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Original Research

Therapeutic effiacy of T cells expressing chimeric antigen receptor derived from a mesothelin-specific scFv in orthotopic human pancreatic cancer animal models

Hyeon Ho Lee[a]; Irene Kim[a]; Un Kyo Kim[a]; Suk San Choi[a]; Tae Yang Kim[a]; Dahea Lee[a]; Youngeun Lee[a]; Jaehee Lee[a]; Jinhui Jo[a]; Young-Tae Lee[a]; Ho Jeong Lee[b]; Sun Jin Kim[b,*]; Jong Seong Ahn[a,*]

[a] GC Cell, Inc, 107, Ihyeon-ro 30beon-gil, Giheung-gu, Yongin, Gyeonggido 16924, Republic of Korea
[b] Platbio, Inc, Platbio, Inc, #1501, Ace Gwanggyo Tower2, 91 Changnyong-daero 256beon-gil, Yeongtong-gu, Suwon, Gyeonggido 16229, Republic of Korea

Abstract

Novel CAR T cells targeting mesothelin (MSLN) expressed on pancreatic cancer cells were developed to overcome the limit of the clinical efficacy of CAR T cell therapy for pancreatic cancer patients. Optimal single-chain variable fragments (scFv) binding to MSLN were selected based on the binding activity and the functional effectiveness of various scFv containing CAR-expressing T cells. Engineered MSLN CAR T cells showed successful anti-tumor activity specific to MSLN expression level. Furthermore, MSLN CAR T cells were evaluated for the anti-cancer efficacy in orthotopic mouse models bearing pancreatic cancer cells, MIA Paca-2, MSLN-overexpressed MIA Paca-2 or endogenously MSLN-expressing AsPC-1. Mice were randomized into control, mock treated, MS501 BBz treated, MS501 28z treated or MS501 28BBz treated group. Mice were monitored by weekly IVIS imaging and tumors were harvested and analyzed by immunohistochemical analyses. MSLN CAR T cells produced the therapeutic effect in orthotopic animal models with complete remission in significant number of mice. Histopathological analysis indicated that CD4+ and CD8+ MSLN CAR T cells infiltrated pancreatic tumor tissue and led to cancer cell eradication. Our results demonstrated the anti-tumor efficacy of MSLN CAR T cell therapy against pancreatic cancer, suggesting its therapeutic potential.

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Keywords: Chimeric antigen receptor, Mesothelin, Single-chain variable fragment, Pancreatic cancer, Orthotopic mouse model, Adoptive immunotherapy

Introduction

The major histocompatibility complex (MHC)-antigen complex of TCRs elicits an immune response that makes tumor cells lose or downregulate MHC expression on their surface as an immune escape mechanism [1–3]. To overcome these mechanisms, adoptive cancer immunotherapy based on chimeric antigen receptor (CAR)-engineered T cells is a promising strategy [4,5]. CAR is an artificial TCR composed of a single-chain variable fragment (scFv) derived from an antigen-binding site that is linked to hinge, transmembrane, and signaling domains [6,7]. CAR T cells directly recognize specific antigens and kill tumor cells independent of MHCs [8–11]. Although five types of CAR T cells approved by the U.S. Food and Drug Administration showed successful therapeutic efficacy against hematological B-cell malignancies, the effectiveness of CAR T cells against different solid cancers remains to be established [12]. Considering the patients with solid tumor multiple metastases, significant portion of CAR T cells systemically administered must be effectively delivered to the lesions (trafficking) and overcome immune-attenuation by the microenvironment (interference by local immune cells).

Mesothelin (MSLN) is a glycoprotein expressed on cell surfaces. It is expressed at low levels in normal tissues but is overexpressed in several cancers, including mesothelioma, lung cancer, pancreatic cancer, triple-negative breast cancer, and ovarian cancer [13–17]. Therefore, it has been explored as a potential target for CAR T cell therapy in MSLN-expressing solid cancers [18]. Based on a phase 1 study, Beatty et al. reported that mRNA-engineered CAR T cells induce an anti-tumor response against

* Corresponding authors.
E-mail addresses: sunkim@platbio.com (S.J. Kim), jongsahn@gccorp.com (J.S. Ahn).
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pancreatic carcinoma metastasis [19]. Haas et al. also reported that patients with malignant pleural mesothelioma (MM), ovarian cancer, and pancreatic ductal adenocarcinoma (PDAC), who were injected with meso-CAR T cells, did not show any improvement of CAR T cell persistence more than 28 days with or without cyclophosphamide pretreatment (NCT02159716). Low persistence and infiltration were observed and attributed to the immune response against the murine scFv [20]. Therefore, subsequent clinical trials have used CAR T cells with a humanized anti-MSLN scFv fused to 4-1BB and CD3ζ signaling domains (NCT03054298). However, unmet need to treat the pancreatic cancer patients justifies the further development of MSLN CAR T cells with more improved efficacy of scFv functions.

In this study, optimal scFvs with a strong binding activity for MSLN were selected and engineered MSLN CAR T cells were tested for cytotoxicity *in vitro,* as well as in an *in vivo* pancreatic orthotopic mouse model, to assess the efficacy of CAR T cell treatment strategies. Especially orthotopic pancreatic cancer models demonstrated therapeutic significance of the biologically effective trafficking and therapeutic efficacy. We suggest MSLN CAR T cells can be the promising potential adoptive immunotherapy for the patients with MSLN expressing PDAC .

Materials and methods

Cell lines and cell culture

MIA PaCa-2, AsPC-1, HeLa, OVCAR-3, and HEK293T cell lines were purchased from the American Type Culture Collection (American Type Culture Collection, Manassas, United States). Mesothelin-overexpressing MIA PaCa-2 (MIA PaCa-2/MSLN) cell line was obtained from the Mogam Biotechnology Institute (Yongin, Korea). Firefly luciferase (Fluc) or MSLN Fluc vectors were designed, cloned, and packaged into lentivirus. Lentiviral transduction was performed in MIA PaCa-2 and AsPC-1 cells. For the cell culture method, please refer to Supplementary Material.

Animals

Six-week old female NOG mice (NOD.Cg-Prkdc^{scid}Il2rg^{tm1Sug}/ShiJic) were purchased from the Central Institute for Experimental Animals (CIEA) (Kawasaki, Japan). The mice were housed and maintained in specific pathogen-free conditions in facilities according to the institutional guidelines for animal care. All animals were cared for and treated humanely according to the guidelines for the welfare and use of animals in cancer research, and experimental procedures were approved by the Animal Care and Use Committee of Woojung Bio (IACUC2019-4-31).

Expression of MSLN scFv

The MSLN-specific IgG was obtained from the Mogam Biotechnology Institute. For full details on MSLN-specific IgG, please refer to the patent [21]. The scFv-encoding genes were amplified using PCR and fused into the pET-22b(+) vector via an in-fusion reaction. The pET-22b(+) vector carrying the scFv-encoding gene was transformed into *E. coli* BL21 strain. Expressed scFv was purified from the culture broth using TALON metal affinity resin (Takara, Otsu, Japan).

Analysis the binding activity of scFv to MSLN

For the analysis of the binding activity of scFv to the recombinant MSLN, the sandwich ELISA was performed using purified scFv coated plates and anti-MSLN biotinylated-antibody (R&D Systems, Minneapolis, MN, USA). For the analysis of scFv binding activity to MSLN on the cells, MSLN-bearing cells were treated with 6x His-tagged anti-MSLN scFv. After subsequent incubation with PE-anti-6x His Tag (BioLegend, San Diego, USA), flow cytometry analysis was performed. For more detail information, please refer to Supplementary Materials.

CAR expressing lentivirus production

MSLN-CAR constructs included the signal peptide for human CD8α, scFv, and the cytoplasmic domain of human CD3 zeta, as well as CD8α or CD28 hinge domains and CD28 or 4-1BB costimulatory domains. All MSLN CAR constructs were incorporated into either the HIV-based lentiviral vector pCDH-MSCV-MCS-EF1α-copGFP or pLVX-EF1α-IRES-Puro using the In-Fusion HD cloning kit (Clontech, Mountainview, CA, USA). EF1α-MSLN CAR vector and pPACKH1 packaging vectors (System Biosciences, California, USA) were transfected on HEK293T cells. Produced lentivirus was quantified by serial dilution and transduction to HEK293T cells. For more detail information, please refer to Supplementary Materials.

CAR T cell production

Human peripheral blood mononuclear cells (PBMCs) were obtained from healthy donors under the approval of Public Institutional Bioethics Committee designated by the MOHW (Approval No. P01-201805-31-001). Enriched CD4 or CD8 positive T cells were activated with Transact (Miltenyi Biotec, Bergisch Gladbach, Germany) and transduced with MSLN CAR expressing lentivirus. MSLN CAR expression and various features of CAR-T cells were analyzed by flow cytometry. For more detail information, please refer to Supplementary Materials.

Cytotoxicity assay

Luciferase labeled MIA PaCa-2, MIA PaCa-2/MSLN, and AsPC-1 as the target cells were co-cultured with CAR T cells according to effector to target ratio. After 24 h, CAR T cytotoxicity was analyzed using the luciferase activity assay. For more detail information, please refer to Supplementary Materials.

Cytokine array

Target cells and CAR T cells were co cultured at a ratio of 1:10 for 24 h and the culture supernatants were collected. Cytokine array was performed with BDTM Cytometric Bead Array Human Th1/Th2/Th17 Cytokine kit (BD Biosciences, San Jose, CA, USA) according to manufacturer's instructions. Data were collected and analyzed by flow cytometry.

Vector copy number (VCN) assay

VCN assay was performed using droplet digital PCR (ddPCR; Bio-rad, Hercules, CA, USA). The mixture for ddPCR was prepared according to the guidelines of Bio-Rad Droplet Digital PCR system. Mixtures were separated into aliquots in the ddPCR 96-well plate and AutoDG ddPCR system (Bio-Rad) was used to form a droplet. PCR was performed under the following thermocycling conditions: 95 °C for 5 min; 40 cycles of 95 °C for 30 s, 60 °C for 1 min; 4 °C for 5 min; 90 °C for 5 min; 4 °C. The results were analyzed using QX200 Droplet Reader (Bio-Rad). VCN value was determined as the ratio of HIV-1 packaging signal copy number per μl to TERT copy number per μl multiplied by a factor '2'. TERT and HIV-1 packaging signal sequence-specific primer information is listed in Supplementary Materials.

Establishment of a pancreatic orthotopic model and CAR T cell treatment

The experimental animal ethics committee of WOOJUNGBIO (Suwon, Korea) approved the animal study protocol (Approval No. WJALC2019-04-31). The luciferase-labeled AsPC-1, MIA PaCa-2, or MIA PaCa-2/MSLN

cells were introduced into the pancreatic lobes of NOG mice. The tumor formation was confirmed using an IVIS® Lumina LT series III *In Vivo* Imaging System (PerkinElmer, Waltham, MA, USA). After confirmation of tumor formation, CAR T cells were injected by intra peritoneal (IP) or intra venous (IV) route. The tumor growth was monitored by weekly IVIS imaging system. For pathological analyses, tumor-bearing mice were sacrificed before showing complete tumor regression. For more detail information, please refer to Supplementary Materials.

Pathological analysis

For histological analysis, paraffin embedded pancreas tissues were sectioned and stained with standard hematoxylin and eosin (H&E). And the immunohistochemistry for MSLN, CD4 and CD8 was performed in paraffin embedded pancreas tissue sections. For the immunofluorescence staining for CD31 and Ki67, the frozen pancreas tissue sections were used. The slides were observed and analyzed using an Olympus BX-53 microscope equipped with a digital camera (Olympus, Melville, NY, USA). For more detail information, please refer to Supplementary Materials.

Statistics

GraphPad Prism software was used for obtaining statistical values. All data were analyzed using one-way ANOVA. *, **, and *** indicates that the *p*-value range is 0.01–0.05, 0.001–0.01, and 0.0001–0.001, respectively.

Results

scFv can bind to MSLN and MSLN-expressing cell lines

To construct CAR, we generated scFvs from MSLN-specific IgG, binding affinities of which were confirmed via surface plasma resonance (SPR) [21], and confirmed scFvs of 28 kDa by western blot analysis (Fig. S1). The designated MS501, MS503, MX175, and C2G4 scFvs bound to recombinant MSLN, while other tested scFvs did not (Fig. 1A). MS501 and MX175 showed slightly higher binding activity than MS503 or C2G4. To verify the binding activity of scFvs to MSLN on the surface of MSLN-expressing cells, we used MSLN-expressing cell lines (MSLN-overexpressing MIA PaCa-2 and endogenous MSLN-expressing OVCAR-3 cells, as well as HeLa cells) and analyzed MSLN expression in these cell lines (Fig. 1B). We assessed the binding activity of the scFvs to MSLN-expressing cell lines using flow cytometry. Our data showed that 76, 69, and 68% of MS501, MS503, and C2G4 scFvs positively bound to MIA PaCa-2/MSLN cells, respectively; whereas MX175 scFv rarely bound to the MSLN-expressing cells. However, the binding of each scFv to OVCAR-3 or HeLa cells was quite different. While MS501 firmly bound to both OVCAR-3 and HeLa cells, MS503 scFv showed very low binding to OVCAR3 cells and C2G4 scFv showed relatively low binding to HeLa cells (Fig. 1C), indicating that each scFv has a different binding activity with the MSLN on tumor cells. Meanwhile, immunohistochemical (IHC) analysis showed that MS501, MS503 and C2G4 scFvs showed significant binding to the pancreatic cancer tissue compared to normal pancreatic tissue (Fig. 1D). Based on these results, MS501, MS503, and C2G4 scFvs were selected for further study.

CAR-Jurkat cells were activated specifically in response to MSLN

We engineered the 2nd generation CAR using the selected MSLN-specific scFvs. The CAR constructs contained the CD28 hinge and transmembrane domains, and CD28 and CD3ζ signaling domains (Fig. 2A). The CARs were effectively transduced into Jurkat cells using a lentiviral vector expressing GFP. The transduction efficiencies of MS501 CAR, MS503 CAR, and C2G4 CAR were 79%, 55%, and 71%, respectively (Fig. 2B). The CAR proteins that were 53 kDa in weight were confirmed by western blot analysis (Fig. 2C). Furthermore, CAR expression was assessed every 2, 3 days by flow cytometry, which confirmed that the expression was maintained for more than 60 days (Fig. 2D). These results indicated successful establishment of CAR-expressing Jurkat cells (CAR Jurkat) and revealed that the levels of CAR in MS501 or C2G4 CAR-Jurkat cells were superior to those in MS503 CAR-Jurkat cells. To determine whether the activation of MSLN CAR-Jurkat cells was specific to MSLN, non-transduced, mock, or CAR-Jurkat cells were co-cultured with MIA PaCa-2 or MIA PaCa-2/MSLN at an E:T ratio of 10:1 for 24 h. The MSLN CAR Jurkat cells expressed very high levels of CD69, which means CAR-Jurkat cells were significantly activated after co-culture with MIA PaCa-2/MSLN compared to those co-cultured with MIA PaCa-2 cells (Fig. 2E). But C2G4 CAR-Jurkat cells were relatively less activated than MS501 or MS503 CAR-Jurkat cells. On the other hand, MS501 CAR-Jurkat cells produced significantly higher amounts of IL-2 than MS503 and C2G4 CAR-Jurkat cells (Fig. 2F). Based on these results, we finally selected MS501 scFv for further study.

Efficacy of MSLN-CAR T cells against MSLN-expressing pancreatic cancer cell lines in vitro

We generated a CAR construct by integrating various components of the selected scFv domain. The 2nd generation CAR, MS501 BBz, MS501 28z with 4-1BB or CD28 costimulatory domains and the 3rd generation CAR, MS501 28BBz with both domains were obtained (Fig. 3A). CAR T cells were generated by transduction of each MSLN CAR lentivirus and analyzed for CAR expression via flow cytometry. CAR expression was the highest in MS501 BBz cells (Fig. 3B). The VCN of MS501 CAR-T cells was approximately 1.6, which was significantly lower than the recommended value of 5 (Fig. 3C). Although there was no significant difference in CD4/CD8 ratios among MS501 BBz, 28z, and 28BBz, CAR-positive T cells had a higher number of CD4+ T cell subsets than CD8+ T cell subsets (Fig. S2 A–D). Overall, CD62L+CD45RO+ central memory (Tcm) cells were observed in the effector/memory subtype, and the expression of CD366 and CD279 exhaustion markers was not significantly different between CAR variants (Fig. S2 E–H). We investigated the specificity and efficacy of MS501 CAR T cells against MSLN-expressing target cells *in vitro*. FLuc-tagged MIA PaCa-2/MSLN and AsPC1, which endogenously express MSLN, were used as CAR T cell targets, and MIA PaCa-2 cells were used as negative controls. Flow cytometry analysis revealed approximately 0, 90, and 65% of MSLN expression in MIA PaCa-2, MIA PaCa-2/MSLN, and AsPC-1 cells, respectively (Fig. 3D). MSLN-CAR T cells showed MSLN-specific and dose-dependent high cytotoxicity in MIA PaCa-2/MSLN cells, while no cytotoxicity was observed in MIA PaCa-2 cells. (Fig. 3E). Cytokines produced by MSLN CAR T cells were measured after co-culture with target cells. Expression of interleukin (IL)-2, interferon-γ (IFN-γ), tumor necrosis factor-α (TNF-α), IL-4, IL-6, and IL-10 in the culture medium was analyzed using a cytokine array. In the MIA PaCa-2/MSLN co-culture, expression of IL-2, IFN-γ, and TNF-α was significantly higher and that of IL-4, IL-6, and IL-10 was slightly increased compared to that in the mock group (Fig. 3F). In MIA PaCa-2 cell co-culture, cytokine production by MSLN-CAR T cells did not increase, which meant that cytokine secretion was dependent on MSLN expression. Also, we found that MSLN-CAR T cells were activated in response to MSLN expression (Fig. S3).These data showed efficient anti-tumor efficacy of MS501 CAR T cells specifically for the target. Therefore, we would like to determine whether a clinical trial on CAR T cell therapy is possible through a preclinical study.

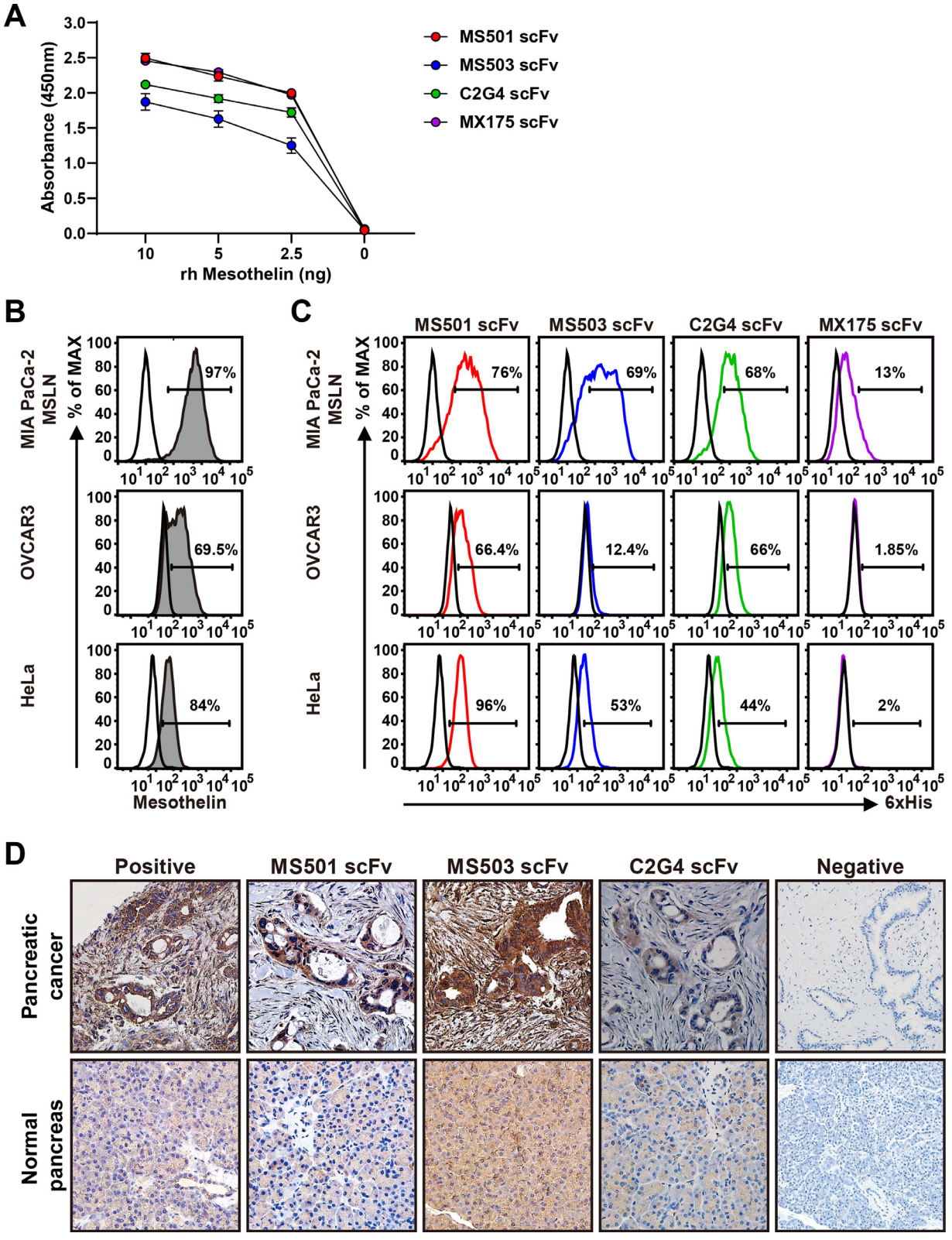

Fig. 1. The binding assays of scFvs **A,** The binding activity of scFvs to the recombinant human mesothelin protein was analyzed by ELISA. **B,** MSLN expressions in MIA PaCa-2/MSLN, OVCAR3 and HeLa cells were determined by flow cytometry analysis. **C,** The binding activity of scFvs to MSLN expressing cell lines was analyzed by the flow cytometry. **D,** Immunohistochemistry (IHC) of human pancreas cancer or normal tissue sections from pancreatic ductal adenocarcinoma (PDAC) patients was performed with scFvs. Magnification, 200x for negative control samples and 400x for other samples.

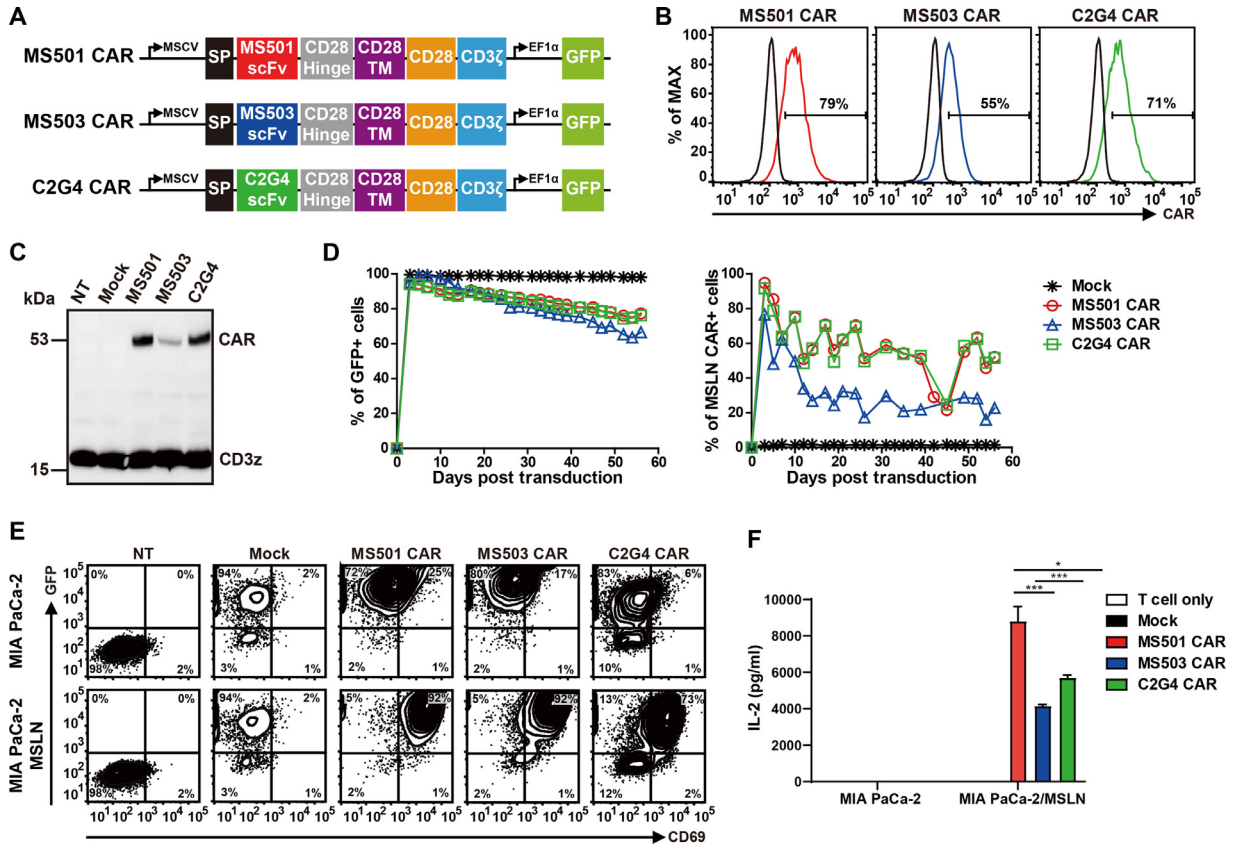

Fig. 2. Using three selected scFvs, the lentivirus constructs were produced and transduced to Jurkat cell. CAR expression and activation of CAR-Jurkat cells were analyzed. **A,** Schematic representation of the MSLN CAR lentivirus constructs. **B,** The MSLN-CAR expression level of lentivirus transduced CAR-Jurkat cells was analyzed by flow cytometry. **C**, The MSLN-CAR proteins expressed on CAR-Jurkat cells were confirmed by western blot analysis. Endogenous CD3ζ, which served as a control, was detected at 16 kDa in all Jurkat cells. **D,** The CAR and GFP expression of CAR-Jurkat cells was observed for 60 days. **E,** Activation of CAR-Jurkat cells was analyzed with CD69 increasment by flow cytometry after co-cultivation with pancreatic cancer cell line MIA PaCa-2 or MIA PaCa-2/MSLN. **F,** IL-2 concentrations in the supernatant collected from the co-culutre conducted in Fig. 2, E weredetected by ELISA. All experiments were analyzed using One-way ANOVA with Bonferroni correction. ***, $p < 0.001$; ****, $p < 0.0001$.

Therapeutic efficacy of MSLN CAR T cells in an orthotopic pancreatic cancer animal model with MSLN-overexpressing or MSLN low/non-expressing human pancreatic cancer cells

We evaluated the anti-tumor efficacy and specificity of MS501 CAR T therapy in a mesothelin-overexpressing pancreatic cancer mouse model. MIA PaCa-2 or MIA PaCa-2/MSLN pancreatic adenocarcinoma cancer cell lines expressing FLuc and GFP were orthotopically injected into the pancreas of immunodeficient NOD.Cg-Prkdcscid Il2rgtm1Sug/Jic (NOG) mice. T or CAR T cells (1.0×10^7) were administered intraperitoneally (i.p.) or intravenously (i.v.) 11 and 25 days after tumor cell administration (Fig. 4A). CAR expression and cytotoxicity of MS501 BBz, 28z, and 28BBz CAR T cells were confirmed before administration (Fig. S4, A and B). Tumor growth was monitored by weekly bioluminescence imaging (BLI).

Mice bearing MSLN low/non-expressing MIA Paca-2 tumors, the vehicle group showed the most aggressive tumor growth and progression of disease. Treatment with mock T cell and MS501 BBZ showed the tendency of slowed tumor growth and disease progression, statistically significant difference was not observed. MS501 28z and MS501 28BBZ produced better therapy without statistical significance (Fig. 4B). However, mice bearing MSLN-overexpressing MIA Paca-2 tumors, all MS501 CAR T cells produced a significant therapy with eradication of tumors regardless of the administration route (Figs. 4C and S4C). Clinical signs and xenogeneic graft-versus-host disease (GvHD)-like symptoms, such as rough fur and alopecia (e.g., fur loss), were commonly observed in animals administered with mock T and CAR T cells (Table S1A and B). Toxicity effects, such as body weight loss, were observed in mice treated with MS501 28BBz CAR T cells, while no weight loss was detected in mice treated with MS501 BBz and 28z CAR T cells (Fig. S4D). Histological analyses were performed with specimens harvested at 8 and 12 weeks after MS501 CAR T cell injection. Hematoxylin and eosin (H&E) staining showed existence of tumor cells correlated with IVIS signals in MSLN-negative tumors (Fig. 4D) and MSLN-positive cancer cells that were treated with MS501 CAR T cells (Fig. 4E). These results confirmed the potent anti-tumor efficacy of MS501 CAR-expressing T cells against MSLN-positive human pancreatic cancer.

We further evaluated the therapeutic efficacy of MS501 CAR T cells using an orthotopic pancreatic cancer mouse model with AsPC-1, human pancreatic adenocarcinoma cell line, which endogenously expresses mesothelin (Fig. 5A). CAR expression and cytotoxicity of MS501 BBz and 28z CAR T cells were confirmed before administration and 1×10^6 CAR-positive T cells were injected intravenously into tumor-bearing mice 11 days after tumor cell injection (Fig. S5A and B). GvHD-like symptoms, including rough fur and alopecia, were recorded (Table S2A and B). Mice treated by MS501 BBz and 28z CAR T cells showed the significant therapeutic efficacy compared to vehicle and mock T cells treated groups (Fig. 5B). In particular, MS501 28z CAR T treatment induced complete remission in five weeks after

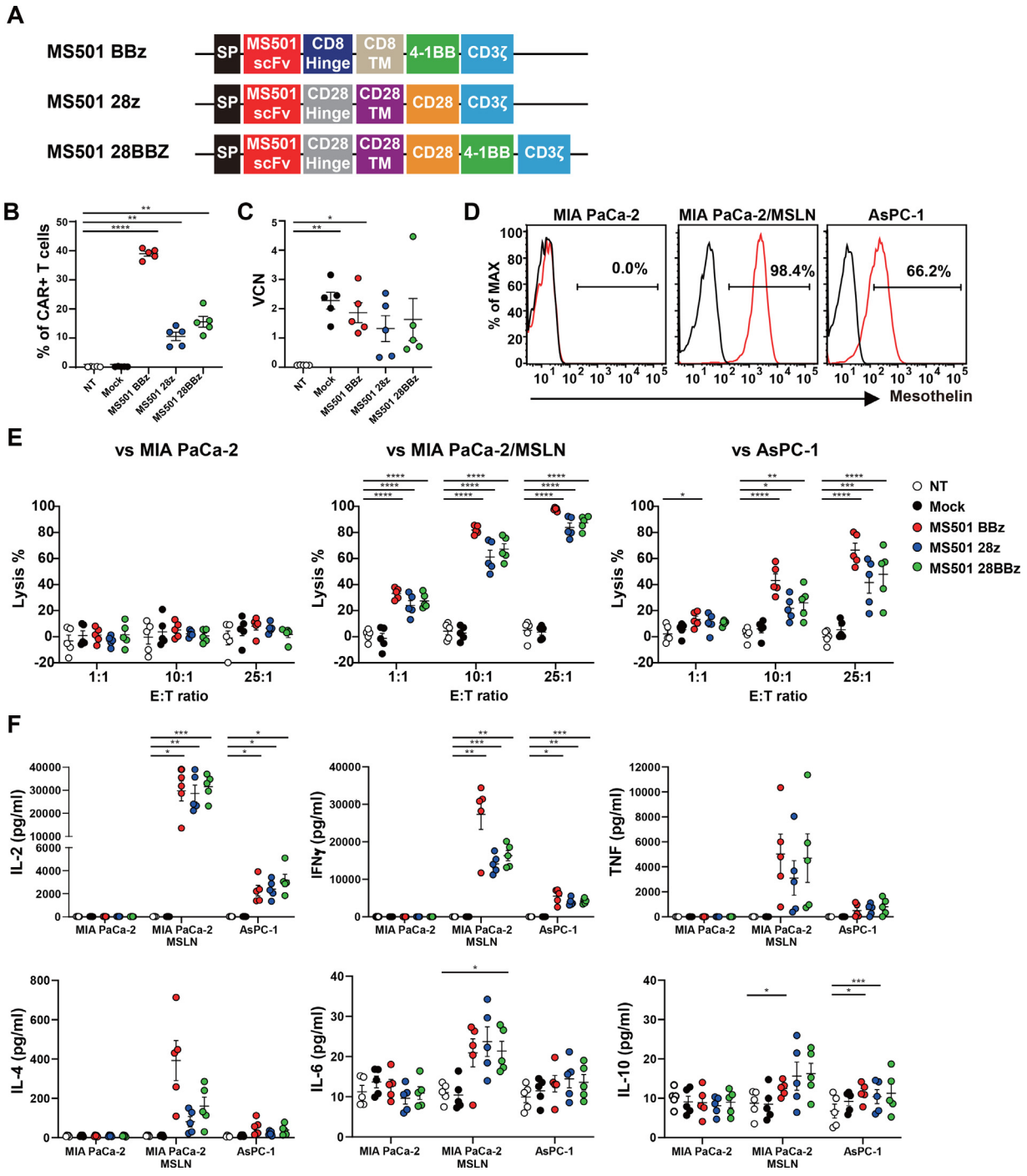

Fig. 3. Cell characteristics, cytotoxicity and cytokine secretion ability of the 2nd or 3rd generation MS501 CAR T cells. **A,** Schematic representation of the MSLN CAR lentivirus constructs including selected scFv MS501. SP=signaling peptide, TM=transmembrane **B,** CAR^+ T cells were observed by flow cytometry analysis at day 14. **C,** Vector copy number of each CAR T cells was verified by ddPCR. **D,** MSLN expression level of Fluc-tagged MIA PaCa-2, MIA PaCa-2/MSLN and AsPC-1 cells was analyzed by flow cytometry. **E,** T cells were co-cultured with each target cells expressing firefly luciferase at the indicated effector to target ratios for 24 h. The percentage of target cell lysis was calculated using luciferase activity. **F,** The secretion of cytokines from T cells or CAR T cells were quantitated by cytokine array using the co-culture supernatant. All experiments were analyzed using One-way ANOVA with Bonferroni correction. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$.

T cell injection, and therapeutic effects were maintained for 12 weeks after administration (Fig. 5B).

We obtained pancreatic tissue from tumor-bearing mice 21 days after T cell injection and performed H&E staining and IHC analysis. Histological analysis showed that MS501 BBz and 28z CAR T cells induced tumor elimination, which correlated with the results of bioluminescence imaging (Fig. 5C). We performed immunofluorescent (IF) staining for CD31 (endothelial cell marker) and Ki67 (proliferation marker) in pancreatic cancer

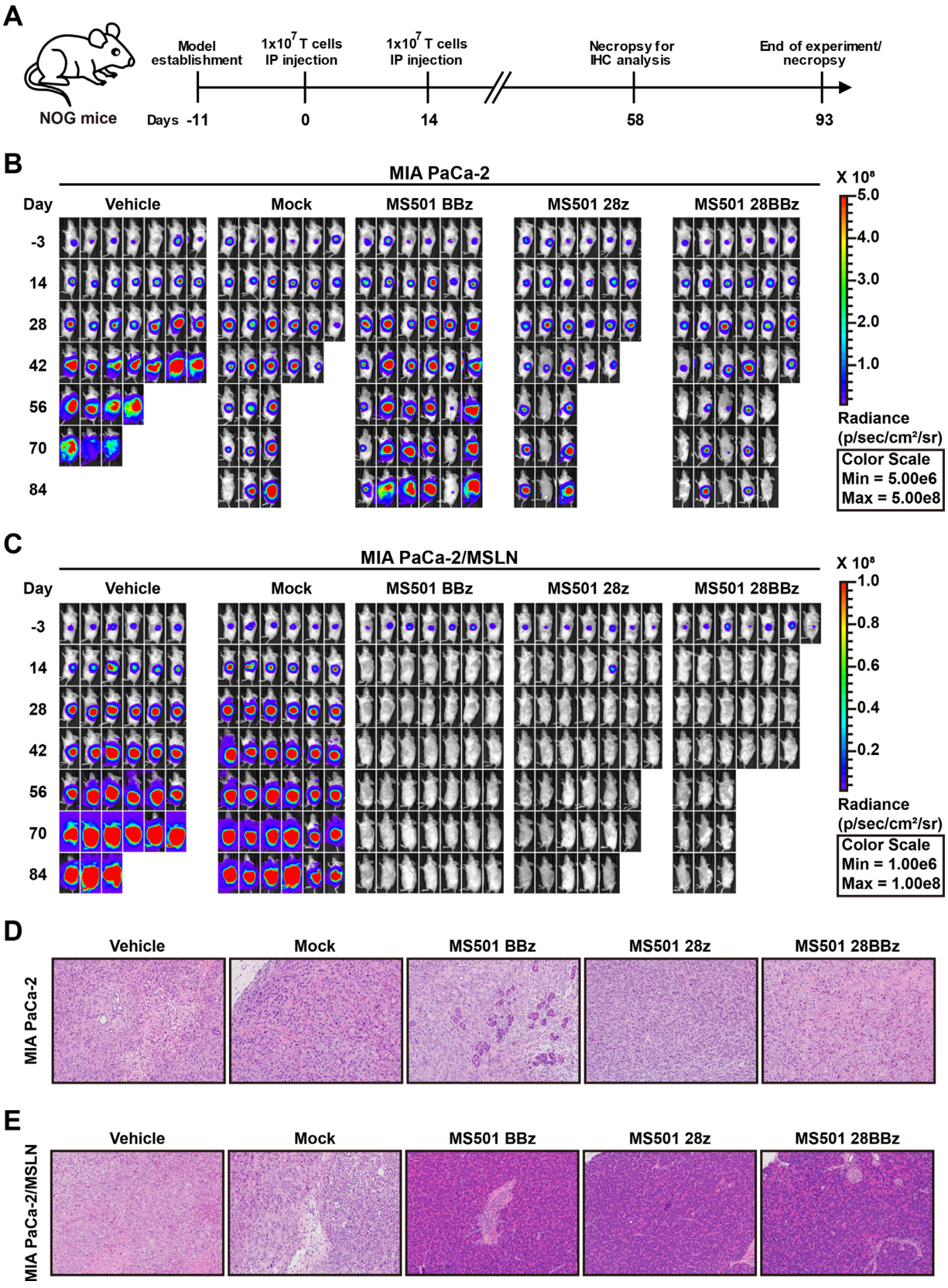

Fig. 4. Antitumor activity of MSLN CAR T cells in orthotopic cancer mouse models of MSLN-overexpressing pancreatic cancer cell line. **A,** Experimental schema of the MIA PaCa-2 or MIA PaCa-2/MSLN orthotopic tumor models. Tumor-bearing mice were injected IP with PBS ($n = 6$-7), Mock T cells ($n = 6$), MS501 BBz T cells ($n = 6$-7), MS501 28z T cells ($n = 6$-7), or MS501 28BBz T cells ($n = 6$-7). Tumor growth of MIA PaCa-2 Fluc (**B**) or MIA PaCa-2/MSLN Fluc (**C**) was monitored by BLI. Histological images using H&E staining of pancreas sections from MIA PaCa-2(**D**) and MIA PaCa-2/MSLN(**E**) pancreatic cancer mouse model. Magnification, 200x.

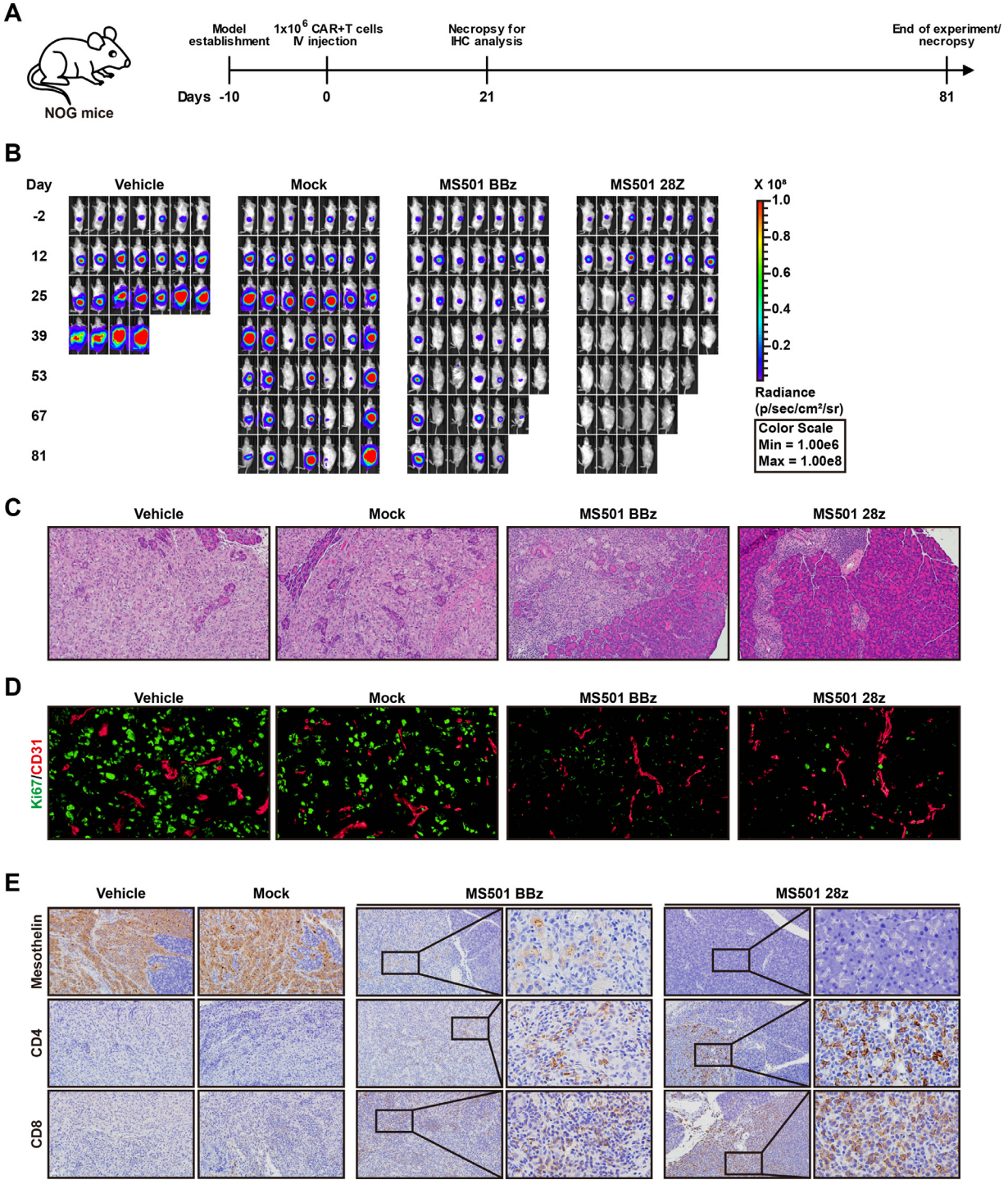

Fig. 5. Therapeutic efficacy of MS501 BBz and 28z T cells in orthotopic mouse models bearing AsPC-1 pancreatic cancer cell line with endogenous mesothelin expression. **A,** Experimental schema of the AsPC-1 orthotopic tumor models. Tumor-bearing mice were injected IV with PBS, Mock T cells, MS501 BBz T cells, or MS501 28z T cells ($n = 7$ per group). **B,** Tumor growth of AsPC-1 Fluc was monitored by BLI. **C,** Histological images using H&E staining of pancreas sections from AsPC-1 pancreatic cancer mouse model. **D,** Representative immunofluorescence staining images of Ki67 and CD31 expression in mouse pancreas tissues. **E,** Representative immunohistochemistry images of mesothelin, CD4, and CD8 expression in pancreas tissue of tumor-bearing mice. Magnification, 200x and 400x.

tissues, which showed fewer proliferative tumor cells in mice treated with MS501 CAR T cells than mock T cells (Fig. 5D). Additionally, we examined the expression of CD4, CD8, and MSLN to visualize tumor-infiltrating T cells and residual MSLN-positive tumor cells in pancreatic cancer tissues using IHC. Tumor-infiltrating CD4 and CD8 lymphocytes were detected in pancreatic tissues of mice treated with MS501 CAR T cells (Fig. 5E). Moreover, we observed the clearance of MSLN-expressing tumors in mice injected with MS501 28z CAR T cells and low MSLN expression in mice treated with MS501 BBz CAR T cells. These observations were consistent with our bioluminescence results, further demonstrating the superior anti-tumor effects of CD28-based MS501 CAR T cells compared with those of 4-1BB-based MS501 CAR T cells, and the optimal signaling domain of MS501 CAR constructs.

The specificity of MS501-mediated cytotoxicity was confirmed using the MIA PaCa-2 cancer model (Fig. S5C). Histological analysis showed no off-target effects of MS501 BBz and 28z CAR T cells (Fig. S5D), and no difference was observed in Ki67, CD4, CD8, and MSLN expressions between mice treated with mock, MS501 BBz, and 28z CAR T cells (Fig. S5E and F). Furthermore, there were no significant changes in body weight in mice treated with PBS, mock T cells, MS501 BBz T cells, or MS501 28z T cells (Fig. S5G). Overall, CAR constructs containing novel scFv specific for MSLN demonstrated high therapeutic efficacy against MSLN-expressing pancreatic cancer.

Discussion

Recently, clinical trials conducted to evaluate the efficacy of CAR T cells for treating solid tumors showed limited outcomes. The clinical efficacy of solid tumor treatment is affected by a complex interplay of several factors [7]. Sufficient number of biologically active CAR T cells should reach the metastatic lesions, that is, targeting and trafficking. Therefore, scFv selection is the most important factor in designing CARs, for scFv binds to its antigen directly [22]. Some studies on primary epithelial cancer cells and myeloid leukemia have suggested that CARs with high-affinity scFvs display stronger anti-tumor activity than those with low-affinity scFvs [23,24]. In addition to the binding activity, the epitope position on the target molecules can impact CAR efficacy [25]. We developed a novel scFv specific for MSLN and demonstrated the anti-tumor effects of CAR T cells and their potential as a cellular therapy for the treatment of pancreatic cancer. Although we did not directly compare the binding site and affinity of different scFvs, we hypothesize that this novel scFv displays different activity and targets a different MSLN epitope. We selected three scFvs from human antibodies that showed appropriate binding activity to target antigens upon evaluation using ELISA for antigen molecule, flow cytometric analysis for antigen on cells, and immunohistochemistry for antigen on tissues.

Costimulatory signaling is essential for improved CAR T cell therapy efficiency. It enhances the proliferation, persistence, safety, and function of CAR T cells. The most widely used costimulatory domains in addition to the CD3ζ domain is CD28 or 4-1BB [26]. Expansion of CD28-based CAR T cells peaks within 7–17 days after injection, but rarely lasts for more than 60 days [27,28]. This expansion is also associated with fast T cell proliferation and facilitates T cell differentiation into cells with effector memory phenotypes [29]. The CAR expression of 4-1BB-based CAR T cell was also shown to peak 7–14 days after injection and last for several months, with 4-1BB-based CAR T cells presenting low pharmacokinetics, central memory differentiation, and long-lasting response [30,31]. These contrasting results suggest that the CAR T efficacy is determined by a complex interplay among various factors. Therefore, we established a strategy to select ideal scFvs using a designated signaling domain CAR. And then selected scFvs were included in various CAR structures, including combinations of costimulatory domains. By evaluating various CAR T cell constructs, we selected candidate CAR T cells for a clinical study.

We compared the function of three different types of anti-MSLN scFvs by transfecting Jurkat cells with MSLN CARs that included the CD28 costimulatory domain. Jurkat cells are immortal human leukemic T cells widely used in *in vitro* assays, including the identification of T cell activation and signaling mechanisms [32,33]. Co-cultures of MSLN-expressing tumor cells and MSLN CAR Jurkat cells showed different levels of CD69 and IL-2 expression depending on the scFv. Based on these results, scFv MS501 was selected for generating the 2nd generation CAR T cells with CD28 or 4-1BB costimulatory domains, while the 3rd generation CAR T cells included both domains. The *in vitro* anti-tumor efficacy of these three types of CAR T cells against PDAC cell lines was comparable. Although MS501 BBz MSLN-CAR T showed slightly higher cytotoxicity, it was not significantly different from that of other MS501 CAR T generations. We observed that the major population of MS501 CAR T cells was CD62L+CD45RO+ central memory T (Tcm) cells, which is known as the long-term persistent phenotype correlated with robust anti-tumor activity [34,35].

We evaluated the anti-tumor efficacy of the three CAR T cells *in vivo*. We used an orthotopic mouse model that was generated using the human pancreatic cancer cells MIA PaCa-2, which did not express detectable MSLN, MIA PaCa-2/MSLN overexpressing cell, and AsPC-1 cells which endogenously express MSLN. We found that MSLN-CAR T cells targeted and killed tumors with detectable MSLN expression. We could also confirm effective trafficking of MSLN CAR T cells, which showed biologically significant anti-tumor efficacy by both of intravenous and intraperitoneal administration. When MSLN-overexpressing tumor-bearing mice were treated with a high dose of CAR T cells, complete remission of pancreatic cancer was observed in all groups treated with MSLN-CAR T MS501 BBz, 28z, and 28BBz. These results demonstrated that the MSLN-CAR T cells had higher anti-tumor efficacy than that of previously reported MSLN-targeting CAR T cells, which showed anti-tumor effects on a variety of solid tumors, including ovarian, gastric, and pancreatic cancer [(36–39]. While the 3rd generation CAR T MS501 28BBz showed off-target toxicity, as well as high tumor-killing efficacy, the 2nd generation CAR T MS501 BBz and 28z had comparable or slightly higher tumor-killing efficacy and very low off-target toxicity. Thus, we concluded the 2nd generation CAR T cells had sufficient anti-tumor efficacy with a lesser degree of gene manipulation compared with the 3rd generation CAR T cells.

To reflect the biological heterogeneity of cancer cells, we adopted AsPC-1 cells, which express endogenous MSLN at a variety levels compared to the MIA PaCa-2/MSLN cells, which relatively express homogeneously very high level of MSLN. IVIS signals were lost too fast in MIA PaCa-2/MSLN orthotopic mouse model experiments (Fig. 4C), we injected fewer CAR T cells into AsPC-1 mice model and we could confirm dose-dependency that the IVIS signals slowly decreased and were not detected approximately five weeks after CAR T injection. This information will be very helpful for the design of the clinical protocol. We observed tumor-infiltrating CAR T cells and that CAR T cells intensively infiltrated MSLN-positive tumor tissues and very few infiltrating cells were detected in mock CAR T treatment group. However even though not significant, it must be noted that mock CAR T cells also showed anti-tumor effects against MSLN high or low/non expressing cells likewise we observed the therapeutic efficacy of non-targeting CAR T cells against the solid tumors in current clinical trials. These results demonstrated that the MS501 CAR T cells had efficient MSLN-expressing, tumor targeting, and penetration ability. Also, it confirmed that CAR T cell infiltration was an important factor for the therapeutic efficacy of CAR T therapy, as previously reported [40–43].

In conclusion, we found that MS501 CAR T cells had several positive features that contributed to the success of the CAR T therapy against solid tumors, including sufficient anti-tumor efficacy for low antigen-expressing tumors, stable CAR expression, low VCN, and minimal off-target toxicity. Therefore, we suggest that MSLN-targeting MS501 CAR T therapy can be a promising therapeutic option for patients with pancreatic cancer. Further

development of assays for biological activities of MSLN CAR T cells, the MSLN expression threshold of tumors and optimal regimen are needed to determine the criteria of inclusion and exclusion criteria of MSLN CAR T cells and patient selection.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

CRediT authorship contribution statement

Hyeon Ho Lee: Methodology, Formal analysis, Investigation, Visualization, Writing – original draft. **Irene Kim:** Methodology, Formal analysis, Investigation, Visualization, Writing – original draft. **Un Kyo Kim:** Methodology, Formal analysis, Investigation. **Suk San Choi:** Methodology, Formal analysis, Investigation, Visualization, Writing – original draft. **Tae Yang Kim:** Methodology, Formal analysis, Investigation. **Dahea Lee:** Methodology, Formal analysis, Investigation. **Youngeun Lee:** Methodology, Formal analysis, Investigation, Writing – original draft. **Jaehee Lee:** Methodology, Formal analysis, Investigation. **Jinhui Jo:** Methodology, Formal analysis, Investigation. **Young-Tae Lee:** Conceptualization, Writing – original draft. **Ho Jeong Lee:** Methodology, Formal analysis, Investigation, Visualization. **Sun Jin Kim:** Conceptualization, Writing – review & editing, Supervision. **Jong Seong Ahn:** Conceptualization, Writing – review & editing, Supervision.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.neo.2021.12.005.

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