226 and OVCAR3 were maintained in RPMI-1640 (Corning, USA) medium. DMEM and RPMI media were supplemented with 10% FBS (Denville, USA) while OVCAR3 media was supplemented with 20% FBS and human insulin (1: 10,000; Sigma, USA). All media were also supplemented with 1% L-glutamine, penicillin, and streptomycin (Life Technologies) and maintained at 37°C in a 5% CO2 humidified incubator.

H226, PANC1, and OVCAR3 cells were lentivirally transduced to express mesothelin variant 1/green fluorescent protein (GFP) fusion protein. The cells were sorted using BD FACS Aria cell sorter II (BD Bioscience, USA). The sorted cells were then lentivirally transduced with PD-L1 and sorted again for cells positive for both PD-L1 and mesothelin after staining cells with human anti-PD-L1 (Biolegend, USA) and anti-mesothelin (R&D systems) antibodies. The double sorted cells were then lentivirally transduced with luciferase with puromycin as a selection marker. All experiments were performed in the stably transduced cells expressing mesothelin, PD-L1, and luciferase. Henceforward, H226, PANC1 and OVCAR3 cells transduced with mesothelin, PD-L1, and luciferase will be referred to as H226-MSLN-PDL1, PANC1-MSLN-PDL1 and OVCAR3-MSLN-PDL1 respectively. All tumor cells were routinely tested for mycoplasma contamination (MP0035, Sigma).

Lentiviral car construct generation and production

The mesothelin specific CAR consists of anti-mesothelin scFv (HN1, humanized) linked to the CD8/4-1BB/CD3 ζ domains. The CAR construct HN1CAR/αPDL1Fc was generated by inserting the anti-PD-L1scFv-IgG1-Fc DNA fragment between the XbaI and NotI restriction sites of MSLN-targeted CAR vector, as shown in Figure 1. The third generation Lentiviral (LV) system was used to generate recombinant LVs. The viral particles were titrated with Lenti-X Go-Stix (Tekara, USA) and viral preparations in serum-free RPMI-1640 media were frozen at -80°C.

T cell isolation and proliferation

Peripheral blood leukopak were isolated from the blood of a healthy donor (70500; Stem cell Technologies, USA) and T cells were isolated using EasyStep Human T cell isolation kit (17951, Stem Cells Technologies, USA). The T cells were seeded on OKT3/CD8 α coated plate and maintained in IL-2 (20 IU/mL). Two days after seeding, T cells were transduced with viral particles containing mesothelin CAR and control vectors by centrifugation for 90 min on plates coated with retronectin (15 µg/mL) at 2000 rpm break free at 32°C. Transduced T cells were maintained in RPMI-1640 supplemented with 1% L-glutamine, penicillin and streptomycin (Life Technologies), 20% FBS and IL-2 (20 U/mL). After 3-4 days, transduction efficiency was determined by using flow cytometric analysis (CANTO II, BD Biosciences, USA).

Cytotoxicity assay

For measuring specific cytotoxicity, 2×10^5 H226, PANC1 and OVCAR3 target cells (all expressing mesothelin, PD-L1, and luciferase) were first irradiated to slow down the cell growth and then co-cultured with mesothelin CAR-T cells (effector cells) at various effector to target ratio (E:T) in 96-well round bottom plates at a final volume of 200 µL for 24 h. The co-cultured cells were added with D-firefly luciferin potassium salt (75 µg/mL) and measured for relative luminometer units (RLU) with a luminescence counter (Packard Biosciences Company, USA). The 1X lysis buffer was added to the row incubated only with the tumor cells to calculate the maximum lysis and the row incubated without effector cells were considered as a spontaneous death. Percent cell lysis was calculated using the formula: (Spontaneous death RLU-experimental RLU)/(Spontaneous death RLU-Maximum death RLU)100. RLU=Relative luciferase units.

Flow cytometry

The surface expression of mesothelin and PD-L1 antigens on H226, PANC1, and OVCAR3 cancer cells were detected using human mesothelin PE-conjugated antibody (Clone#420211, R&D systems, USA) and APC-conjugated anti-human PD-L1 (CD274 (B7-H1); Clone:29E.2A.3, BioLegend). The cells were sorted using BD FACS ARIA flow cytometer (BD Biosciences) and approximately 50,000 to 200,000 cells were sorted for each cell line. For evaluation of CAR expressions, the transduced T cells were stained with anti-human IgG(Fab')2 (R&D Systems, USA). tEGFR expression was measured with anti-EGFR (C2259 Cetuximab, Absolute Antibodies Ltd., Oxford, UK) and conjugated secondary antibody. BV510 (BioLegend) was used to stain the live cells. LAG-3, TIM-3, PD-1, CD69, OX40, CD45RA, CCR-7, CD3, CD4 and CD8 antibodies (BioLegend) were used to determine the T cell status and phenotype. All data were acquired using BD FACS CANTO II flow cytometer (BD Biosciences) and analyzed using FlowJo software.

Cytokines release assay

Secretion of Cytokine (IFNgIL-2, and TNF α) by mesothelin CAR-T cells was evaluated using ELISA (BioLegend, USA) and ProcartaPlex multiplex immunoassay (Invitrogen, CA, USA) following the manufacturer's protocol. Briefly, the effector (2 × 105) and irradiated target cells were co-cultured in a 24-well plate in 1:1, 5:1 and 20:1 (E:T) ratio for 24 hours. The supernatants were collected after 24 hours and measured by ELISA and Luminex (magnetic-bead fluorescent immunoassays were run on MAGPIX™).

Western blot for car and α pdl1fc expression

CUTLL1-T cells were transduced with HN1CAR and HN1CAR/α PDL1Fc lentiviral particles, and tEGFR positive cells were then sorted out few days after transduction. Following, 1 × 107 sorted CUTLL1 cells were cultured in a T25 flask and 3 days later, cells were harvested, lysed and CAR expression was determined by western blot using anti-CD3 ζ (BD, cat. No. 556366). Cell culture media was also collected, filtered through a 0.02-µm inorganic membrane filter (Millipore, Billerica, MA SLGP033RB), and concentrated 100X using Amicon Ultra-15 Centrifugal Filter Units. The concentrated media were analyzed by western blot using anti-human IgG followed by IRDye® 800CW Goat anti-Human IgG (H+L) (Licor, cat. 925-32232) antibody. All samples were analyzed under a reducing condition.

Detection of human PD-L1 on cell surface by flow cytometry

1×10^5 H226-MSLN-PD-L1 and PANC1-MSLN-PD-L1 cells were incubated on ice for 30 mins with 50 µL of 10X concentrated (using Amicon Ultra-15 Centrifugal Filter Units) culture media of HN1CAR and HN1CAR/α PD-L1Fc CAR-transduced T cells, or with 50 µL of purified α PDL1-Fc or human IgG at the indicated concentration. Cells were washed twice with PBS, followed by staining with viability dye and conjugated anti-human PD-L1 or anti-human IgG and then analyzed by flow cytometry.

PD-L1 inhibition assay

Commercial PD-L1 (1 µg/well) protein was used to coat 96-well ELISA plate. 50 µl mixture of 10 ng PD-1-biotin and Mock, hIgG, purified α PDL1-Fc protein from HN1CAR/α PD-L1Fc supernatant or commercial α PD-L1 antibody (Biolegend, Cat. 329702) at indicated concentration was added into each coated well and incubated at room temperature for 2 hours. Diluted streptavidin-HRP was added to each well after washing and incubated at room temperature for 1 hour with shaking. After 3X wash, 100 µL TMB HRP substrate was added until blue color was developed. OD450 was measured after 100 µL and 1N sulfuric acid was added to stop the reaction. OD of sample/(OD of Mock-OD of background) represents inhibition percent activity.

Surface Plasmon Resonance (spr)-binding assays

The assay was carried out at 25°C using a Biacore T100 (USC NanoBiophysics Center). CM5 sensor chips were activated by a 7 min injection of a 1:1 mixture of N-hydroxysuccinimide and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride at a flow rate of 10 µL/min. The PD-L1-His tag protein was prepared at a concentration of 10 µg/mL in 10 mM acetate buffer, pH 4.0, and immobilized to the sensor chips by amine linkage, with the typical immobilization levels being 100 response units. For blocking sensor chips, 70 µL of 1M ethanolamine–HCl buffer, pH 8.5, was injected and the chips were further washed with 10 µL of 10 mM glycine–HCl buffer, pH 1.5. For analyzing the binding and equilibrium affinity of purified α PDL1-Fc from T cell supernatant, 7 serial dilutions of α PDL1-Fc starting from 200 µg/mL, and α PD-L1 antibody starting from 100 µg/mL were injected at a flow rate of 30 µL/min over the sensor chips coupled with PD-L1 protein. The data were analyzed using a model for 1:1 binding.

ELISA to detect anti-PDL1Fc binding

CUTLL1-T cells were transduced with HN1CAR orHN1CAR/α PDL1Fc virus and sorted for tEGFR positive cells. 1 × 107 seEGFR+CUTLL1 cells were cultured in 10 mL complete RPMI 1640 media in T25 flasks. 300 µL supernatants of each group at the indicated time point were harvested and analyzed by human IgG Fc ELISA. Briefly, 100 ng/mL anti-Human Polyvalent Immunoglobulins (G,A,M) antibody produced in goat (sigma, I1761) was coated overnight. Serial dilution of standard hIgG-Fc (1000 pg/mL, 500 pg/mL, 250, 125, 62.5, 31.25 pg/mL and blank) and several dilutions of cell supernatants were added and incubated at RT for 2 hours. After several washes, HRP-anti human IgG-Fc (ThermoFisher) was added and incubated at RT for 1 hour. Freshly made TMB substrate was finally added.

In vivo experiments

All animal studies were conducted per approval from the University of Southern California's Institutional Animal Care regulations and Use Committee (IACUC). Ten to twelve-week-

old NOD/SCID γ mice (The Jackson Laboratory) were inoculated with 1 × 106 luciferase-expressing tumor cells subcutaneously (s.c.) at the right flanks (5 mice per group). Three days later, mesothelin CAR-T cells (5 × 106 cells re-suspended in 100 µL of PBS per mouse) or 100 µL PBS was injected intravenously (i.v.) *via* tail-vein for each respective CAR-T or control groups. Tumor burden was monitored weekly using the Xenogen IVIS (Caliper Life Sciences) imaging instrument. Mice were anesthetized using a mild dose of isoflurane and administered intravenously (i.v.) *via* tail-vein with 15 mg/mL D-luciferin (GoldBio, USA) per gram of body weight and imaged 90 seconds later. The therapeutic effects of mesothelin CAR-T cell treatment were evaluated based on bioluminescence signals compared to control groups. Image acquisition was done on a 25-cm field of view at a medium binning level at 1-second to the 1-minute exposure time.

RESULTS

Generation and characterization of the novel CAR-T cells

Monoclonal antibody HN1 binds to human mesothelin on pancreatic, ovarian, mesothelioma and lung cancer cells and blocks the interaction of mesothelin with CA125/MuC16 [27]. In addition, the HN1 immunotoxin showed a high cytotoxic activity against mesothelin positive cancers [27]. Thus, we engineered a bicistronic lentiviral vector to express the anti-mesothelin (HN1) scFv linked to 4-1BB and CD3-ζ signaling domains (HN1CAR) in the first cassette and a truncated, non-functional EGFR (tEGFR) in a second expression cassette after an internal ribosome entry site (IRES) as a marker for gene transduction and potential cell ablation with an anti-EGFR antibody (Figure 1A). To develop more effective CAR-T therapy for mesothelin+ tumors that could block T cell exhaustion, we further engineered the HN1CAR to co-express anti-PD-L1scFv-IgG1Fc (αPDL1Fc) by inserting its DNA sequence with a T2A linker upstream of HN1CAR DNA. The resultant construct was designated as HN1CAR/αPDL1Fc (Figure 1A). The human T-cells were efficiently transduced with the CAR lentiviral particles with transduction efficiencies above 85% (Figure 1B). The transduced T cells with the various CAR lentiviral particles proliferated at comparable rates with HN1CAR/αPD-L1Fc CAR-T cells slightly slower (no statistical difference) (Figure 1C). Similar transduction efficiency was obtained for all subsequent functional assays. The lung mesothelioma (H226), pancreatic (PANC1) and ovarian (OVCAR3) cells lines were transduced with lentivirus and retrovirus to stably express human mesothelin and PD-L1 respectively, to make sure that all target cells show base-line expression of tumor-associated antigen mesothelin and PD-L1 expression (Figure 1D). All subsequent experiments were performed on target cells positive for both mesothelin and PD-L1.

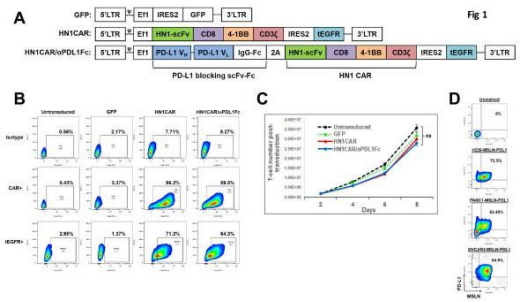


Figure 1: Generation of recombinant lentiviral vectors expressing chimeric antigen receptor T (CAR-T) cells. (A) Schematic representation of anti-mesothelin CARs. Mesothelin-targeted CAR constructs with or without co-expression of αPD-L1Fc was engineered. A control construct expressing GFP was also generated. (B) Expression of HN1 scFv and tEGFR was examined on human primary T cells transduced with CARs by flow cytometry. Transduction efficiencies are indicated in %. (C) Proliferation rate was determined post-transduction in T cell. (D) Cell surface expression of mesothelin and PD-L1 was determined by flow cytometry on mesothelioma (H226), pancreatic (PANC1) and ovarian (OVCAR3) cells after lentivirally and retrovirally transduced with mesothelin and PD-L1 respectively. Error bars are standard error.

We first investigated whether αPDL1Fc can be secreted from the HN1CAR/αPDL1Fc-T cells blocking PD-1/PD-L1 interaction. We used a human T-cell lymphoma line CUTLL1 [28] because it can be efficiently transduced with LV and showed no phenotypic differences compared to transduced T cells from healthy donor. The CUTLL1 transduced with HN1CAR/αPDL1Fc viral particles showed HN1CAR and αPDL1Fc in cell lysates or in the supernatant over time, as detected by Western Blot or ELISA, suggesting the efficient transduction as well as secretion of αPD-L1-Fc antibodies by the CAR-T cells (Figures 2A and 2B). The flow cytometry analysis revealed that purified αPDL1Fc from HN1CAR/αPDL-CUTLL1 effectively bound to the surface PANC1-MSLN-PDL1 or H226-MSLN-PDL1, but parental PANC1 or H226, implying αPD-L1Fc interaction with PD-L1 on the surface of PANC1-MSLN-PDL1 or H226-MSLN-PDL1. Surface plasmon resonance binding analysis confirmed that at various concentrations protein A/G-purified αPD-L1Fc efficiently bound to PD-L1 (Figures 2C and 2D). Compared with commercial αPD-L1 antibody, αPDL1-Fc showed a similar binding affinity, but much longer dissociation time (Figure 2D). Furthermore, the purified αPD-L1-Fc could block the interaction between PD-L1 and PD-1 as evidenced by comparative ELISA (Figure 2E). The supernatant derived from CUTLL1 cells transduced by HN1CAR/αPDL1 blocked PD-L1 to be detected by the anti-PD-L1 antibody on tumor cell surface further confirming secreted αPD-L1-Fc binding PD-L1 on the cell surface (Figure 2F). These data suggest that T cells transduced with HN1CAR/αPDL1 secrete αPDL1Fc and block the interaction between PD-1 and PD-L1 axis.

Phenotypic characterization of mesothelin CAR-T cells

To characterize primary T cells transduced with HN1CAR/αPD-L1Fc, T cells isolated form healthy PBMCs were

transduced with HN1CAR/α PD-L1Fc viral particles and expanded for 5 days. Flow cytometry analysis revealed that more than 90% of cultured cells were CD3-positive, a marker of T cells while a majority of the T cells were positive for CD4 (56%), a marker of helper T cells or CD8 (35%), a marker for cytotoxic T cells (Figure 3A). Surface markers CD45RA and CCR-7 were used to define naïve T cells and memory T cell subsets. Results showed that while ~44% of HN1CAR/α PD-L1Fc-CAR-T cells expressed CD45RA, indicating a naïve T cell phenotype, ~40.5% HN1CAR/α PD-L1Fc-CAR-T cells were differentiated into central memory T cells (Tcm, CD45RA-CCR7+) and 14.8% differentiated in effector memory T cells (Tem, CD45-CCR7-) (Figure 3B). The HN1CAR/α PD-L1Fc-T cells were further analyzed for expressions of activation markers (CD69 and OX40) or exhaustion markers (PD-1, Tim-3 and Lag3). Majority of the CAR-T expressed CD69 (66.2%) or OX40 (68.8%,), while expressions of PD-1 and TIM-3 were lower (9.3% or 8.1%). LAG-3 expression was relative high (53%) (Figures 3C and 3D). Similar T cell phenotype was observed for untransduced, GFP and HN1CAR cells (data not shown). Moreover, the proliferation rate of the CARs was not greatly reduced post-transduction compared to the untransduced T cells (Figure 1C). These data suggest that transduction of the T cells with CAR viral particles did not greatly impact the phenotype and growth rate of T cells.

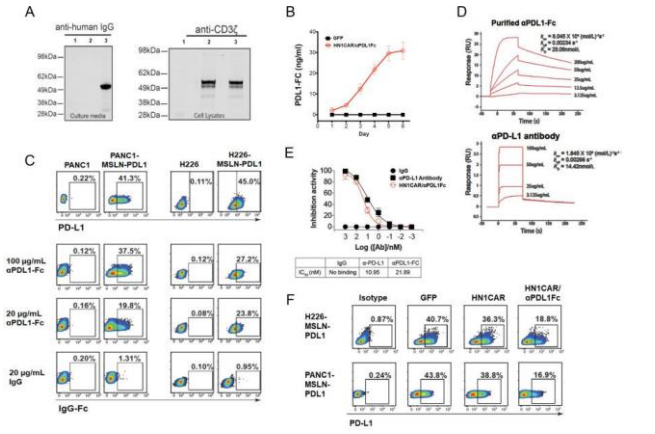


Figure 2: α PD-L1Fc is secreted and blocked interaction between PD-1 and PD-L1. (A) CUTLL1 T cells were transduced with the indicated viral particles. The clarified supernatants were precipitated with protein A/G beads and then analyzed with anti-human IgG and, whole cell lysate analyzed with anti-CD3ζ *via* western blot. 1=GFP, 2=HN1CAR, 3=HN1CAR/α PD-L1Fc (B) ELISA assay showing that HN1CAR/α PDL1Fc-T cells secrete α PD-L1Fc over time. (C) H226 or H226-MSLN-PDL1 and PANC1 or PANC1-MSLN-PDL1 cells were confirmed to express PD-L1with commercial anti-PD-L1 (Top). The tumor cells were incubated with purified α PD-L1Fc or human IgG antibodies at indicated concentrations. The cells were stained with anti-human IgG Fc and analyzed by flow cytometry (bottom). (D) Surface plasmon resonance (SRP)-binding assay comparing binding affinity of purified α PD-L1Fc or commercial anti-PD-L1 to PD-L1. (E) The purified α PD-L1-Fc antibody was compared with a commercial α PD-L1 for competitive inhibition of PD1/PD-L1 interaction *via* a competitive ELISA. (F) H226-MSLN-PDL1 and PANC1-MSLN-PDL1 cells were incubated with supernatants of different CAR-T cells followed by detection by a conjugated α PD-L1 antibody. The cells were analyzed by flow cytometry. Experiments were repeated and consistent results were acquired.

Co-expression of α pd-l1fc enhances cytokine secretion and cytotoxicity of msln-targeted car-t cells

We then examined the ability of CAR-T cells to release cytokines (IFN γ, IL-2, and TNF α) in response to antigen-specific stimulation. The CAR-T cells were co-cultured with the irradiated mesothelin+PD-L1+ target cells (H226, PANC1, and OVCAR3) at various E:T ratio for 24 hours. HN1CAR-T cells co-expressing α PD-L1Fc displayed increased cytokine production against all target cells, despite that the degree of cytokine secretion varies among target cells, as manifested by ELISA (Figures 4A-4C) and Luminex (Protocarta Plex multiplex immunoassay). We further tested the cytotoxicity of the CAR-T cells toward the luciferase-expressing target cells (H226, PANC1 and OVCAR3).

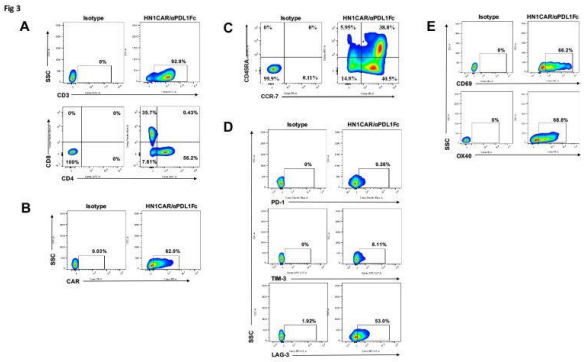


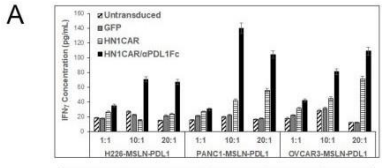
Figure 3: CAR-T cell phenotype post-transduction. (A) HN1CAR/α PDL1Fc-T cells were stained with CD3, CD4 and CD8 T cell markers. (B) CAR+ T cells were gated. (C) CAR+ T cells were further analyzed with CD45RA and CCR-7 for memory phenotypes (D) CAR+ T cells were analyzed with PD-1, TIM-3 and LAG-3 for defining T cell exhaustion. (E) CAR+ T cells were analyzed with CD69 and OX40 for defining T cell activation. All experiments were done in duplicates and one of the representative experimental data is shown.

In the short-term bio-luminescence cytotoxicity assay, HN1CAR co-expressing α PD-L1Fc demonstrated increased cytolytic activity against all the three target cells compared to the construct having HN1CAR alone even at a low E:T ratio (Figures 5A and 5B). The control GFP or untransduced cells did not exhibit any significant cytolytic activity against all the three target cell lines (Figures 5A and 5B). These data support that HN1CAR-T cells with α PD-L1Fc blocking PD1/PD-L1 interaction display more potent effector activity.

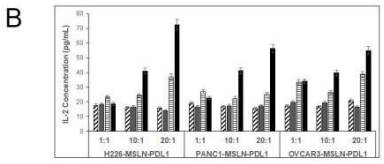
Enhanced anti-tumor activity of α pd-l1fc expressed mesothelin car-T cells in xenograft mouse models

We next investigated anti-tumor activity of HN1CAR/α PDL1Fc-T cells in xenograft mouse models. NOD/SCID γ mice were subcutaneously (s.c.) inoculated with mesothelin+PD-L1+luciferase+ H226 or PANC1 cells (1 × 106 cells per mouse) on their right flanks followed by an intravenous (i.v.) injection of 5 × 106 HN1CAR/α PD-L1Fc-T cells (89% CAR), HN1CAR-T cells (82% CAR), control GFP-T cells or untransduced-T cells on day 3. Mice treated with HN1CAR/α PDL1Fc-T cells showed significantly lower tumor luminescence over the course of the experiment as compared to the mice in the HN1CAR-T cell groups despite that HN1CAR-T cells displayed substantial anti-tumor effect compared with the

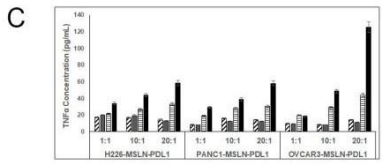
mice in the two control T-cell groups (Figures 6A, 6B and 6D). The anti-tumor effect exhibited by mice treated with HN1CAR/ α PDL1Fc-T cells become evident by day 21 and 28 (Figures 6A and 6D). The lower tumor luminescence in mice treated with HN1CAR/ α PDL1Fc-T cells was associated with an improved overall survival rate ($p<0.001$) (Figures 6C and 6F). The modest anti-tumor effect was observed with mice inoculated with mesothelin+PD-L1+luciferase+ OVCAR3 cells. These results suggest that HN1CAR/aPDL1Fc-T cells can more efficiently regress the growth of mesothelin+tumor cells *in vivo*.



IFNγ (20:1)		Untransduced	GFP	HN1CAR
H226-MSLN-PDL1	HN1CAR/ αPDL1Fc	0.015983	0.001593	0.008228
PANC1-MSLN-PDL1		0.0028163	0.0029344	0.0048284
OVCAR3-MSLN-PDL1		0.003775	0.006157	0.031291



IL-2 (20:1)		Untransduced	GFP	HN1CAR
H226-MSLN-PDL1	HN1CAR/ αPDL1Fc	0.034547	0.03195	0.052004
PANC1-MSLN-PDL1		0.001618	0.002446	0.005335
OVCAR3-MSLN-PDL1		0.050753	0.043772	0.080908



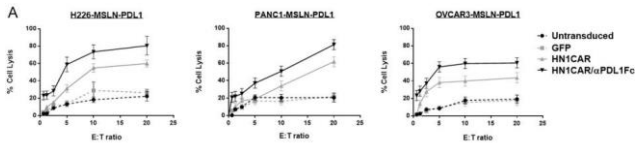
TNF-α (20:1)		Untransduced	GFP	HN1CAR
H226-MSLN-PDL1	HN1CAR/ αPDL1Fc	0.003448	0.003164	0.026491
PANC1-MSLN-PDL1		0.003164	0.002446	0.051443
OVCAR3-MSLN-PDL1		0.00034341	0.0003328	0.0053964

Figure 4: Cytokine secretion activity of CAR-T cells. The ability of HN1CAR/ α PDL1Fc-T and control T cells to release IFN γ , IL-2 and TNF α were analyzed by ELISA A-C) after 24 hours of co-culture with H226-MSLN-PDL1, PANC1-MSLN-PDL1 or OVCAR3-MSLN-PDL1 target cells at 1:1, 10:1 and 20:1 (E:T) ratios. The p-value at the 20:1 (E:T) ratio was calculated and displayed below each ELISA graph. Error bars are the standard error. All experiments were done in triplicates.

DISCUSSION

In this study, we investigated the mesothelin HN1CAR-T cell therapy to treat solid tumors such as mesothelioma, pancreatic and ovarian cancers. Another mesothelin-specific CAR SS1, derived from the mouse anti-mesothelin scFv, exerted anti-tumor activity against mesothelin-expressing cancer cells [29,30]. In the current study, we generated a second generation of mesothelin-specific CAR using HN1 scFv, a human version, that specifically recognizes the epitope of mesothelin on tumor cells and blocks the interaction of mesothelin and MuC16 (CA125) [27]. The PD-1 and PD-L1 interaction have been shown to be crucial for immune tolerance. Effective blocking of either PD-1 or PD-L1 overcomes immune tolerance in patients with advanced cancer including lungs, pancreatic and ovarian cancers [31]. Monoclonal antibodies (mAb) that target PD-1/PD-L1 axis have shown therapeutic efficacy in clinical trials [32,33]. Moreover, an Fc-linked anti-PD-L1 scFv triggered tumor regression in mice [34,35]. In addition, CAR-T co-expressing anti-PD-L1 scFv showed an enhanced anti-tumoral affect [36,37]. To improve the efficacy of CAR-T cell therapy in solid tumors, Rafiq and colleagues generated armored CAR-T cells against MuC16 or CD19 that secrete PD-1 blocking scFv and showed induced cytotoxicity and bystander T-cell activity both *in vitro* and *in vivo*. In addition, these CAR-Ts were only detected in tumor microenvironment suggesting increased efficacy and reduced toxicity [37]. In order to counteract the negative effect of tumor microenvironment on CAR-T cells, we designed CAR with scFv specific to mesothelin for high affinity against mesothelin positive cancers and to secrete anti-PD-L1-scFv-Fc antibodies to block CAR-T cell exhaustion (Figure 1A). The scFv-Fc format offers several advantages over scFv including longer half-life and Fc-mediated effector function [38]. The HN1CAR together with α PD-L1Fc was more effective than HN1CAR alone in regressing tumor growth suggesting that addition of α PD-L1Fc attributed to block inhibitory signals and allowed antigen recognition (Figures 4-6).

Our results support the feasibility of targeting solid tumors expressing higher levels of mesothelin. We have provided evidence that engineering CAR-T cells to secrete the human antibody against checkpoint blockade at the tumor site can efficiently diminish their ability to exhaust the T cells (Figure 3). The Fc-linked PD-L1 integrated into HN1CAR-T cells can maintain their capacity to undergo proliferation and mAb secretion when co-cultured with mesothelin positive cancer cells (mesothelioma, pancreatic and ovarian) and efficiently stimulate target cell lysis (Figure 5) and secrete higher levels of IL-2, IFN γ and TNF α cytokines (Figure 4). In a xenograft mouse model of human mesothelioma, pancreatic and ovarian cancers, α PD-L1Fc co-expressing HN1CAR-T cells had the ability to secrete antibodies at the tumor site as witnessed by the more prominent tumor regression in all three mouse models compared to the HN1CAR without α PD-L1Fc (Figure 6). However, OVCAR3-bearing mice treated with HN1CAR-T cells co-expressing α PD-L1Fc did not achieve a significant anti-tumor effect even at an early stage and only achieved a modest anti-tumor effect at a later stage suggesting that infused CAR-T cells (5×10^6) may not be sufficient to control this aggressive tumor and repeated injections or more CAR-T cells may be required to combat OVCAR3 tumors. Nonetheless, HN1CAR-T cells co-expressing α PD-L1Fc were able to significantly delay the tumor growth in at least two of three tested mesothelin-positive cancer cells. It seems CAR-T cells were eventually get exhausted and thus suggests that repeated CAR-T injections were required for the continued proliferation of CAR-T cells for tumors to regress in all three mouse models.



B

		Untransduced	GFP	HN1CAR	
H226-MSLN-PDL1	HN1CAR/ αPDL1Fc	0.033852152	0.009312183	0.000549523	20:1
		0.023968871	0.001368189	0.00149125	10:1
PANC1-MSLN-PDL1		1.77E-10	1.20642E-05	1.15548E-05	20:1
		3.88752E-11	4.26356E-06	3.59643E-09	10:1
OVCAR3-MSLN-PDL1		0.00120315	0.050167969	0.05055891	20:1
		0.005579416	0.00390103	0.041818689	10:1

Figure 5: Cytolytic activity of CAR-T cells. (A) The cytolytic activity of CAR-T cells were analyzed on H226-MSLN-PDL1, PANC1-MSLN-PDL1 and OVCAR3-MSLN-PDL1 luciferase positive target cells by bio-luminescence assay at indicated Effector: Target (E:T) ratios. (B) Table showing the p-values of HN1CAR/ α PDL1Fc-T vs. controls at 20:1 and 10:1 (E:T) ratios. Error bars are standard deviation. All experiments were done in triplicates.

There is ample evidence that combinational immunotherapy is more effective in both pre-clinical and clinical settings. There are several mesothelin based clinical trials are under investigation summarized in these reviews [15,39]. The early phase I clinical trial with a combination of mesothelin CAR-T and CD19 CAR-T cell therapy may prolong the duration of mesothelin CAR-T cells in the body of the pancreatic cancer patients by depleting CD19+ B cells, thereby, promoting the elimination of antibody-mediated clearance of mesothelin CAR-T cells (NCT02465983). The combination of anti-CTLA-4 and anti-PD-1 mAb therapy leads to a greater pre-clinical anti-tumor effect [40-42] and in clinical trials in advanced melanoma patients [43]. In addition, blocking PD-1 enhanced the anti-HER2 T cells in breast cancer patients [44]. Moreover, the human anti-carbonic anhydrase IX (CAIX) CAR with secreting human anti-PDL1 antibodies resulted in regressed renal cell carcinoma tumors in mice xenografts [35]. The efficiency of mesothelin CAR-T cell therapy has also been investigated spanning all three generations of CAR in preclinical or clinical settings in pancreatic, mesothelioma and lung cancers [3,14,15,30,45,46]. Combinational CAR-T cells with cyclophosphamide (NCT02414269, NCT02159716) or CAR-T with cyclophosphamide and CD19-CAR-T cells (NCT02465983) were also investigated in clinical trials for mesothelin positive cancer patients. In addition, the therapeutic delivery of mesothelin targeted CAR-T cells was investigated pre-clinically [46] as well as in phase I clinical trial in patients with primary or secondary pleural malignancies (NCT02414269) [47]. The risk of on-target off-tumor toxicity was also examined by delivering transient mesothelin CAR *via* mRNA electroporation [13,48] (NCT01355965). Our results also provide evidence that the combination of anti-HN1 and blocking PD-1/PD-L1 interaction can lead to greater anti-tumor efficacy against mesothelin positive cancer cells.

We have not performed *ex vivo* studies though multiple groups have shown that T cell exhaustion could get reverted by using bicistronic lentivector CAR that was engineered to produce human monoclonal antibody [35,37]. In addition, preclinical studies have shown that CAR-T cells could induce tumor killing either given exogenously or CAR-T secreting PD-1/PD-L1 blocking antibodies [35,37,49]. However, these studies were limited to xenograft or orthotopic mouse models that do not mimic human tumor microenvironment. We have also seen all three xenograft mouse models ultimately succumb to disease suggesting that a humanized version of mouse models is needed to recapitulate the human tumor microenvironment. In addition, we have not determined whether the CAR-T cells secreting PD-L1 scFv-Fc antibodies are trafficking to the tumor site or circulate out of tumor in mice. Although Rafiq et al. have shown that the CAR-T secreting PD-1 scFv (not scFv-Fc) are localized to the tumor site, however, they could detect the antibodies *in vivo* only for one day [37]. We have not demonstrated the *in vivo* secretion of PD-L1 scFv-Fc antibodies, though; we could detect the antibodies secretion up to 6 days *in vitro* suggesting that scFv-Fc format offers longer half-life over scFv (Figure 2B). Lastly, the anti-tumor efficacy also depends on *in vivo* T cell differentiation. Less differentiated memory cells are more effective in eliminating tumors.

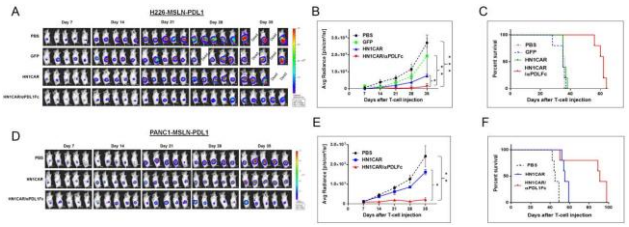


Figure 6: Mesothelin targeted CAR-T cells enhances anti-tumor efficacy in xenograft mouse models. NSG mice were grouped and injected on their right flanks with 1 × 106 H226-MSLN-PDL1 luciferase cells (A) or PANC1-MSLN-PDL1 (D). On day 3rd, the mice were treated with 5 106 indicated CAR-T cells or 100 µL of PBS (5 mice each group). Tumor growth was quantified by bioluminescence imaging (BLI) after 90 seconds of luciferin injection *via* tail vail on day 3 (data not shown) 7, 14, 21 and 35 after CAR-T injections. (B, E) The graph shows the mean of 5 mice for each group. H226-MSLN-PDL1: ***p value<0.000005; **p value<0.004; *p value<0.002; PANC1-MSLN-PDL1: **p value<0.005; *p value<0.01 at the time point on day 35th. Error bars are the standard error. C&F) Kaplan-Meier survival curve showing the percent survival. The p value (H226-MSLN-PDL1: p value<0.0017; PANC1-MSLN-PDL1: p value<0.002) was calculated by Mantel-Cox test. The median survival for HN1CAR/ α PDL1Fc was 62 days while 35-36 days for controls (H226-MSLN-PDL1) and 91 days while 45 days for controls (PANC1-MSLN-PDL1).

Therefore, several approaches have been put forward to maintain the T cell central memory phenotype to booster the anti-tumor affects both in preclinical and clinical settings [50-52]. We have observed that after transduction the CAR-T cells showed higher levels of activating signals and lower expression of exhaustion markers (Figure 3) and a high percentage of the CAR-T cells displayed the central memory phenotype (Cd45RA-CCR7+). However, the CAR-T cells (when infused with a half million) failed to fullly eradicate the inoculated tumor in the mouse models, implying the CAR-T cells are unable to maintain their less-differentiated phenotype and ultimately gets exhausted in the mice (Figure 6).

CONCLUSION

In conclusion, our results demonstrate that second-generation anti-mesothelin HN1CAR-T cells secreting anti-PD-L1 IgG-Fc antibodies enhanced anti-tumor efficacy and increased cytokine

production. This CAR design may diminish CAR-T cell exhaustion and improve treatment for solid tumors. We propose that CAR-T cells targeting tumor antigen as well as secreting antibody to block checkpoint signaling may circumvent the exhaustion of CAR-T cells. We foresee that a combination of CAR and anti-PD1/PD-L1 into a single construct should be among the next steps to explore to achieve higher anti-tumor efficacy in solid tumors.

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CONFLICT OF INTEREST

The authors declare no potential conflicts of interest.

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