Development of Anti-Human Mesothelin-Targeted Chimeric Antigen Receptor Messenger RNA–Transfected Peripheral Blood Lymphocytes for Ovarian Cancer Therapy

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CD19-targeted chimeric antigen receptor (CAR) engineered T/natural killer (NK)-cell therapies can result in durable clinical responses in B-cell malignancies. However, CAR-based immunotherapies have been much less successful in solid cancers, in part due to “on-target off-tumor” toxicity related to expression of target tumor antigens on normal tissue. Based on preliminary observations of safety and clinical activity in proof-of-concept clinical trials, tumor antigen-specific messenger RNA (mRNA) CAR transfection into selected, activated, and expanded T/NK cells may permit prospective control of “on-target off-tumor” toxicity. To develop a commercial product for solid tumors, mesothelin was selected as an antigen target based on its association with poor prognosis and overexpression in multiple solid cancers. It was hypothesized that selecting, activating, and expanding cells *ex vivo* prior to mRNA CAR transfection would not be necessary, thus simplifying the complexity and cost of manufacturing. Now, the development of anti-human mesothelin mRNA CAR transfected peripheral blood lymphocytes (CARMA-hMeso) is reported, demonstrating the manufacture and cryopreservation of multiple cell aliquots for repeat administrations from a single human leukapheresis. A rapid, automated, closed system for cGMP-compliant transfection of mRNA CAR in up to 20×10^9 peripheral blood lymphocytes was developed. Here we show that CARMA-hMeso cells recognize and lyse tumor cells in a mesothelin-specific manner. Expression of CAR was detectable over approximately 7 days *in vitro*, with a progressive decline of CAR expression that appears to correlate with *in vitro* cell expansion. In a murine ovarian cancer model, a single intraperitoneal injection of CARMA-hMeso resulted in the dose-dependent inhibition of tumor growth and improved survival of mice. Furthermore, repeat weekly intraperitoneal administrations of the optimal CARMA-hMeso dose further prolonged disease control and survival. No significant off-target toxicities were observed. These data support further investigation of CARMA-hMeso as a potential treatment for ovarian cancer and other mesothelin-expressing cancers.

Keywords: cell therapy, immunotherapy, chimeric antigen receptor (CAR), mesothelin, ovarian cancer

INTRODUCTION

OVARIAN CANCER IS the fifth leading cause of cancer death in women, with a 5-year survival rate of only 25%. This dismal prognosis reflects the limitations of current management strategies for ovarian cancer. Because there are no effective screening tests,

ovarian cancer patients typically have advanced disease that has spread throughout the peritoneum at diagnosis. The standard of care for ovarian cancer remains upfront surgical debulking followed by adjuvant treatment using platinum-based chemotherapy. Although patients are often initially rendered tumor free,^{1–3} all patients ultimately relapse

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with platinum-resistant disease.⁴ Responses to single agent chemotherapy for recurrent platinum-resistant disease are low, and overall survival for these patients is only about 1 year. New approaches to therapy are urgently needed.

Multiple studies in epithelial ovarian cancers^{4–7} have shown that the presence of tumor infiltrating lymphocytes (TILs) correlates with increased patient survival, suggesting that immune-based therapies could impact outcomes in this disease. The major immunotherapy platforms evaluated for ovarian cancer to date include vaccines, immune checkpoint antagonists, and adoptive T-cell therapies.^{8–10} One type of adoptive T-cell therapy utilizes chimeric antigen receptor (CAR) T cells—T cells engineered to express CARs—that specifically target tumor associated antigens (TAA).¹¹ CAR T cells provide both the antigen specificity of a monoclonal antibody and the antitumor activity of cytotoxic T cells in one product.

Multiple clinical trials have demonstrated that the adoptive transfer of CAR T cells specific for the CD19 cell surface antigen can result in significant disease regressions in leukemia patients.¹² An essential feature of these products is the long-term expression of CAR by the viral transduction of highly purified, activated, and expanded T cells. This requires a complex, inefficient, and costly manufacturing process that includes approximately 1–2 weeks of *ex vivo* T-cell expansion.¹³ Furthermore, the stable expression of CAR may result in significant “on-target off-tumor” toxicity, particularly in solid tumors that express the antigen target. In the case of CD19+ malignancies, the deletion of normal CD19+ B cells can be clinically managed as needed by long-term intravenous (i.v.) immunoglobulin therapy to restore immunoglobulins in treated patients.¹⁴ Additional significant toxicities associated with CAR T-cell therapy include tumor lysis syndrome, cytokine release syndrome (CRS), and neurotoxicities related both to CRS and to the conditioning regimen used.^{15,16} These difficulties, coupled with additional challenges posed by the inefficient homing and migration of CAR T cells to the solid tumor microenvironment and its immunosuppressive milieu, have largely restricted the development of CAR T-cell immunotherapies to CD19-expressing hematologic malignancies.^{17,18}

Expanded T cells transfected with messenger RNA (mRNA) encoding CAR (CAR-T) have the potential to mitigate “on-target off-tumor” toxicities. Initial preclinical^{19–21} and human proof-of-concept studies in metastatic mesothelioma and pancreatic cancer^{22,23} support the safety and anti-

tumor activity of limited dosing with mRNA CAR-transfected and expanded T cells. In contrast to transduced CD19-CAR T cells, which are “living drugs” with the potential to expand *in vivo* and establish a memory response, mRNA-transfected CAR T cells are transient and do not persist, and thus would require multiple infusions for antitumor activity.²² The short half-life of mRNA-transfected T cells may provide a prospective way to control potential “on-target off-tumor” toxicity by simply withholding further infusions of the cell product.

The possible advantages of mRNA CAR-transfected immunotherapies led to the further development of a streamlined, cGMP-compliant strategy for manufacturing peripheral blood mononuclear cells (PBMCs) transfected with antigen-specific mRNA CAR without prior expansion or activation, CARMA, as a potential platform for treating multiple cancers. Mesothelin was selected as the first target tumor antigen²⁴ to develop for several reasons. First, it is widely overexpressed by multiple solid cancers.^{25–28} Second, mesothelin has shown promise as a target for antibody-directed therapies.^{29,30} Third, mesothelin-specific CAR T cells have shown evidence of safety and clinical activity in mesothelioma and pancreatic cancer in an early clinical trial.²³ However, the product in this proof-of-principle trial contained mouse mesothelin sequences, which could result in neutralizing antibodies to the cellular product. Thus, a CARMA product was developed utilizing human mesothelin sequences to decrease the potential for immunogenicity and allow for repeated administration in humans.

This study characterizes anti-human-mesothelin CAR mRNA transfected directly into unselected, unexpanded PBMCs that have not been activated (CARMA-hMeso) first *in vitro*, and then *in vivo* using a preclinical murine model of ovarian cancer. CARMA-hMeso demonstrated high viability and CAR-expression *in vitro*, with the ability to recognize and kill mesothelin-expressing tumor cells at low effector-to-target (E:T) ratios. There were no detectable differences in the killing of tumor cells when anti-human-mesothelin CAR mRNA was transfected into PBMCs or adherence-depleted PBMCs (peripheral blood lymphocytes [PBLs]), whether fresh or cryopreserved, or whether activated or not. In nude mice challenged with murine ovarian cancer cells transfected with human mesothelin, it was demonstrated that a single intraperitoneal (i.p.) injection of human CARMA-hMeso cells resulted in a dose-dependent inhibition of tumor growth, with longer survival compared to untreated control and CARMA-CD19 (irrelevant CAR)-treated groups. Weekly administration of

CARMA-hMeso for 3 or 6 weeks improved disease control, resulting in longer survival compared to a single administration of CARMA-hMeso. No overt toxicities were noted following a single i.p. administration of up to 1×10^8 CARMA-hMeso cells, a single i.v. administration of up to 4×10^7 CARMA-hMeso cells, or a total of six weekly i.p. administrations of 5×10^7 CARMA-hMeso cells. Together, these results support further evaluation of the safety and efficacy of CARMA-hMeso cells for the treatment of ovarian cancer and other mesothelin-expressing solid tumors in humans.

MATERIALS AND METHODS

Gene constructs used for electroporation

The CAR is schematically shown (Fig. 1A) and described below. All CAR molecules were cloned into the pcDNA3.1+ plasmid backbone (Thermo Fisher Scientific) and contained a CD8 α signal peptide, defined single chain variable fragment (scFv), CD8 hinge/transmembrane domain (TM), and 4-1BB and CD3 ζ intracellular domains; H and M designators indicate human and murine scFv, respectively:

α CD19CAR-M:	Signal peptide	scFv- α CD19	TM	4-1BB	CD3 ζ
α MesoCAR-M:	Signal peptide	scFv- α Meso-M	TM	4-1BB	CD3 ζ
α MesoCAR-H:	Signal peptide	scFv- α Meso-H	TM	4-1BB	CD3 ζ

Figure 1. The structure of three different antigen-specific mRNA chimeric antigen receptors (CARMA): two specific for mesothelin with human single chain variable fragment (scFv; CARMA- α hMeso-H) or murine scFv (CARMA- α hMeso-M), and one specific for CD19 with murine scFv (CARMA- α CD19-M). Isolated peripheral blood mononuclear cells (PBMCs) that have not been further selected, expanded, or activated are electroporated with CARMA using the cGMP-compliant MaxCyte GT[®] System[™], and then either cryopreserved or used fresh in experiments.

The sequences of the scFv components used in the construction of the CAR cassettes were obtained from published sources. The Meso-H scFv sequence was obtained from the 2007 *Cancer Letters* publication,³¹ the Meso-M scFv sequence was obtained from its U.S. Patent, and the α CD19-scFv sequence was obtained from FMC63-28z NCBI. The sequence of the final construct was confirmed by sequencing (GenScript).

mRNA synthesis

mRNAs encoding different CARs were produced by *in vitro* transcription using the XbaI-linearized DNA template. mRNA was synthesized in accordance with the manufacturer's protocol provided with the mMachine mMessage T7 Ultra Kit. mRNA was confirmed by 1% agarose gel, as recommended by the kit. The mRNA was isolated from the gel, formulated in double-distilled water with 0.1 mM of EDTA, aliquoted, and cryopreserved at -80°C .

Cells and cell lines

Healthy volunteer donor leukapaks obtained from 10–15 L blood volume processing were purchased from Key Biologics, and shipped overnight on wet ice packs. Density gradient centrifugation using Ficoll Paque Plus (cat# 17-1440-03; Thermo Fisher Scientific) was performed to isolate the PBMCs. PBLs were obtained by collecting cells in suspension after 2 h culture of isolated PBMCs in five-tier cell factory (cat# 05-539-098; Thermo Fisher Scientific).

The K562-Meso cell line was developed by transfecting wild-type K562 cells with a plasmid encoding human mesothelin and the G418 resistance gene (cat# RC202532; Origene). K562 cells expressing human mesothelin were then selected by treatment with G418. Single cell clones were further selected by limiting dilution assay. The selected clones were expanded and assayed for expression of human mesothelin using purified rat monoclonal IgG2A α -human mesothelin as primary (cat# MAB32625; BD Biosciences) and α -rat IgG-PE as secondary antibodies (cat# F0105B; R&D Systems, Inc.). One clonal line was selected and designated as K562-Meso.

OP-1 cells were a gift from Dr. Dario Campana.³² The cells were used to measure the target cell killing efficiency of α CD19CAR-M-transfected PBLs for the nonspecific control used in the animal studies. Wild-type and human mesothelin-engineered murine ovarian cancer cell line Defb29 Vegf-luc/Hmeso cells, all with GFP integration, were developed, as previously described.³³

Transfection of mRNA CAR

PBLs or PBMCs were washed in MaxCyte's Electroporation (EP) Buffer (cat# B201-100) at $>20\times$ volume relative to the volume of the cell pellet. Washed cells were resuspended in MaxCyte EP buffer at 5×10^8 cells/mL and kept on ice. mRNA was added to this cell suspension to a final concentration of 200 $\mu\text{g/mL}$. The resulting cell and mRNA mixture was immediately transfected using the MaxCyte GT[®] System[™], a cGMP-compliant electroporation system. Post transfection, the cells were allowed to recover for 20 min at 37°C and were subsequently incubated in RPMI-1640 culture medium supplemented with 10% fetal bovine serum (FBS) and 2 mM of L-glutamine for an additional 30 min. The resulting cells were then either cryopreserved or used immediately for functional studies.

Cryopreservation and thawing of cells

Cells were cryopreserved in CryoStor[®] freeze media (Biolife Solutions) at a concentration of $1\text{--}4\times 10^8$ cells/mL, stored at -80°C overnight, and transferred to long-term storage in the vapor phase of a liquid nitrogen freezer. Cryopreserved cells were quickly thawed in a 37°C water bath. Immediately post thaw, $1\times$ Dulbecco's phosphate-buffered saline (PBS) supplemented with 0.1% human serum albumin was added to the cells: (1) initially dropwise for the half volume of thawed cells, (2) subsequently dropwise for $1\times$ volume of the thawed cells, and (3) then to a total of $10\times$ volume of the thawed cells. Resulting cells were centrifuged and resuspended in either PBS for direct injection in the animal studies or in culture medium of RPMI-1640 supplemented with 10% FBS and antibiotics if used for *in vitro* studies.

Detection of CAR expression

The detection of CAR expression was performed using biotinylated rabbit anti-human IgG (H + L) Fab (code# 309-065-082; Jackson ImmunoResearch Laboratories, Inc.) as the primary antibody for α MesoCAR-H and biotinylated rabbit anti-murine IgG (H + L) Fab (code# 115-605-072; Jackson ImmunoResearch Laboratories, Inc.) as the primary antibody for α MesoCAR-M or α CD19CAR-M. After the cells were washed, they were stained with a secondary streptavidin antibody conjugated to PerCP (BD Pharmingen), and fluorescence-activated cell sorting (FACS) analysis was performed to detect CAR expression. In some studies, CD3-FITC and CD8-PE (BD Pharmingen) were used in addition to the secondary antibody for detecting the CAR expression.

In vitro cell killing assay

The target cell killing assay has been previously described.³⁴ Briefly, to evaluate killing of the K562-Meso target cells, the target cells were first loaded with calcein-AM (cat# C1430; Molecular Probe). After washing three times, 100 μL target cells at $10^5/\text{mL}$ (10^4 cells per well) were mixed with 100 μL human effector PBL at concentrations to reach the desired E:T ratios in duplicate culture wells of a U-bottom 96-well plate. After culturing the E:T cell mixtures overnight at 37°C in 5% CO₂, cells were assayed for cytotoxicity using 20 s of time-controlled FACS acquisition and analysis. To evaluate killing of the target Defb29 Vegf-luc/Hmeso human ovarian cancer cells, the cells were plated in a flat-bottom 96-well plate 1 day prior the addition of human PBL effector cells. Cells from three wells were trypsinized for counting to determine the cell number per well just before the addition of effector cells. Effector cells (control or hMeso-transfected Defb29 Vegf-luc) at the desired E:T ratio were placed in duplicate wells. Following overnight culture, the mixture was rinsed with PBS and detached from the wells by addition of 100 μL trypsin to dissociate the viable target cells from the wells. Following detachment, 100 μL culture medium was added to neutralize the trypsin, and cells were assayed for cytotoxicity using 20 s of time-controlled FACS acquisition and analysis. The ratio of decrease in viable number of target cells in effector cultures versus control cultures was used to calculate the cytotoxicity using the equation: $\% \text{Lysis} = (N_{\text{NonEffector}} - N_{\text{Mix}}) \times 100 / N_{\text{NonEffector}}$, where $N_{\text{NonEffector}}$ is the viable target cell number without effector and N_{Mix} is the viable target cell number with effector.

Biodistribution study

The biodistribution of CARMA-hMeso cells was evaluated following i.p. administration in NSG mice. NSG mice were purchased from Jackson Laboratories. On day 0 (D0), NSG mice ($N=5$) received a single i.p. injection of 5×10^7 cryopreserved control PBLs or CARMA-hMeso cells. The biodistribution of control and CARMA-hMeso cells in the peripheral blood was evaluated by PE anti-human CD3 (BD Pharmingen) and FITC anti-human CD45 (BD Pharmingen) by FACS analysis on D1, D2, D4, and D7. On D8, the mice were euthanized, and the biodistributions of control and CARMA-hMeso cells in peritoneal washings and the spleen were evaluated by FACS analysis.

Animal tumor model studies

A series of four animal studies was performed, using transfected and cryopreserved CARMA-hMeso cells from different donors.

Tumor cell line. The Defb29 Vegf-luc/Hmeso ovarian cancer cell line was generated by transducing human mesothelin and luciferase into Defb29 Vegf-luc mouse ovarian tumor cells and was maintained in RPMI 1640 supplemented with 10% fetal calf serum and 100 IU/mL of penicillin, as previously described.³³ The expression of mesothelin on Defb29 Vegf-luc/Hmeso cells (ID8 H-Meso) and Defb29 wild-type control cells (ID8-WT) was confirmed by using primary rat anti-human mesothelin antibody (cat# MAB32625R&D Systems) with secondary anti-rat IgG-PE (cat# F0105B; R&D Systems). FACS (FACSCalibur; BD Biosciences) analysis was performed to detect the mesothelin expression.

Adoptive cell therapy experiments. Nude mice were challenged with 3×10^5 or 1×10^4 Defb29 Vegf-luc/Hmeso cells via i.p. injection on D0. On D3, the baseline luminescence intensity from the mouse abdomen was imaged using a Xenogen IVIS 200 (*In Vivo* Imaging System). The CARMA-hMeso cells were thawed and given as a single injection of a range of cell doses on D4 by i.p. or retro-orbital (r.o.) administration. Mice were then imaged weekly. In some experiments, mice were treated with CARMA-hMeso cells i.p. or r.o. weekly for 3 or 6 weeks. The survival data were recorded. The dose of tumors, the schedule of imaging, and the day of the treatment were based on previous publications.^{33,35}

All animal experiments were approved by the Johns Hopkins University Animal Care and Use Committee. Athymic nude mice were purchased from the Jackson Laboratory. Data are presented as mean $\pm$ standard deviation.

RESULTS

Comparison of CARMA-mMeso- and CARMA-hMeso-mediated tumor cell lysis activity *in vitro*

CARMA-mMeso selected, activated, and expanded T cells have been reported to have antitumor activity.³⁶ CARMA-hMeso is a cellular immunotherapy product that utilizes CAR mRNA encoding a human origin scFv instead of a mouse origin scFv (known as CARMA-mMeso) as part of the CAR, which may have the advantage of reduced immunogenicity. The manufacturing process for CARMA-hMeso was fine-tuned to reduce time-consuming *ex vivo* manipulation. To confirm that CARMA-hMeso and CARMA-mMeso have similar antitumor activity against mesothelin-expressing target cells, their ability to kill human K562 cells expressing human

mesothelin was evaluated in an *in vitro* cytotoxicity assay. Human mesothelin expression on K562-hMeso target cells was confirmed by flow cytometry (data not shown). The CAR T-cell viability was similar following transfection with either mouse mesothelin CAR mRNA or human mesothelin CAR mRNA (Supplementary Fig. S1A; Supplementary Data are available online at www.liebertpub.com/hum). Both CARMA-hMeso and CARMA-mMeso cells show *in vitro* cytotoxicity toward K562-Meso target cells (Supplementary Fig. S1B). This functional activity confirms that CARMA-hMeso gives comparable antitumor activity to that previously reported for CARMA-mMeso cells.

CARMA-hMeso cells induce target-specific killing of human mesothelin expressing mouse ovarian tumor cells *in vitro*

The ability of CARMA-hMeso cells to recognize and kill Defb29 Vegf-luc/Hmeso tumor cells was evaluated *in vitro*. The expression of human mesothelin on Defb29 Vegf-luc/Hmeso cells was confirmed via FACS analysis (data not shown). When Defb29 Vegf-luc/Hmeso cells were co-cultured in the absence of effector cells or in the presence of control PBL effector cells (PBLs that were not transfected with hMeso targeted CAR mRNA), the Defb29 Vegf-luc/Hmeso cells continued to grow in monolayers (Supplementary Fig. S2A). However, when cultured in the presence of CARMA-hMeso cells, the Defb29 Vegf-luc/Hmeso cells were lysed, and the only remaining cells in the wells were CARMA-hMeso cells with small cell aggregates consistent with an activated T-cell morphology. The antigen-specific killing of Defb29 Vegf-luc/Hmeso cells, but not Defb29Vegf-luc cells, by CARMA-hMeso was incrementally increased across a range of E:T ratios (Supplementary Fig. S2B). No difference was found in the extent of cell lysis with directly transfected PBL or T cells that were expanded and activated by CD3/CD28 beads and then transfected (Supplementary Fig. S3).

Product characterization of transfected resting PBLs

For the clinical product, one manufacturing run using a single patient leukapheresis generates multiple cryopreserved aliquots of cellular product to enable repeat administrations. Therefore, the effect of cryopreservation was tested on CARMA-hMeso products by comparing the same CARMA product, one fresh (without cryopreservation) and the other cryopreserved. No significant differences in viability, transfection efficiency, or the phenotype of

cell populations of the product were observed (Supplementary Fig. S4A). Furthermore, fresh and cryopreserved CARMA-hMeso cells showed comparable ability to kill mesothelin-expressing tumor cells *in vitro* (Supplementary Fig. S4B).

Biodistribution of CARMA-hMeso cells following i.p. injection in NSG mice

The biodistribution of CARMA-hMeso cells was evaluated following i.p. administration in NSG mice (Fig. 2). NSG mice were utilized for the biodistribution study because nude mice retain an active innate immune system that could result in increased clearance of CARMA-hMeso cells. Following a single i.p. injection, CARMA-hMeso cells were observed in the peripheral blood beginning on D2 (<1%) and increased through D7 (approximately 3%), the last time point evaluated. On D8, approximately 80% of the CARMA-hMeso cells were detectable in peritoneal washings, and approximately 4% were detectable in the spleen. *In vitro* data show that CAR-hMeso expression is not detected by D7. So, the additional persistence of the autologous PBLs that have lost the CAR-hMeso expression is not likely to serve any killing function. The biodistribution for control PBL and CARMA-hMeso cells was similar, confirming the MaxCyte transfection process does not negatively impact cell trafficking in NSG mice.

Intraperitoneal injection of CARMA-hMeso cells delays ovarian tumor growth *in vivo*

To evaluate the activity of CARMA-hMeso cells *in vivo*, nude mice were challenged i.p. with 3×10^5 Defb29 Vegf-luc/Hmeso cells. On D3 before treatment with CARMA-hMeso cells, there was no difference in tumor burden as reflected by the luminescence intensity among groups (Fig. 3). In mice treated with nonspecific CAR T cells (CD19 CAR) at doses of 1×10^7 and 1×10^8 cells, there was a very strong luciferase signal in the abdomen that was indistinguishable from the luciferase signal observed in mice treated with PBS alone. Mice injected with 1×10^7 CARMA-hMeso cells showed similar tumor signals in the abdomen, which indicated the treatment had no effect at this low cell dose. In contrast, mice injected with 1×10^8 CARMA-hMeso cells showed significantly lower tumor signals in the abdomen at all time points. To determine if it is necessary to incubate the CARMA-hMeso cells *in vitro* to allow time for hMeso CAR expression prior to injection, CARMA-hMeso cells were placed in a 37°C incubator with 5% CO₂ for 3 h prior to injection. There was no difference in efficacy between

Figure 2. Biodistribution of CARMA-hMeso cells following intraperitoneal (i.p.) injection in NSG mice. Cryopreserved control peripheral blood lymphocytes (PBLs) or CARMA-hMeso cells at 5×10^7 were injected i.p. into NSG mice on day 0 (D0). **(A)** On D1, D2, D4, and D7, blood was collected and profiled by staining with anti-human CD3-PE and CD45-FITC and analysis by flow cytometry. On D8, **(B)** peritoneal washings and **(C)** splenocytes were stained with anti-human CD3-PE and CD45-FITC and analyzed by flow cytometry. Significant differences were observed in the blood on D2 ($p=0.012$) and D4 ($p=0.0028$), but there was no statistically significant difference on D7 ($p=0.098$). No statistically significant differences were observed in the peritoneum on D7 ($p=0.0603$) or in the spleen on D8.

CARMA cells recovered in the incubator and CARMA cells injected immediately after thawing. Thus, all remaining animal studies were performed using CARMA-hMeso cells immediately post thaw. Notably, it was observed that Defb29 Vegf-luc/Hmeso produces very aggressive tumors, and mice challenged with 3×10^5 tumor cells were dead after just 1 month. Due to the high lethality of Defb29 Vegf-luc/Hmeso cells, mice were challenged with a lower dose of tumor cells (1×10^4) for the remaining experiments.

Figure 3. Injection i.p. of CARMA-hMeso cells controlled tumor growth in a mouse hMeso-expressing ovarian tumor model in nude mice. Mouse ovarian tumors expressing hMeso were initiated by i.p. injection of 3×10^5 Defb29 Vegf-luc/Hmeso into nude mice on D0. On D3, the baseline luminescence intensity from the mouse abdomen was imaged using IVIS. The CARMA-hMeso cells were thawed and injected i.p. on D4, and the mice were imaged once a week. **(A)** Luminescence intensity of mouse imaging of increasing doses of the CARMA-hMeso cells used. Phosphate-buffered saline (PBS) and non-specific CARMA cells (CD19 CARMA) were used as controls. Cells were injected immediately after thawing or rested in a 37°C incubator for 3 h. **(B)** Bar graph of luminescence intensity over a period of 13 days. Student's *t*-tests were done to compare each group with the PBS group. Only significant values are shown.

CARMA-hMeso cells were also prepared from PBLs following removal of adherent cells (e.g., monocytes and macrophages) by culturing PBMC in a five-tier cell factory for 2 h. To determine if antitumor effects could also be observed when adherent cells are present, PBMCs were transfected with the CARMA-hMeso-PBMCs and evaluated for antitumor activity in mice. Mice were challenged i.p. with 1×10^4 Defb29 Vegf-luc/Hmeso ovarian tumor cells on D0. On D4, PBS or 5×10^7 CARMA-hMeso-PBMCs were injected i.p. Mice treated with CARMA-hMeso-PBMCs showed stronger antitumor effects than mice treated with PBS control (Supplementary Fig. S5).

CARMA-hMeso cells have antitumor activity when given i.p. but not i.v.

The antitumor activity of CARMA-hMeso cells was evaluated following i.p. or i.v. (via r.o.) administration (Fig. 4). When compared to the control group, mice treated with both 1×10^7 and 1×10^8 CARMA-hMeso cells by i.p. injection had significant antitumor effects at all time points, and injection of 1×10^8 cells was more effective than the injection of 1×10^7 cells. Treatment i.v. was evaluated by injecting 2×10^7 or 4×10^7 CARMA-hMeso cells r.o. The tumor burden of mice treated with either dose r.o. was similar to control untreated mice, suggesting that the administration of meso-CAR T cells i.v. was ineffective. To evaluate this further, 2×10^7 CAR T cells r.o. were injected for

three consecutive days. Repeat i.v. administration did not result in either a lower tumor burden or a survival benefit in treated mice.

Weekly i.p. administration of CARMA-hMeso cells was more effective than a single i.p. administration

A single i.p. injection of CARMA-hMeso cells effectively controlled ovarian tumor growth *in vivo*, but this treatment effect was short-lived. To overcome the limitation of transient expression of the CAR on T cells, the study tested whether repeated i.p. injections of CARMA-hMeso cells would result in more effective ovarian tumor growth control. Nude mice were challenged i.p. with 1×10^4 Defb29 Vegf-luc/Hmeso ovarian tumor cells on D0. Tumor-bearing mice were then given 1×10^7 or 5×10^7 CARMA-hMeso cells i.p. on D4, D11, and D18. A weekly administration schedule was selected based on *in vitro* data demonstrating target cell lysis activity through approximately 7 days of *in vitro* culture (data not shown). Mice treated with a single injection of 1×10^7 or 5×10^7 CARMA-hMeso cells were injected on D4. Weekly administration of 5×10^7 CARMA-hMeso cells resulted in better tumor control than a single injection of 1×10^7 or 5×10^7 CARMA-hMeso cells (Fig. 5A). Furthermore, six weekly i.p. injections of 5×10^7 CARMA-hMeso cells was more effective in controlling tumor growth than three weekly i.p. injections of 5×10^7 CARMA-hMeso cells (Fig. 5B). These results

Figure 4. Intravenous injection of CARMA-hMeso cells did not have treatment effects on ovarian cancer *in vivo*. Defb29 Vegf-luc/Hmeso cells (1×10^4) were injected i.p. into nude mice on D0. On D3, the baseline luminescence intensity from the mouse abdomen was imaged using IVIS. The CARMA-hMeso cells were thawed and injected on D4 by i.p. or retro-orbital (r.o.) injection with different cell numbers, and treated mice were imaged once a week. Two days after the first injection, the continuous injection was administered daily for a total of three injections. The non-treatment group was the control group. **(A)** Imaging was obtained once weekly for 4 weeks, until D27. **(B)** Bar graph of luminescence intensity over a period 27 days. Student's *t*-tests were used to compare each individual group to the non-treatment group. **(C)** The survival of the mice was measured.

indicate that serial weekly i.p. injections of CARMA-hMeso cells more effectively decreased tumor burden and improved survival than a single administration of these cells, and that higher doses of CARMA-hMeso cells were more effective at controlling ovarian tumor growth *in vivo* than lower doses of CARMA-hMeso cells.

DISCUSSION

Here, the development of a cellular immunotherapy product, CARMA-hMeso, is described. CARMA-hMeso is produced by transiently transfecting PBLs with CAR mRNA produced *in vitro* from a plasmid containing the following nucleotide sequences: CD8 α signal peptide, human origin

scFv targeting human mesothelin, CD8 hinge/transmembrane domain, and the 4-1BB and CD3 ζ intracellular domains. CARMA-hMeso T cells in the PBLs bind to mesothelin-expressing cells, activating T-cell dependent antitumor activity through signaling via CD3 ζ and the costimulatory molecule 4-1BB. The study demonstrates that this cellular product can lyse ovarian tumor cells expressing human mesothelin *in vitro*, and control the growth of murine ovarian tumor cells expressing human mesothelin *in vivo* in a mouse model. Six key findings critical for product development and clinical translation emerged from these studies. First, the fully human CAR specific for human mesothelin (CARMA-hMeso) has similar lytic activity as the mouse CAR specific for human me-

Figure 5. Repeated i.p. injection of CARMA-hMeso cells showed better activity in a mouse hMeso-expressing ovarian tumor model compared to a single injection. Mouse ovarian tumors expressing hMeso were initiated by i.p. injection of 1×10^4 Defb29 Vegf-luc/Hmeso cells into nude mice ($N=10$ /group) on D0. **(A)** Tumor-bearing mice were treated by i.p. injection (once weekly, three doses) with PBS (control) or CARMA-hMeso cells at 1×10^7 on D4 ($1 \times 1e7$); 5×10^7 on D4 ($1 \times 5e7$); 1×10^7 on D4, D11, and D18 ($3 \times 1e7$), or 5×10^7 on D4, D11, and D18 ($3 \times 5e7$). Survival of tumor-bearing mice is shown through D90. **(B)** Tumor-bearing mice were treated by i.p. injection with phosphate PBS; 5×10^7 control cells (Control T); or CARMA-hMeso cells at 5×10^7 on D4 ($1 \times$ CAR T), 5×10^7 on D4, D11, and D18 ($3 \times$ CAR T), or 5×10^7 on D4, D11, D18, D25, D32, and D39 ($6 \times$ CAR T). Survival of tumor-bearing mice is shown through D130.

sothelin (CARMA-mMeso), with the advantage that fully human CARMA-hMeso should be less immunogenic. Second, expression of the CAR is transient *in vitro*, potentially providing a means of mitigating potential “on-target off-tumor” toxicity *in vivo* in humans. It was not possible to evaluate toxicity in the mouse models, since CARMA-hMeso does not recognize murine mesothelin. Third, there were no differences in the composition and potency of PBLs transfected fresh and cryopreserved, or PBLs transfected fresh and evaluated without cryopreservation. Fourth, there was no apparent

impact of adherent cells on the ability of CAR-transfected PBLs to control tumor growth *in vivo*. Fifth, weekly i.p. administrations of CARMA-hMeso control tumor growth more effectively than a single i.p. administration. Finally, i.p. administration of CARMA-hMeso effectively controlled tumor growth, but i.v. administration did not.

The use of transiently transfected CAR mRNA has the potential advantages of reduced toxicity to normal tissue triggered by retroviral transfections, and reduced target-specific off-tumor toxicity due to the transient pattern of CAR expression.³⁷ The

platform developed here also confers the ability to target solid tumors based on antigen expression, and also provides a cost-effective, automated, cGMP-compliant clinical/commercial manufacturing process. There have been studies of mRNA CAR-transfected cells specific for a range of targets other than mesothelin, including CD20,³⁸ CD33,³⁹ NKG2D,⁴⁰ folate receptor,⁴¹ and GD2.⁴² Modified cells specific for these targets are known to induce hematopoietic stem cell toxicity or other organ-specific toxicities. These and other challenges associated with optimizing mRNA CAR approaches to permit meaningful clinical responses, as demonstrated in a study comparing mRNA CAR versus viral vector CAR delivery targeting the GD2 antigen,⁴² suggest that systematic scientific studies are warranted.

Here, some of these challenges were specifically addressed by deploying a nonviral approach applicable to multiple solid cancers using mRNA CAR transfection to permit prospective control of “on-target off-tumor” toxicity. Collaborating with investigators at the University of Pennsylvania and the National University of Singapore, translation of mRNA CAR transfection has been enabled into *ex vivo* selected, activated, and expanded T cells/natural killer cells into preclinical models and human clinical trials targeting multiple tumor-associated antigens for both hematological malignancies and for solid cancers.^{40,43–47} Preliminary results from these studies indicate that mRNA CAR is a safe, nonviral strategy for engineering tumor-targeted immunotherapy. This approach has resulted in measurable antitumor activity in animals and preliminary evidence of clinical activity in man.^{22,23}

Human proof-of-concept trials of mRNA CAR transfected into selected, activated, and expanded T cells (mRNA CARTs) have provided supportive evidence for the use of CARMA. In these studies, a dose of 1×10^9 cells (>95% CAR+) was well-tolerated and did not result in any detectable “on-target off-tumor” toxicity. CAR protein expression was detectable *in vivo* but decreased incrementally over time due the dilutional impact of cell division. In some patients, a measurable reduction in tumor burden was observed about 2 months after cell administration.^{22,23} Preclinical studies have shown that that repeat dosing of mRNA CARTs results in improved antitumor activity, and may promote long-term survival.^{20,45,46}

The initial human clinical trials used a CAR molecule that included mouse scFV anti-human mesothelin sequences (mMeso), which share homology and cross-react with human-mesothelin

(hMeso). Initial evaluation of repeat dosing of mRNA-CART-mMeso in a patient who experienced a reduction in tumor burden after one dose of cells resulted in anaphylaxis,⁴⁸ likely due to human anti-mouse antibody that developed as a result of the initial treatment. Multiple cycles of treatment could thus not be evaluated in this mRNA-CART-mMeso trial. To mitigate the risk of developing anti-drug antibodies and facilitate repeat dosing, a fully human scFv that targets human mesothelin for CARMA-hMeso product manufacture was selected and tested. Notably, even though CARMA-hMeso uses a human mesothelin construct, no significant differences in K562-Meso tumor target cell lysis were observed when CARMA-mMeso or CARMA-hMeso effector cells were evaluated in an *in vitro* tumor killing assay.

The CARMA product platform capitalizes on the ability to transfect mRNA encoding CAR molecules rapidly into PBMCs and the deployment of a cGMP and regulatory compliant automated closed system for cell processing.⁴⁹ With the leukapheresis product in hand, the entire manufacturing process can be completed in a few hours, generating multiple cryopreserved cell aliquots suitable for repeat dosing. The process developed does not require use of viral vectors or extensive *ex vivo* manipulations and expansion over multiple days. The product characterization studies here showed that there were no statistically significant differences in viability, transfection efficiency, or ability to cryopreserve transfected CARMA-hMeso cells. In addition, fresh and cryopreserved CARMA-hMeso cells showed comparable cytotoxicity for tumor cells *in vitro*.

Most of the *in vivo* studies presented here used CARMA-hMeso cells prepared from PBLs following removal of adherent cells with a 2h panning step. However, one study evaluated PBMCs transfected directly with the CARMA-hMeso-PBMCs. In this study, CARMA-hMeso-PBMCs also demonstrated *in vivo* antitumor activity. These findings suggest that it may be possible to decrease the overall cell processing time for future CARMA products further, but additional head-to-head comparisons between CARMA-hMeso-PBLs and CARMA-hMeso-PBMCs from the same donor preparation are needed to confirm these findings.

The CARMA strategy was used here for the treatment of human mesothelin-expressing mouse ovarian tumor cells in nude mice. Mesothelin was selected as the target antigen for CARMA-hMeso based on its high expression in human ovarian cancers and low expression in normal human tissues. Although data for other immunotherapies targeting mesothelin suggest that this target is

safe,⁵⁰ “on-target off-tumor” toxicity could not be evaluated, since CARMA-hMeso does not recognize mouse mesothelin. CARMA-hMeso therapy resulted in decreased tumor burdens and increased survival in treated mice, with larger doses and repeat administrations resulting in better tumor growth control and improved survival. The need for repeat dosing to improve efficacy likely reflects the transient pharmacodynamics of CARMA-hMeso.

The mechanism of action of CARMA is more similar to the “hit and run” mechanism of ADCC-enhanced monoclonal antibodies or bi-specific antibodies such as BiTEs⁵¹ than to the long-lived mechanism of conventional viral-vector transduced CD19 CARTs,¹⁵ which relies on creating a niche for engraftment and expansion of CARTs.¹⁶ Accordingly, CARTs frequently have associated toxicities related to “on-target-off-tumor” effects, the induction of CRS, neurotoxicity, and/or other changes that may require lymphodepleting regimens for patient conditioning.¹⁴ CARMA-hMeso may also require patient conditioning or lymphodepleting regimens,⁴⁶ but it is possible that less aggressive regimens could be effective, since the modified cells intrinsically have a limited life-span. This requires further study.

Although no benefit was observed following i.v. administration of CARMA-hMeso in the mouse tumor model, the tumors were confined to the abdomen, which may not fully reflect metastatic ovarian cancer in humans where tumor cells may be observed outside the peritoneum. Thus, both i.p. and i.v. administration may be important to achieve optimal responses in humans.^{22,23} Furthermore, the use of nude mice instead of NSG mice may have contributed to the lack of effects because nude mice have an active innate immune system that may have helped clear the CARMA-hMeso cells delivered i.v. The biodistribution study in NSG mice did show distribution of CARMA-hMeso cells into the blood, spleen, and

distant lymph nodes at 4 days after a single i.p. administration.

CARMA represents a unique platform for the rapid manufacture and delivery of tumor-targeted immunotherapies using fully human CAR mRNA transfected into PBLs, potentially allowing for repeat cycles of treatment. With the CARMA platform, multiple aliquots of CARMA can be rapidly manufactured within hours from a single leukapheresis using a cGMP and regulatory compliant, automated, closed system, resulting in a consistent, high-quality product in a practical time frame and at reasonable cost. These data suggest that the manufacturing of CARMA-hMeso cells is feasible, and can produce multiple doses of tumor antigen-specific CARMA for repeated administration. Together, these *in vitro* and animal studies support the evaluation of i.p. CARMA-hMeso cells as a treatment for patients with platinum-resistant ovarian cancer.

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