Successful Eradication of Established Peritoneal Ovarian Tumors in SCID-Beige Mice following Adoptive Transfer of T Cells Genetically Targeted to the MUC16 Antigen

Alena A. Chekmasova1, Thapi D. Rao1, Yan Nikhamin1, Kay J. Park2, Douglas A. Levine3, David R. Spriggs1, and Renier J. Brentjens1,4

Abstract

Purpose: Most patients diagnosed with ovarian cancer will ultimately die from their disease. For this reason, novel approaches to the treatment of this malignancy are needed. Adoptive transfer of a patient's own T cells, genetically modified ex vivo through the introduction of a gene encoding a chimeric antigen receptor (CAR) targeted to a tumor-associated antigen, is a novel approach to the treatment of ovarian cancer.

Experimental Design: We have generated several CARs targeted to the retained extracellular domain of MUC16, termed MUC-CD, an antigen expressed on most ovarian carcinomas. We investigate the in vitro biology of human T cells retrovirally transduced to express these CARs by coculture assays on artificial antigen-presenting cells as well as by cytotoxicity and cytokine release assays using the human MUC-CD+ ovarian tumor cell lines and primary patient tumor cells. Further, we assess the in vivo antitumor efficacy of MUC-CD–targeted T cells in SCID-Beige mice bearing peritoneal human MUC-CD+ tumor cell lines.

Results: CAR-modified, MUC-CD–targeted T cells exhibited efficient MUC-CD–specific cytolytic activity against both human ovarian cell and primary ovarian carcinoma cells in vitro. Furthermore, expanded MUC-CD–targeted T cells infused through either i.p. injection or i.v. infusion into SCID-Beige mice bearing orthotopic human MUC-CD+ ovarian carcinoma tumors either delayed progression or fully eradicated disease.

Conclusion: These promising preclinical studies justify further investigation of MUC-CD–targeted T cells as a potential therapeutic approach for patients with high-risk MUC16+ ovarian carcinomas.

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Ovarian cancer is the sixth most common cancer worldwide and the seventh leading cause of cancer-related deaths in women (1, 2). Despite multimodality therapy with surgery and chemotherapy, most patients with ovarian carcinomas have a poor prognosis. For this reason, alternative approaches to treating this disease are urgently needed.

Infusion of a patient's own T cells genetically targeted ex vivo to antigens expressed on the surface of tumor cells is a promising novel approach to the adoptive immunotherapy of cancer, and one that has only recently been explored in earnest in the clinical setting. T cells may be genetically modified to target tumor-associated antigens (TAA) through the retroviral introduction of genes encoding artificial T-cell receptors (TCR) termed chimeric antigen receptors (CAR). CARs are most commonly composed of a single-chain fragment length antibody (scFv), derived from a murine monoclonal antibody (mAb) targeting a given TAA, fused to a transmembrane with or without an additional cytoplasmic signaling domain (typically derived from CD8, CD28, OX-40, or 4-1BB), fused to the TCRζ chain cytoplasmic signaling domain (3–13). When expressed by the T cells, the resulting construct, on engagement with the targeted antigen, induces T-cell activation, proliferation, and lysis of targeted cells. To date, preclinical studies using CAR-modified T cells have shown promising results in a wide variety of malignancies (3, 4, 11, 14–18). More recently, this approach has been investigated in phase I clinical trials (6, 19–21).

Ovarian carcinomas seem to be relatively immunogenic tumors capable of inducing an endogenous immune response because long-term prognosis of patients is markedly influenced by the degree and quality of the endogenous immune response to the tumor. Specifically, it has been well documented that the presence of endogenous effector T cells within the ovarian cancer tumor microenvironment...
Eradication of Ovarian Carcinomas with Targeted T Cells

Translational Relevance

Ovarian carcinomas seem to be immunogenic tumors because increased numbers of tumor-infiltrating T cells present in the pretreatment tumor are associated with an enhanced survival following surgery and chemotherapy. Patient T cells may be genetically modified to recognize tumor-associated antigens through the introduction of chimeric antigen receptors (CAR) specifically targeted to these antigens. We have generated a CAR, 4H11-28z, specific to the retained extracellular portion (MUC-CD) of MUC16, a glycoprotein overexpressed on most ovarian carcinomas. We show that T cells, when modified to express the 4H11-28z CAR, specifically lyse human ovarian cancer cells in vitro, and further show efficient antitumor efficacy in orthotopic xenograft tumor models. Collectively, these data support the translation of these preclinical findings to the clinical setting in a planned phase I trial treating patients with relapsed or refractory ovarian cancer.

directly correlates to prolonged patient survival (22–25). In contrast, increasing numbers of immunosuppressive CD4+ CD25+ regulatory T cells (Tregs) within the tumor, which in turn presumably abrogate the antitumor activity of infiltrating effector T cells, correlate with shorter patient survival (26–29). In fact, it seems that it is the ratio of Tregs to effector T cells within the tumor microenvironment that ultimately dictates whether the endogenous immune response to the cancer is of benefit or detriment to the patient (24, 28). In this setting, the ability to generate and subsequently expand a population of tumor-targeted effector T cells ex vivo, which are subsequently infused back into the patient, may in turn skew the Treg to effector T-cell ratio to one more favorable to eradicating the disease.

Mucins are important biomolecules for cellular homeostasis and protection of epithelial surfaces (1). MUC16 is one such mucin that is overexpressed on most ovarian carcinomas and is an established surrogate serum marker for the detection and progression of ovarian cancers (30–33). MUC16 is a glycosylated mucin composed of a large cleaved and released domain, termed CA-125, consisting of multiple repeat sequences, and a retained domain (MUC-CD), which includes a residual nonrepeating extracellular fragment, a transmembrane domain, and a cytoplasmic tail (34). Because the antigen is otherwise only expressed at low levels in the uterus, endometrium, fallopian tubes, ovaries, and serosa of the abdominal and thoracic cavities, MUC16 is a potentially attractive target for immune-based therapies.

The fact that most of the extracellular domain of MUC16 is cleaved and secreted limits the utility of MUC16 as a target antigen on ovarian carcinomas. To date, all reported mAbs to MUC16 bind to epitopes present on the large secreted CA-125 fraction of the glycoprotein, with none known to bind to the retained extracellular fraction (MUC-CD) of the antigen (35–37). Because the MUC-CD fraction of the antigen is retained on the cell surface, generating T cells specific to this portion of MUC16 may largely overcome the limitation of MUC16 as a target for adoptive cellular immunotherapy. To this end, we have previously generated a series of murine mAbs specific to the retained MUC-CD extracellular domain (38). Using a hybridoma that expresses one such mAb, 4H11, we have successfully constructed several CARs specific to the MUC-CD antigen.

In this report, we show highly efficient retroviral transduction of these MUC-CD–targeted CARs into human T cells, with resulting T cells able to specifically target and lyse MUC-CD+ tumor cell lines in vitro. In addition, we show significant cytotoxicity mediated by 4H11-28z patient T cells and healthy donor T cells targeting primary ascites-derived ovarian carcinoma cells. In vivo studies in immunocompromised SCID-Beige mice bearing established peritoneal orthotopic MUC-CD+ human ovarian tumors show marked antitumor efficacy of MUC-CD–targeted T cells, following either i.p. or i.v. injection. These data serve as a rationale for future clinical trials using this approach in patients with high-risk ovarian carcinomas.

Materials and Methods

Cell lines and T cells

The OV-CAR3 and SK-OV3 tumor cell lines were cultured in RPMI 1640 (Invitrogen) supplemented with 10% heat-inactivated fetal bovine serum (FBS), nonessential amino acids, HEPES buffer, pyruvate, and 2-Mercaptoethanol (Invitrogen). T80 cells originated from normal ovarian surface epithelium, which were immortalized by transfection with SV40 large T antigen, were kindly provided by Dr. Robert C. Bast (The University of Texas M. D. Anderson Cancer Center, Houston, TX) (39). T80 cells were cultured in 1:1 ratio of MCDB 105 medium (Sigma-Aldrich Co.) and Medium 199 (Invitrogen) supplemented with 10% heat-inactivated FBS. The PG13, HeLa, and gpg29 retroviral producer cell lines were cultured in DMEM (Invitrogen) supplemented with 10% FCS. NIH-3T3 artificial antigen-presenting cells (AAPP), described previously (3), were cultured in DMEM supplemented with 10% heat-inactivated donor calf serum. Human T cells were isolated from peripheral blood of healthy donors under Institutional Review Board–approved protocol 95-054 using BD Vacutainer CPT tubes (Becton Dickinson) as per the manufacturer’s instructions.

T cells were cultured in RPMI 1640 supplemented with 20 IU/mL interleukin (IL)-2 (Novartis Pharmaceuticals), and where indicated, medium was supplemented with 10 ng/mL IL-15 (R&D Systems). Primary ovarian cancer cells derived from ascites specimens were cultured in RPMI 1640 supplemented with 10% FBS. All media were supplemented with 2 mmol/L L-glutamine (Invitrogen), 100 units/mL penicillin, and 100 μg/mL streptomycin (Invitrogen).
Isolation of patient ascites-derived ovarian cancer cells

Primary human ascites-derived cancer cells were obtained from ovarian cancer patients undergoing surgery for newly diagnosed ovarian carcinomas under Institutional Review Board–approved protocol 97-134. The tumor cells were isolated from ascites fluid of patients by centrifugation at 600 g for 10 minutes at room temperature. Cells were washed once with 1× PBS and cultured in FBS-supplemented RPMI 1640 in tissue culture flasks. After 5 days in culture, nonadherent cells were removed, retaining the tumor cell–enriched adherent fraction for further study.

Generation of the MUC-CD–targeted 4H11z and 4H11-28z CARs

The heavy and light chain variable regions of the 4H11 scFv were derived from the hybridoma cell line 4H11. Using cDNA generated from 4H11 RNA, we isolated the VH coding region by RACE (rapid amplification of cDNA ends) PCR using modified primers as described elsewhere (40, 41). The VH chain variable region was cloned by standard PCR using modified primers as described by Orlandi et al. (42, 43). The resulting VH and VL fragments were subcloned into the TopoTA PCR 2.1 cloning vector (Invitrogen) and sequenced. The VH and VL fragments were subsequently ligation to a (Gly4Ser)3 spacer domain, generating the 4H11 scFv, and fused to the human CD8 leader peptide (CD8L) by overlapping PCR (9, 42). To construct the MUC-CD–targeted 4H11 CARs, the coding region of the CD8L-4H11 scFv was fused to the human CD8 hinge and transmembrane domains (to generate the 4H11z CAR) or, alternatively, to the CD28 transmembrane and cytoplasmic signaling domains (to generate the 4H11z-28z CAR), fused to the TCR CD3ζ signaling domain (3, 9, 44). The resulting CAR constructs were subsequently subcloned into the modified Moloney murine leukemia virus retroviral vector SFG (45). VSV-G–pseudotyped retroviral supernatants derived from transduced gpg29 fibroblasts were used to construct stable PG13 gibbon ape leukemia virus envelope-pseudotyped retroviral-producing cell lines (42).

Retroviral gene transfer

Isolated healthy donor peripheral blood mononuclear cells were activated with phytohemagglutinin at 2 μg/mL (Sigma), whereas patient T cells derived from fresh ascites samples were isolated, activated, and expanded with Dynabeads ClinExVivo CD3/CD28 beads (Invitrogen) following the manufacturer’s recommendations. Activated T cells were retrovirally transduced on retronectin-coated nonissue culture plates as described previously (46). Gene transfer was assessed on day 7 by fluorescence-activated cell sorting (FACS).

To generate the relevant NIH-3T3 murine fibroblast AAPCs, a MUC-CD construct encoding the retained extracellular, transmembrane, and cytoplasmic domains of the MUC16 antigen was initially subcloned into SFG retroviral vector, SFG(MUC-CD). 3T3(MUC-CD) AAPCs were generated by retroviral transduction of SFG(MUC-CD) into wild-type NIH-3T3 fibroblasts, whereas 3T3(MUC-CD/B7.1) AAPCs were generated by retroviral transduction of previously established 3T3(B7.1) fibroblasts (42, 47). Enriched cell lines were isolated by FACS.

OV-CAR3(MUC-CD), SK-OV3(MUC-CD), HeLa(MUC-CD), OV-CAR3(MUC-CD/GFP-FFLuc), and SK-OV3 (MUC-CD/GFP-FFLuc) cell lines were generated by retroviral transduction with SFG(GFP-FFLuc) (48) and/or SFG (MUC-CD) VSV-G–pseudotyped retroviral supernatants derived from gpg29 fibroblasts as described elsewhere (45). T80(MUC-CD) cells were generated by transfection of the MUC-CD gene using the Vitality phrGFP II C vector expression system (Stratagene). Resulting tumor cells were sorted by FACS for MUC-CD expression.

In vitro analyses of CAR+ human T cells

To assess in vitro expansion and cytokine release on stimulation, 3 × 10^6 transduced T cells were cocultured for 7 days after retroviral transduction in six-well tissue culture plates (BD Biosciences) on confluent NIH-3T3 AAPCs in the absence of supplemented cytokines. For in vivo studies, transduced T cells were expanded by coculture on 3T3(MUC-CD/B7.1) AAPCs in RPMI 1640 supplemented with 20 IU/mL IL-2 and 10 ng/mL IL-15 as described previously (3, 44).

Western blot analysis of CAR expression

Western blot analysis of T-cell lysates under reducing conditions with 0.1 mol/L DTT (Sigma) was done as previously described (47).

Cytotoxicity assays

In vitro–modified T-cell cytotoxicity was assessed using the DELFIA EuTDA assay (Perkin-Elmer LAS, Inc.) following the manufacturer’s recommendations. Cytotoxicity was assessed at 2 hours at serially diluted E:T ratios. Effector T cells in these assays represent the CD8+ CAR+ T-cell fraction.

Cytokine detection assays

Cytokine assays were done as per the manufacturer’s specifications using a multiplex Human Cytokine Detection assay to detect IL-2 and IFN-γ (Millipore Corp.) using the Luminex IS100 system. Cytokine concentrations were assessed using LUM1 software (Luminex Corp.).

In vivo SCID-Beige mouse tumor models

In all in vivo studies, 8- to 12-week-old Fox Chase C.B.-17 (SCID-Beige mice; Taconic) were injected i.p. with 3 × 10^6 tumor cells. Subsequently, CAR+ T cells, at indicated doses, were injected either i.p. or i.v. on either day 1 or 7 following tumor cell injection. Mice were monitored for distress as assessed by increasing abdominal girth, ruffled fur, and decreased response to stimuli. Distressed mice were euthanized. All murine studies were done in the context of an Institutional Animal Care and Use Committee–approved protocol (00-05-065).
Bioluminescent imaging of GFP-FFLuc+ human ovarian tumor cells in SCID-Beige mice

Bioluminescent imaging (BLI) was done using Xenogen IVIS imaging system with Living Image software (Xenogen). Briefly, GFP-FFLuc+ tumor-bearing mice were injected by i.p. with d-luciferin (150 mg/kg; Xenogen) suspended in 200 µL PBS and imaged under 2% isoflurane anesthesia after 10 minutes. Image acquisition was done on a 25-cm field of view at medium binning level for 0.5-minute exposure time (3, 44).

Flow cytometry

All flow cytometric analyses of T cells and tumor cells were done using a FACSscan cytometer with CellQuest software (BD Biosciences). T cells were analyzed using CAR-specific polyclonal goat Alexa Fluor 647 antibody (Molecular Probes); phycoerythrin-labeled anti-human CD4, CD8, B7.1, and CD45RO (Caltag Laboratories); phycoerythrin-conjugated anti-human CD62L (eBioscience). MUC-CD expression was measured by FACS with Alexa Fluor 647–labeled 4H11 antibody (generated and labeled in the Memorial Sloan-Kettering Cancer Center mAb core facility).

Carboxyfluorescein diacetate succinimidyl ester labeling of CAR+ T cells

CAR+ T cells were stained with carboxyfluorescein diacetate succinimidyl ester (CFSE) using the CellTrace CFSE cell proliferation kit following the manufacturer's recommendations (Molecular Probes).

Assessment of in vivo T-cell persistence

SCID-Beige mice were infused i.p. with OV-CAR3 (MUC-CD) tumor cells and, 7 days later, treated by i.p. infusion with CAR+ T cells. Subsequently, mice were sacrificed at days 2, 14, 21, and 28 following T-cell infusion. Peritoneal washes were collected using 10 mL PBS, RBCs were lysed with ACK lysing buffer (Lonza), and remaining cells were washed with 2% FBS/PBS and analyzed by FACS for persistence of human CD3+ T cells.

Statistics

Survival data assessed by log-rank analysis using Prism 5.0 (GraphPad Software) and cytokine secretion data assessed by Student's two-tailed t test. In the comparison between survival of i.p. versus i.v. modified T-cell infusion, two experiments were done with different follow-up times. For combined analysis, the stratified log-rank statistic (stratified by experiment) was used to test whether the survival rates differed between the i.p. and i.v. treatment groups.

Results

We have constructed SFG retroviral vectors encoding first-generation (4H11z) and second-generation costimulatory (4H11-28z) CARs targeted to the MUC-CD antigen using the 4H11 hybridoma, which generates a mAb specific to the MUC-CD antigen (Fig. 1A). We confirmed expression of appropriately sized CAR proteins by Western blot analysis of resulting PG-13 retroviral producer cells (SFG-4H11z and SFG-4H11-28z) probed with a ζ chain–specific antibody (data not shown).

To assess the function of the first-generation 4H11z CAR, healthy donor T cells isolated from peripheral blood were retrovirally transduced to express the 4H11z CAR and control T cells modified to express the irrelevant CD19-targeted 19z1 CAR (Fig. 1B). The function of the 4H11z CAR was assessed by proliferation of 4H11z-transduced T cells following coculture on 3T3(MUC-CD/B7.1) AAPCs. Results show specific proliferation of 4H11z-transduced T cells when compared with 19z1-modified T cells (Fig. 1C). These data are consistent with 4H11z CAR–mediated specific binding to the MUC-CD antigen and subsequent T-cell activation.

Because most malignancies fail to express costimulatory ligands, we further modified the 4H11z CAR to express the CD28 transmembrane and cytoplasmic costimulatory signaling domains, generating the 4H11-28z CAR (Fig. 1A). Results show specific proliferation of 4H11-28z-transduced T cells following coculture on 3T3(MUC-CD/B7.1) AAPCs, whereas proliferation on either 3T3(MUC-CD) or 3T3 (MUC-CD/B7.1) AAPCs was similar to that of control T cells (Fig. 1D). The second-generation 4H11-28z CAR is capable of inducing both a primary activating signal (termed "signal 1") through the ζ chain and a costimulatory signal (termed "signal 2") through the CD28 cytoplasmic domain, which in turn allows for efficient T-cell proliferation in the absence of exogenous costimulatory ligands, thus enabling T-cell proliferation following coculture on either 3T3(MUC-CD) or 3T3 (MUC-CD/B7.1) AAPCs in the absence of exogenous cytokines. As expected, the second-generation 4H11-28z T cells markedly expanded when compared with 4H11z T cells on coculture with 3T3(MUC-CD) AAPCs (P = 0.0047). In contrast, both 4H11z and 4H11-28z T cells expanded equally well on 3T3(MUC-CD/B7.1) AAPCs (P = 0.18; Fig. 2A). Costimulation mediated by the 4H11-28z CAR was further verified by analysis of day 2 tissue culture supernatants from coculture experiments on 3T3(MUC-CD) AAPCs, showing significantly enhanced IL-2, a cytokine typically secreted in the context of T-cell costimulation, and IFN-γ secretion when compared with control 19-28z T cells and first-generation 4H11z T cells (Fig. 2B).

We next assessed the ability of 4H11-28z T cells to expand following weekly restimulations on 3T3(MUC-CD/B7.1) AAPC monolayers in the context of exogenous IL-2 and IL-15 (3). In this setting, 4H11-28z T cells expanded equally well as compared with 4H11z T cells on coculture with 3T3(MUC-CD) AAPCs, showing significantly enhanced IL-2, a cytokine typically secreted in the context of T-cell costimulation, and IFN-γ secretion when compared with control 19-28z T cells.

In vitro MUC-CD–specific activation of 4H11-28z T cells

To assess MUC-CD specificity of 4H11-28z T cells, we initially conducted a series of cytotoxicity assays using...
the MUC-CD+ OV-CAR3 and SK-OV3 cell lines with or without further genetic modification with MUC-CD. As expected, 4H11-28z+ T cells efficiently lysed MUC-CD+ but not unmodified tumor cell lines, consistent with MUC-CD specificity mediated through the 4H11-28z CAR (Fig. 3A). To further verify these findings, we conducted similar studies on nonovarian carcinoma cell lines, HeLa, and an immortalized normal ovarian surface epithelial cell line, T80, showing similar results (Supplementary Fig. S1). We next assessed the activation of CAR-modified T cells, as measured by proliferation and cytokine secretion, by coculture with these same cell lines. Consistent with 4H11-28z CAR specificity for MUC-CD, T-cell proliferation as well as IL-2 and IFN-γ secretion only occurred in the setting of MUC-CD+ cell lines cocultured with 4H11-28z+ T cells (Fig. 3B and C; Supplementary Fig. S1).

In vivo antitumor activity of MUC-CD–targeted T cells in SCID-Beige mice
To assess the in vivo antitumor activity of 4H11z+ and 4H11-28z+ T cells, we next generated an orthotopic xenograft ovarian cancer tumor model by i.p. injection of OV-CAR3(MUC-CD) tumor cells into SCID-Beige mice. If left untreated, these mice developed marked ascites and multiple nodular peritoneal tumors by 5 weeks following tumor cell injection (Fig. 4A). All untreated tumor-bearing mice had to be euthanized by 7 weeks following tumor cell injection due to abdominal distention and evidence of distress.

For in vivo studies, cohorts of SCID-Beige mice, injected i.p. with 3 × 10^6 OV-CAR3(MUC-CD/GFP-FFLuc) tumor cells when compared with 19-28z-transduced T cells (Fig. 3D). Moreover, in three of three cases, we found that patient ascites-derived T cells modified to express the 4H11-28z CAR similarly lysed matched autologous primary ovarian carcinoma cells (Fig. 3D).
cells on day 1, were initially treated with dose-escalating levels of CAR-modified T cells by i.p. injection on day 2. FACS analysis of modified in vitro-expanded T cells used in our in vivo studies showed most infused T cells with a retained central memory phenotype as assessed by CD62L, CCR7, CD28, and CD45RO expression (Supplementary Fig. S2; ref. 49). These studies showed a dose-dependent in vivo antitumor response to MUC-CD-targeted T-cell therapy at a treatment dose of $1 \times 10^7$ 4H11-28z+ T cells found to be the minimum dose required to achieve a meaningful long-term survival at day 70, with all mice treated at lower dose levels showing 100% mortality (Supplementary Fig. S3). Based on these findings, we chose the highest tested dose level of $3 \times 10^7$ CAR+ T cells for further studies. We subsequently repeated these studies with larger cohorts of mice treated at this dose level of 4H11z+ or 4H11-28z+ T cells. For negative controls, tumor-bearing mice were either untreated or treated with T cells modified to express the irrelevant CD19-targeted 19z1 CAR. Both MUC-CD–targeted T-cell–treated cohorts showed statistically significant enhanced survival when compared with untreated or 19z1+ T-cell–treated control cohorts, with no statistically significant difference in survival when comparing the 4H11z+ and 4H11-28z+ T-cell–treated cohorts (Fig. 4B; Supplementary Fig. S4A).

As a further in vivo control for 4H11-28z MUC-CD specificity, we repeated these studies using SCID-Beige mice bearing i.p. MUC-CD− SK-OV3(GFP-FFLuc) or SK-OV3 (MUC-CD/GFP-FFLuc) human ovarian carcinoma tumors similarly treated with $3 \times 10^7$ 4H11-28z+ T cells 1 day after tumor cell injection. As expected, treatment with 4H11-28z+ T cells failed to result in any long-term survival of SK-OV3(GFP-FFLuc) tumor-bearing mice, whereas 40% of mice bearing SK-OV3(MUC-CD/GFP-FFLuc) tumors remain alive at day 70 (Supplementary Fig. S4B) with no evidence of disease as assessed by BLI (data not shown).

To determine whether systemically infused MUC-CD–targeted T cells successfully traffic to peritoneal tumors,

![Image](https://example.com/image.png)

**Fig. 2.** In vitro comparison of T cells modified to express the first-generation 4H11z CAR to T cells modified to express the second-generation costimulatory 4H11-28z CAR. A, CAR+ T cells were cocultured on MUC-CD monolayers with (right) or without (left) B7.1. CAR+ T cells (1 × 10^6 per well) were cocultured on AAPC monolayers in 12-well tissue culture plates in cytokine-free medium. Total viable T-cell counts were assessed on days 2, 4, and 7 by trypan blue exclusion assays. B, antigen-specific cytokine secretion by MUC-CD− targeted T cells. C, expansion of CAR+ T cells following three cycles of stimulation on 3T3(MUC-CD/B7.1). Arrows indicate first and second cycles of restimulation on AAPCs. D, FACS analysis of the CAR+ T-cell fraction of 4H11-28z+ T cells increased following each weekly cycle of stimulation. I, FACS following initial transduction; II, FACS at 7 d following first stimulation on AAPCs; III, FACS at 7 d following second stimulation on AAPCs.
we next compared i.p. to i.v. infusion of 4H11-28z+ T cells in SCID-Beige mice 1 day after i.p. infusion of OV-CAR3 (MUC-CD/GFP-FFLuc) tumors. Both i.p. and i.v. 4H11-28z+ T-cell–treated mice exhibited statistically enhanced survival when compared with untreated or 19-28z+ T-cell–treated control cohorts as assessed by BLI imaging (Fig. 5A) as well as overall survival (Fig. 5B; Supplementary Fig. S5). Stratified log-rank analysis of merged data from two independent experiments showed statistically equivalent antitumor efficacy between i.p. and i.v. MUC-CD–targeted T-cell–treated cohorts (P = 0.092).

We further confirmed trafficking of i.v. infused CFSE-labeled 4H11-28z+ T cells to the peritoneum by FACS analysis of single-cell suspensions of macerated OV-CAR3(MUC-CD) peritoneal tumor deposits (Fig. 5C). The presence of i.v. injected CFSE-labeled 19-28z+ control T cells and 4H11-28z+ T cells 1 day following infusion into SCID-Beige mice with advanced OV-CAR(MUC-CD) tumors (injected 7 days earlier) reveals a marked population of human CD3/CFSE+ T cells within peritoneal OV-CAR3 (MUC-CD) tumor deposits of 4H11-28z+ but not of control 19-28z+ T-cell–treated mice.

We next treated SCID-Beige mice bearing peritoneal OV-CAR3(MUC-CD/GFP-FFLuc) tumor injected 7 days before adoptive T-cell therapy, a time point at which the mice had evidence of overt disease as assessed by BLI. A subset

Fig. 3. On coculture, 4H11-28z+ T cells specifically expand and lyse MUC-CD+ tumor cells. A, cytotoxicity assays of 4H11-28z+ T cells targeting OV-CAR3(MUC-CD) and SK-OV3(MUC-CD) tumor cells show efficient cytotoxicity mediated by 4H11-28z+ T cells from healthy donors compared with control 19-28z+ T cells. B, antigen-specific proliferation of MUC-CD–targeted T cells after coculture with OV-CAR3(MUC-CD) or SK-OV3(MUC-CD) tumor cells. CAR+ T cells were cocultured with wild-type or MUC-CD–expressing tumor cells at 1:1 ratio for 7 d. Absolute T-cell numbers assessed on days 2, 4, and 7 following coculture with ovarian tumor cells. The T-cell seeding density for T-cell proliferation assay was 1 × 10^6/mL. C, antigen-specific IL-2 and IFN-γ secretion by 4H11-28z+ transduced T cells after 2 d of coculture on ovarian cancer cells. D, healthy donor T cells or autologous T cells isolated from ascites, modified to express the 4H11-28z CAR, lyse primary patient ascites-derived MUC-CD+ tumor cells when compared with T cells modified to express the control 19-28z CAR.
of 4H11-28z+ T-cell–treated mice assessed to be tumor-free by BLI on day 60 after tumor cell infusion was sacrificed, and peritoneal washes were analyzed for the presence of MUC-CD/GFP–FFLuc+ tumor cells by FACS. At this time point, in contrast to control-treated mice sacrificed and analyzed at day 30, peritoneal washings from treated mice showed <0.01% of analyzed cells to be MUC-CD/GFP+ (Supplementary Fig. S6A). Once more, we found that therapy with MUC-CD–targeted T cells initially eradicated most BLI evident disease in all treated mice (Fig. 6A), with 75% of mice ultimately developing relapsed disease at later time points, whereas 25% of treated mice survive at 120 days after tumor cell infusion with no evidence of disease as assessed by BLI (Fig. 6B). Significantly, FACS analyses of tumor cell suspensions obtained from all 4H11-28z+ T-cell–treated mice with relapsed disease showed persistent expression of the MUC-CD antigen (data not shown).

To test for the persistence of modified T cells over time in these studies, additional mice were infused i.p. with OV-CAR3(MUC-CD) tumors and treated on day 7 with modified T cells and sacrificed serially following therapy. We analyzed for the presence of CAR+ T cells in peritoneal washes of 4H11-28z and 19-28z T-cell–treated tumor-bearing mice on days 2, 14, 21, and 28 after T-cell infusion by FACS, showing a decreasing but persistent population of modified T cells out to 28 days after T-cell infusion (the latest time point studied; Supplementary Fig. S6B).

Discussion

Based on analyses of patient tumor samples, ovarian carcinomas seem to be relatively immunogenic tumors. Specifically, researchers have found a direct correlation between prognosis following surgery and chemotherapy and the quantity of tumor-infiltrating T cells (TIL) in pretreatment tumor samples (25, 50, 51). Furthermore, others have described an inverse correlation between prognosis following therapy and pretreatment levels of Tregs within the tumor, which in turn presumably inhibit the antitumor function of tumor-specific effector TILs (26, 28, 52). Both of these findings imply a role for an endogenous effector T-cell response to tumor in controlling disease progression both before and following initial therapy and strongly support the contention that ovarian carcinomas may be susceptible to killing by adoptive infusion of autologous T cells targeted to ovarian tumor cell antigens.

Although endogenous effector TILs are one source for presumably tumor-specific T cells, an alternative approach to adoptive T-cell therapy is to isolate autologous peripheral blood T cells, which in turn may be genetically...
modified ex vivo to target tumor cell antigens. One such genetic approach is to retrovirally transduce patient T cells with CARs targeted to surface-exposed antigens either unique to or overexpressed by the tumor. To this end, promising preclinical studies using this approach in other malignancies have recently been translated to the clinical setting (6, 16, 19, 53). Application of this approach to ovarian carcinomas requires the identification of suitable target antigens expressed on the tumor cell surface. To this end, other investigators have studied this approach in vivo in the preclinical setting using CAR+ T cells targeted to disparate antigens overexpressed on ovarian carcinomas, including the α-folate receptor, the Lewis-Y antigen, Her2/neu, NKG2D ligands (MICA, MICB, and UL-16 binding proteins), mesothelin, and MUC-1 (4, 11, 54–61). Specifically, in an elegant series of studies using a syngeneic orthotopic 1D8 tumor model of ovarian carcinoma in C57BL6 mice, Barber and Sentman (54) show efficient eradication of well-established i.p. tumors when treated with i.p. injections of syngeneic murine T cells modified to express the chNKG2D CAR. Furthermore, Hwu et al. (4) showed significant delays in tumor progression in immunocompromised nude mice bearing orthotopic human IGROV tumors following a single infusion of murine T cells modified to express an α-folate receptor–targeted CAR. In the xenogeneic setting, several groups have shown delayed tumor progression or complete antitumor responses of subcutaneous human ovarian carcinoma cell lines in immunocompromised mice following intratumoral and/or i.v. infusion of human T cells expressing CARs specific to the Her2/neu and Lewis-Y antigens (59, 61, 62). Similarly, Wilkie et al. (60) and Carpenito et al. (57) have recently published reports showing efficient antitumor efficacy in xenotransplant models of human breast and mesothelioma tumors treated with human T cells modified, respectively, with CARs targeted to the MUC-1 and mesothelin antigens, antigens also overexpressed on ovarian carcinomas.

In the clinical setting, Kershaw et al. (6) recently published the results of a phase I dose escalation trial treating patients with relapsed ovarian carcinomas with autologous T cells modified to express a first-generation CAR specific to the α-folate receptor. The authors of this study found that therapy with targeted T cells was well tolerated, but noted a lack of antitumor response in these studies related to poor persistence of modified T cells over time as well as a yet undefined T-cell inhibitory factor in the serum of several treated patients.

In our studies, we have chosen to target the MUC16 glycoprotein, which is overexpressed on most ovarian

Fig. 5. MUC-CD–targeted 4H11-28z+ T cells traffic to peritoneal OV-CAR3(MUC-CD/GFP-FFLuc) tumors following systemic i.v. infusion resulting in efficient antitumor efficacy. A, BLI of tumor progression of representative i.p. and i.v. 4H11-28z+ T-cell–treated mice with ultimately progressive disease following treatment compared with BLI of tumor progression in a representative control 19-28z+ T-cell–treated mouse. Kaplan-Meier survival curve of SCID-Beige mice treated i.p. or i.v. with 4H11-28z+ T cells. Tumor eradication is enhanced after either i.p. or i.v. infusion of 4H11-28z+ T cells when compared with control-treated mice. Both i.p. and i.v. 4H11-28z+ T-cell–treated mice exhibited statistically enhanced survival when compared with 19-28z+ T-cell–treated control cohorts, whereas survival between the i.p. and i.v. treated 4H11-28z+ T-cell cohorts was not statistically significant (P = 0.22). C, systemically injected CFSE-stained 4H11-28z+ T cells traffic to advanced i.p. OV-CAR(MUC-CD) tumors. Presence of i.v. injected CFSE-labeled 19-28z+ control T cells (left) and 4H11-28z+ T cells (right) 1 d following infusion into SCID-Beige mice with OV-CAR(MUC-CD) tumors injected 7 d earlier as assessed by FACS analysis of single-cell OV-CAR3(MUC-CD) tumor suspensions.
carcinomas (1, 30, 32, 33). The utility of MUC16 as a target antigen for adoptive T-cell therapy is compromised by the fact that most of the extracellular portion of this molecule is cleaved by the tumor cell, secreted, and may be detected in the serum as the CA-125 tumor marker. However, following cleavage of this secreted fraction of MUC16, there remains a residual extracellular fraction of the glycoprotein, termed MUC-CD, which is retained on the tumor cell surface and is therefore an attractive target for immune-based therapies. To this end, we used a murine hybridoma, 4H11, generated to the MUC-CD antigen (38) to construct a first-generation (4H11z) as well as a second-generation costimulatory CAR (4H11-28z) specific to MUC-CD. Significantly, the antigen to the 4H11 antibody is highly expressed on most pretreatment ovarian carcinoma tumor samples obtained from patients treated at our institution as assessed by immunohistochemistry (38, 63).

Consistent with previous studies, we found that T cells transduced to express the second-generation 4H11-28z CAR, but not the first-generation 4H11z CAR, efficiently expanded on coculture with 3T3(MUC-CD) fibroblasts in the absence of exogenous costimulation. This conclusion is further supported by the finding that 4H11-28z T cells secreted significantly higher levels of IL-2, a cytokine indicative of T-cell costimulation, and IFN-γ on coculture on 3T3(MUC-CD) fibroblasts when compared with T cells transduced to express the first-generation 4H11z CAR.

Specificity of the 4H11-28z CAR to the MUC-CD antigen was subsequently verified in vitro by comparing 4H11-28z T-cell cytotoxicity and proliferation on a series of MUC-CD− ovarian carcinoma cell lines, as well as HeLa cells and the T80 immortalized normal ovarian surface epithelial cell line, to the same cell lines further genetically modified to express the MUC-CD antigen. To additionally validate the clinical relevance of our findings, we subsequently showed specific in vitro lysis of primary ascites-derived tumor cells isolated from untreated ovarian carcinoma patients by both healthy donor allogeneic 4H11-28z T cells and, more significantly, autologous 4H11-28z patient ascites-derived T cells. These...
data strongly support the contention that treatment with autologous 4H11-based CAR+ T cells has promise in future clinical applications.

To assess the in vivo relevance of our in vitro findings, we next generated several orthotopic human ovarian cancer tumor models in SCID-Beige mice. These studies showed eradication of early as well as more established BLI evident peritoneal tumors following i.p. injection of healthy donor 4H11-28z+ T cells. In the setting of delayed therapy, tumor imaging by BLI could show initial marked eradication of disease. However, loss of bioluminescent signal in these studies did not preclude future relapse of disease because most apparently tumor-free mice, as assessed by BLI at earlier time points of follow-up, developed relapsed disease within the peritoneum over time, consistent with the notion that BLI lacks the sensitivity to measure in vivo minimal residual disease in demonstrated persistent expression of the targeted MUC-CD antigen, consistent with the notion that loss of target antigen expression by the tumor or immune selection of MUC-CD+ tumors was not the cause of tumor relapse. Although the source of relapsed disease in these mice remains speculative, studies of i.p. infused 4H11-28z+ T-cell persistence show rapidly declining numbers of T cells over time. While modified T cells were still detectable in peritoneal washes out to 28 days after T-cell infusion, these numbers were declining, suggesting a loss of modified T-cell persistence as a potential source of disease relapse, which typically occurred at later time points.

We further show trafficking of i.v. injected MUC-CD-targeted T cells to peritoneal tumors by FACS. Significantly, tumor-bearing mice treated with i.v. infused 4H11-28z+ T cells exhibited similar antitumor efficacy when compared with i.p. treated mice as assessed by combined survival data from two separate experiments using stratified log-rank analysis.

Although insightful, these xenotransplant murine tumor models have significant limitations. Specifically, the biology of human T cells in immunocompromised mice may significantly differ from autologous modified patient T cells in the clinical setting, wherein these T cells encounter an intact immune system, which may elicit an immune response to the CAR, and enter into a hostile tumor microenvironment containing immunosuppressive Tregs, immune-inhibitory cytokines (including IL-10 and transforming growth factor-β), and myeloid-derived suppressor cells (64–66). To address these limitations, we are currently generating a more clinically relevant syngeneic immunocompetent murine tumor model of ovarian carcinoma to further study the in vivo biology, immunogenicity, and antitumor efficacy of MUC-CD-targeted T cells.

A further limitation of xenotransplant models is the inability of these studies to address potential unforeseen off-target toxicities that may occur in the clinical setting, wherein infused CAR-modified T cells recognize antigen not only on tumor cells but also on normal tissues. Based on previously published adverse events in recent clinical trials using CAR-modified T cells, this is a very real concern with respect to the clinical feasibility of this adoptive T-cell approach to cancer therapy. Specifically, three patients with metastatic renal carcinoma treated with autologous T cells transduced to express the G250 CAR specific to the TAA carboxyanhydrase IX developed significant liver toxicity (Common Toxicity Criteria grade 4 in patient 1, grade 2 in patient 2, and grade 3 in patient 3) due to an off-target modified T-cell response to carboxyanhydrase IX expressed by bile duct epithelial cells (20, 53). More recently, Morgan et al. reported the death of a patient with metastatic colon cancer treated with ERBB2-targeted autologous 4D5-CD8-28BBz+ T cells. Following an extensive postmortem analysis, the investigators of this study postulate that the patient’s death resulted from off-target recognition of the targeted ERBB2 antigen expressed by normal lung tissue resulting in marked modified T-cell release of inflammatory cytokines, including tumor necrosis factor-α and IFN-γ, leading to pulmonary toxicity, edema, and a subsequent cascading cytokine storm resulting in multiorgan failure and death (67).

Although similar concerns may be raised in the setting of treating patients with autologous T cells targeted to the MUC-CD antigen, we have conducted extensive immunohistochemical studies using the 4H11 antibody to assess for off-target binding to normal human tissues. Significantly, these studies showed no binding of the 4H11 mAb in normal adult colon, rectum, small intestine, ectocervix, ovary, liver, pancreatic ducts, spleen, kidney, brain, and skin tissues. However, 4H11 did weakly bind cytoplasm of endocervical gland cells and thymic corpuscles, the luminal surface of esophageal glands, as well as intracytoplasmic granules of bronchial epithelium and gastric glands (38). Significantly, these studies failed to show membrane-bound antigen on the vascular surfaces of any normal human tissues. Nevertheless, the potential for off-target toxicity in the clinical setting is not precluded by these studies. To this end, we acknowledge the possible requirement that further safety measures be added in the form of additional T-cell modification with a suicide gene vector to enhance safety in the clinical setting.

In conclusion, herein, we present the first published data showing the feasibility of targeting MUC16, an antigen overexpressed on most ovarian carcinomas, through adoptive therapy with genetically modified T cells targeted to the retained MUC-CD portion of the MUC16 antigen. Further, this report is the first to show efficient targeting of T cells in an orthotopic murine model of ovarian cancer, showing efficacy of a single T-cell infusion of modified T cells in the absence of exogenous IL-2 cytokine support. Collectively, these data support the further planned translation of this approach to the clinical setting in the form of a phase I clinical trial in patients with persistent or relapsed ovarian carcinomas following initial therapy with surgery and chemotherapy.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.
Eradication of Ovarian Carcinomas with Targeted T Cells

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References


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Alena A. Chekmasova, Thapi D. Rao, Yan Nikhamin, et al.

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