**In Vivo Persistence, Tumor Localization, and Antitumor Activity of CAR-Engineered T Cells Is Enhanced by Costimulatory Signaling through CD137 (4-1BB)**

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**Abstract**

Human T cells engineered to express a chimeric antigen receptor (CAR) specific for folate receptor-α (FRα) have shown robust antitumor activity against epithelial cancers *in vitro* but not in the clinic because of their inability to persist and home to tumor *in vivo*. In this study, CARs were constructed containing a FRα-specific scFv (MOv19) coupled to the T-cell receptor CD3ζ chain signaling module alone (MOv19-ζ) or in combination with the CD137 (4-1BB) costimulatory motif in tandem (MOv19-BBζ). Primary human T cells transduced to express conventional MOv19-ζ or costimulated MOv19-BBζ CARs secreted various proinflammatory cytokines, and exerted cytotoxic function when cocultured with FRα tumor cells *in vitro*. However, only transfer of human T cells expressing the costimulated MOv19-BBζ CAR mediated tumor regression in immunodeficient mice bearing large, established FRα human cancer. MOv19-BBζ CAR T-cell infusion mediated tumor regression in models of metastatic intraperitoneal, subcutaneous, and lung-involved human ovarian cancer. Importantly, tumor response was associated with the selective survival and tumor localization of human T cells *in vivo* and was only observed in mice receiving costimulated MOv19-BBζ CAR T cells. T-cell persistence and antitumor activity were primarily antigen-driven; however, antigen-independent CD137 signaling by CAR improved T-cell persistence but not antitumor activity *in vivo*. Our results show that anti-FRα CAR outfitted with CD137 costimulatory signaling in tandem overcome issues of T-cell persistence and tumor localization that limit the conventional FRα T-cell targeting strategy to provide potent antitumor activity *in vivo*. Cancer Res; 71(13); 4617–27. ©2011 AACR.

**Introduction**

Immune targeting of tumor antigens that are overexpressed by cancer cells in numerous cancer types with limited expression in normal tissues holds significant promise for widespread clinical application. Folate receptor-α (FRα) is a glycosylphosphatidylinositol-anchored protein that is overexpressed on the surface of cancer cells in a spectrum of epithelial malignancies, including ovarian, breast, renal, colorectal, lung, and other solid cancers but limited in normal tissues (1–8). FRα overexpression is associated with high-grade tumor progression, poor prognosis in ovarian cancer (9), and poor survival in breast cancer (10), and therefore represents an attractive candidate for targeted biological therapy of epithelial-derived cancers, particularly for epithelial ovarian carcinomas (EOC) where approximately 90% of cancers express FRα.

Chimeric antigen receptors (CAR) or "T-bodies" couple the high affinity binding of antibodies with the signaling domains of the T-cell receptor (TCR) CD3ζ chain for specified triggering of T-cell activation similar to the endogenous TCR (11). Despite promising results in neuroblastoma (12, 13) and lymphoma (14, 15), transfer of T cells genetically redirected with CAR to FRα in a phase I clinical trial for the treatment of metastatic ovarian cancer did not induce tumor regression due to the poor persistence of the gene-modified T cells *in vivo*, a lack of T-cell localization to tumor, and the induction of an undefined T-cell inhibitory factor in the serum of several patients (16). Persistence of tumor antigen–specific T cells after adoptive transfer correlates with tumor regression in patients with advanced metastatic cancer (17), where transferred T cells can localize to regressing lesions (18). Transferred T cells persisting in the blood of responding patients express high levels of costimulatory receptors (19, 20) and response to therapy is associated with the transfer of high numbers of T cells expressing costimulatory receptors (21),
suggesting that provision of costimulatory signals is necessary to facilitate improved T-cell survival and antitumor response \textit{in vivo}. The addition of costimulatory domains, including the intracellular domain of CD28, and TNF receptor family members, CD134 (OX-40) and CD137 (4-1BB) into CARs can significantly augment the ability of these receptors to stimulate cytokine secretion and enhance antitumor efficacy in preclinical animal models of solid tumors and leukemia that lack cognate costimulatory ligands (13, 22–24), and thus rationalizes the incorporation of costimulatory modules in the creation of anti-FRα CAR therapy.

Here, we addressed the issue of limited FRα-specific T-cell persistence and tumor activity \textit{in vivo} through the introduction of the CD137 costimulatory signaling domain into a FRα-specific CAR and studied the role of CD137 signaling in FRα-directed CAR T-cell therapy of human cancer. Compared with ‘first-generation’ CAR that provide CD3ζ signaling to T cells but lack cis costimulatory signaling capacity, T cells expressing FRα-specific CAR with a CD137 signaling domain in tandem showed minimally improved antitumor activity \textit{in vitro}, but markedly superior tumor regression capacity in established human ovarian cancer xenograft models, which was associated with enhanced T-cell persistence and tumor localization \textit{in vivo}. Tumor regression and T-cell persistence were both attainable by various routes of T-cell infusion, and intravenous (i.v.) cell infusion mediates the regression of human cancer in xenograft models of advanced intraperitoneal (i.p.), subcutaneous (s.c.), and lung-involved metastatic disease. T-cell persistence and tumor activity \textit{in vivo} were largely antigen-driven; however, provision of CD137 signaling in the absence of specific antigen recognition by CAR could improve T-cell persistence but not antitumor activity \textit{in vivo}. Incorporation of the CD137 signaling domain in FRα-specific CARs thus overcomes the limitation of past CAR approaches by improving the persistence of transferred T cells \textit{in vivo}, and bolstering their accumulation in tumor and antitumor potency.

Materials and Methods

**Anti-FRα chimeric immune receptor construction**

The MOv19 scFv-based (2, 25, 26) chimeric immune receptor was constructed by using pCLPS lentiviral vector backbone constructs previously described (22). CAR construction and lentivirus production are detailed in Supplementary Materials and Methods.

**Human T cells**

Primary human CD4⁺ and CD8⁺ T cells, which were purchased from the Human Immunology Core at University of Pennsylvania, were isolated from healthy volunteer donors following leukapheresis by negative selection. All specimens were collected under a protocol approved by a University Institutional Review Board, and written informed consent was obtained from each donor. T cells were cultured in complete media (RPMI 1640 supplemented with 10% heat inactivated FBS, 100 U/mL penicillin, 100 µg/mL streptomycin sulfate, 10 mmol/L HEPES), and stimulated with anti-CD3 and anti-CD28 monoclonal antibodies (mAb)-coated beads (Invitrogen) as described (27). Twelve to twenty-four hours after activation, T cells were transduced with lentiviral vectors at multiplicity of infection of approximately 5 to 10. CD4⁺ and CD8⁺ T cells used for \textit{in vivo} experiments were mixed at 1:1 ratio, activated, and transduced. Human recombinant interleukin-2 (IL-2; Novartis) was added every other day to a 50 IU/mL final concentration and a cell density of 0.5 × 10⁶ to 1 × 10⁶ cells/mL was maintained. Once T cells seemed to rest down, as determined by both decreased growth kinetics and cell sizing by using the Multisizer 3 Coulter Counter (Beckman Coulter), engineered T-cell cultures were adjusted to equalize the frequency of transgene expressing cells prior to functional assays.

**Functional assays**

Cytokine release assays were carried out by an IFN-γ ELISA Kit (Biolegend) or by Cytometric Bead Array, according to manufacturer’s instructions (BD Biosciences) as described in Supplementary Materials and Methods. Cell-based bioluminescence and ⁵¹Cr release assays of cytolysis were carried out as previously described (28, 29).

**Xenograft model of ovarian cancer**

Mouse studies were carried out as previously described (22, 30) with modifications detailed in Supplementary Materials and Methods.

**Immunohistochemistry**

Fresh frozen tumor samples were sectioned for immunohistochemical analysis as described in Supplementary Materials and Methods.

**Statistical analysis**

Statistical analysis was carried out by 2-way repeated measures ANOVA for the tumor burden (tumor volume, photon counts). Student’s \( t \)-test was used to evaluate differences in absolute number of transferred T cells, cytokine secretion, and specific cytolysis. Kaplan–Meier survival curves were compared by using the log-rank test. GraphPad Prism 4.0 (GraphPad Software) was used for the statistical calculations. \( P < 0.05 \) was considered significant.

Results

**CAR construction**

The mouse anti-human FRα-specific scFv MOv19 was selected on the basis of its high binding affinity for FRα (10⁻⁸–10⁻⁹ M⁻¹; refs. 2, 25, 26). FRα CAR constructs were comprised of the MOv19 scFv linked to a CD8z hinge and transmembrane region, followed by a CD3ζ signaling moiety alone (MOv19-ζ) or in tandem with the CD137 intracellular signaling motif (MOv19-BBζ; Fig. 1A). A signaling deficient FRα-specific CAR containing a truncated CD3ζ intracellular domain (MOv19-Aζ) was designed to assess the contribution of CD3ζ signaling. An anti-CD19 CAR containing CD3ζ and CD137 signaling motifs in tandem (anti-CD19-BBζ) was used as an antigen specificity control (30). CAR constructs were...
subcloned into the pCLPS lentiviral vector where transgene expression is driven off the cytomegalovirus promoter. Using gene transfer technology established for clinical application, lentiviral vectors efficiently transduced primary human T cells to express the anti-FRα CAR (Fig. 1B). T-cell transduction efficiency, as assessed by flow cytometry, was equilibrated for all constructs at approximately 50% in all assays.

**Primary human FRα CAR T cells exert antigen-specific function in vitro**

Because ovarian cancer frequently express FRα (2), a panel of established human ovarian cancer cell lines that express surface FRα at varying levels (SKOV3, A1847, and OVCAR3) was selected for assays (Fig. 1C). Two ovarian cancer lines, C30 and PEO-1, were negative for FRα. Transduced T cells expressing MOv19-BBζ or MOv19-ζ CARs recognized FRα⁺ tumor lines and secreted high levels of IFN-γ, but not when stimulated with FRα⁻ lines (Fig. 1D). FRα-specific CAR T cells also secreted high levels of IL-2 and TNF-α when stimulated with FRα⁺ cancer cells and low but detectable levels of IL-4 and IL-10 (Supplementary Fig. S1). MOv19 CARs functioned in both primary human CD4⁺ and CD8⁺ T cells. In all cases, MOv19-BBζ T cells secreted more IFN-γ than MOv19-ζ T cells after specific stimulation. CD19-BBζ CAR did not produce IFN-γ, except when co-incubated with K562 cells engineered to express surface CD19 antigen, and human T cells expressing MOv19-Δζ CAR did not secrete cytokine when stimulated with FRα⁺ cancer cells (Fig. 1D), showing that antigen specificity and CD3ζ signaling are required for CAR activity in T cells.
To interrogate antigen-specific cytolytic potential, anti-FRα CAR CD8⁺ T cells were cocultured with FRα⁻ AE17 (31), a mouse malignant mesothelioma cell line, or AE17:FRα (an AE17 line derivative transduced to express high surface levels of human FRα). In standard 4-hour chromium release and 24-hour bioluminescence assays, FRα-specific CAR T cells (MOv19-ζ and MOv19-BBζ) specifically lysed AE17:FRα cells but not the parental AE17 line (Supplementary Fig. S2). T cells expressing anti–CD19-BBζ, MOv19-Δζ, or green fluorescent protein (gfp) did not lyse AE17:FRα or AE17 cells. Consistent with cytokine production results, primary human CD8⁺ T cells expressing MOv19-ζ or MOv19-BBζ CAR directly and efficiently lysed FRα⁺ human ovarian cancer cell lines SKOV3 and A1847, but not FRα⁻ lines C30 or 624mel, a melanoma cell line (Fig. 1E). MOv19-BBζ CAR T cells exhibited increased cytotoxicity compared with MOv19-ζ CAR T cells, but not at a level of statistical significance. Thus, human T cells transduced with FRα-specific CAR specifically recognize FRα⁺ human and mouse cancer cells and exert MHC-unrestricted cytotoxic activity in vitro.

**Antitumor activity of primary human FRα CAR T cells in vivo**

CAR functional activity in vitro cannot accurately predict the antitumor potential of transduced human T cells in vivo. The antitumor efficacy of FRα CAR constructs were evaluated in a xenograft model of large, established cancer. Immunodeficient NOD/SCID/IL-2Rγc⁺/(NSG) mice were inoculated s.c. with firefly luciferase (fluc)-transfected FRα⁺ SKOV3 human ovarian cancer cells on the flank and received intratumoral (i.t.) injections of CAR⁺ T cells on days 40 and 45 post-tumor inoculation (p.i.), when tumors were 250 mm² or more in size. Tumors in mice receiving saline, MOv19-Δζ CAR T cells, or gfp T cells progressed beyond the time of T cell transfer as measured by caliper-based sizing and bioluminescence imaging (BLI; Fig. 2A and B). Tumor growth was modestly delayed in mice receiving MOv19-ζ T cells (P = 0.027), compared with all 3 control groups at the latest evaluated time point (38 days after first T-cell dose). In contrast, mice receiving i.t. injection of MOv19-BBζ T cells experienced rapid tumor regression, which was significantly better than MOv19-ζ T cells (P < 0.001), indicating that incorporation of CD137 signals enhances overall antitumor activity in vivo. Tumor-bearing mice treated with MOv19-BBζ–transduced T cells delivered i.v., i.p. injection, or i.t. routes experienced tumor regression (Fig. 2C). Following i.v. or i.p. infusion of MOv19-BBζ T cells, antitumor activity was again observed, though delayed in regression by approximately 7 days relative to i.t. delivery, indicating that although local injection is optimal, systemically infused CAR T cells can marginalize upon adoptive transfer to mediate potent antitumor effects in vivo.

**Persistence of primary human FRα CAR T cells in vivo is increased by 4-1BB signals**

The persistence of transferred tumor-reactive T cells following adoptive T-cell therapy is highly correlated with tumor regression (17). In the experiments described earlier, peripheral blood was collected from tumor-bearing mice 3 weeks after the last T-cell dose and quantified for persistent human CD4⁺ and CD8⁺ T cells (Fig. 2D). CD4⁺ and CD8⁺ T-cell counts were highest in mice receiving MOv19-BBζ CAR T cells, whether delivered by i.t., i.p., or i.v. routes of administration, compared with gfp, MOv19-Δζ, and MOv19-ζ treatment groups. Notably, human T-cell counts in mice receiving MOv19-BBζ CAR T cells by i.v. injection was significantly higher than those in the parallel MOv19-ζ CAR group (P < 0.01), indicating a role for CD137 in T-cell survival in vivo. There was no significant difference in level of T-cell persistence among mice receiving MOv19-BBζ CAR T cells by i.v., i.t., or i.p. injection (P = 0.2), despite a trend toward less cells in the i.v. injection group. Total T-cell counts in the MOv19-ζ treatment group was statistically similar to other control groups including mice receiving saline in the absence of human T-cell injection (Supplementary Fig. S3; P > 0.05), suggesting that antigen specificity alone is not sufficient for T-cell maintenance in vivo. This was primarily attributed to poor CD4⁺ T-cell persistence because circulating MOv19-ζ CAR CD8⁺ T cells persisted at greater numbers than MOv19-Δζ CAR (P = 0.026) or gfp (P = 0.013) cells. Four weeks after last MOv19-BBζ CAR T-cell dose, the absolute number of human T cells persisting in the blood was inversely correlated with tumor burden of each group (Supplementary Fig. S3; r = −0.78). Tumor BLI results were consistent with the size of resected residual tumors (Supplementary Fig. S4). Mechanistically, enhanced persistence of MOv19-BBζ CAR T cells, compared with MOv19-ζ, seemed to be attributed in part to an increased upregulation of antiapoptotic Bcl-X protein expression after antigen stimulation (Supplementary Fig. S3). Thus, tumor regression was associated with the stable persistence of engineered human T cells in vivo and supported by provision of CD137 costimulation.

**Tumor regression and T-cell persistence are antigen-driven in vivo**

To determine whether MOv19-BBζ CAR antitumor activity is antigen-specific, a comparative study was conducted with an anti-CD19–specific CAR also containing the CD137 signaling domain (30). NSG mice with established s.c. SKOV3 fluc⁺ tumor receiving 2 i.t. CAR T-cell injections experienced rapid tumor regression, whereas tumor grew progressively in mice treated with T cells expressing gfp or CD19-BBζ CAR (Fig. 3A), excluding alloreactivity as a mechanism of tumor regression. Mice receiving MOv19-BBζ T cells had significantly higher human CD4⁺ and CD8⁺ T-cell counts than mice in anti-CD19 CAR or gfp groups (Fig 3B; P = 0.009), indicating that tumor antigen recognition drives the survival of the adoptively transferred T cells in vivo. Interestingly, T-cell persistence was reproducibly higher in mice receiving anti-CD19-BBζ CAR T cells than gfp T cells (P = 0.012), suggesting that persistence of CAR T cells can be promoted in part through a CD137-driven process that does not require scFv engagement with antigen. Nevertheless, there was no statistical difference in tumor control between anti-CD19-BBζ CAR and gfp groups (P = 0.065) even at the latest time point studied (day 73), showing that persistence in the absence of antigen specificity is insufficient to mediate tumor response. In this line, CAR
expressing T-cell frequency in the blood of tumor-bearing mice administered MOv19-BBζ T cells was higher than that observed in mice receiving CD19-BBζ CAR T cells, though not at statistical significance (Fig. 3C; P = 0.08). However, coupled with increased T-cell counts, the total number of circulating CAR⁺ T cells persisting 1 month after infusion were significantly higher in mice receiving MOv19-BBζ T cells (76 ± 13 cells/μL; P = 0.013); mice in CD19-BBζ CAR and gfp groups had little to no detectable persistence of CAR⁺ T cells with counts of 12 ± 4 cells/μL and 0 ± 0 cells/μL, respectively (Fig. 3D). Consistent with the increased persistence of MOv19-BBζ T cells in the blood of treated animals, immunohistochemical analysis revealed robust accumulation of human CD3⁺ T cells in regressing SKOV3 lesions 6 weeks after i.v. T-cell administration (Fig. 4). Few CD3⁺ T cells were detected in tumors resected at the same time from mice that received anti–CD19-BBζ CAR or gfp-transduced T cells.

**Tumor regression in the metastatic disease setting**

Advanced ovarian cancer is a disease usually confined to the peritoneal cavity with occasional metastatic spread to the pleural compartment. A xenogeneic model of advanced i.p. metastatic cancer was established to evaluate the functional activity of FRα-specific T cells against tumor localized to a more physiologically relevant compartment. NSG mice that were inoculated i.p. with SKOV3 fLuc⁺ cells efficiently developed peritoneal carcinomatosis which was readily evident 30 days p.i., when MOv19-BBζ or control anti–CD19-BBζ CAR T-cell therapy was administered (Fig. 5A). Within 3 weeks of T-cell transfer, all mice that received control anti–CD19-BBζ...
CAR T cells developed distended abdomens, marked bloody ascites of approximately 5 to 8 mL volume and multiple nodular peritoneal tumors, and had to be euthanized due to tumor-associated, abdominal distention (Fig. 5B and C). By the time of euthanasia of mice treated with MOv19-BBζ CAR T cells, compared with 9 and 12 days in the anti–CD19-BBζ i.p. vs. anti–CD19-BBζ i.v. groups, respectively (MOv19-BBζ i.p., P = 0.0023; MOv19-BBζ i.v. vs. anti–CD19-BBζ i.v., P = 0.0025; Fig. 5D). Two months after treatment with MOv19-BBζ CAR cells via i.p. or i.v. routes, 60% (3 of 5) and 40% (2 of 5) of tumor-inoculated mice remained alive, respectively.

Occasionally, ovarian cancer patients develop lung metastases and pleural ascites formation requiring thoracentesis or other supportive management procedures during disease progression (33). A model of metastatic ovarian cancer of lung was generated by inoculation of NSG mice with SKOV3 fluc+ cells (34). We selected CD137 on the basis of its demonstrated capacity to support of CD8 T-cell expansion (36), and upregulate important anti-apoptotic protein Bcl-xL expression (37), and results showing of lung metastasis in all treated animals 14 days p.i. and 80% (4 of 5) of mice had no evidence of recurrence after 1 month. By contrast, disease progression occurred in all mice receiving anti–CD19-BBζ T cells.

Discussion

CARs combine the high affinity and specificity of antigen-specific antibody, which binds cell surface determinants in a non–MHC-restricted manner, with the potent effector functions of T lymphocytes (11). Genetically retargeting of primary human lymphocytes with CARs recognizing tumor-associated antigens offers a robust and rapid avenue toward the generation of tumor-reactive T cells for therapy. To date, CAR-based therapy has shown promising but often limited clinical activity, despite the reproducible demonstration of strong effector activity in vitro (12–16). Effective adoptive T-cell therapy not only requires antitumor activity, but also in vivo expansion and persistence of the infused tumor-reactive T cells (17). In our study, we have addressed the central issue of limited CAR T-cell persistence and tumor activity in vivo (16) through the introduction of the CD137 (4-1BB) costimulatory signaling domain into a Mov19 scFv-based CAR.

CD137 is a TNF receptor family member that plays an important role in T-cell proliferation and survival, particularly for T cells within the memory T-cell pool (34–36). We selected CD137 on the basis of its demonstrated capacity to support of CD8 T-cell expansion (36), and upregulate important anti-apoptotic protein Bcl-xL expression (37), and results showing
that adoptive transfer of tumor-specific T cells costimulated ex vivo with 4-1BBL supports persistence and antitumor activity in vivo (38). This work builds upon our previous study which showed high costimulatory receptor expression on tumor antigen–specific T cells persisting in the circulation in patients responding to adoptive immunotherapy (19), and correlations between costimulatory receptor expression on transferred T cells and tumor regression (20;21). Like the "first-generation" Mov19-ζ CAR expressing CD3ζ signaling alone, T cells engineered to express a "second-generation" Mov19-BBζ CAR containing CD3ζ signaling and a CD137 signaling domain in tandem preferentially secrete high levels of Th1 cytokines including IFN-γ, TNF-α, and IL-2 upon tumor encounter and exert strong antitumor activity in vitro. Here, IFN-γ cytokine production levels were generally associated with the level of FRα expression by tumor cell targets, and cytolyis of tumor cells by Mov19-ζ CAR and Mov19-BBζ CAR T cells was efficient even at a 3:1 effector to target cell (E/T) ratio in vitro. In all in vitro antitumor assays, engineered T cells expressing Mov19-BBζ CAR outperformed Mov19-ζ CAR T cells, albeit not always to the level of statistical significance. Interestingly, the single exception was in the level of Th2 cytokine secretion induced by tumor stimulation, where FRα engagement by Mov19-ζ CAR T cells induced greater IL-4 and IL-10 production, suggesting that combined CD3ζ and CD137 signaling enforces a Th1 skewed response.

The dichotomy between first- and second-generation CAR vectors was most evident in in vivo studies where CD137 bearing Mov19-BBζ CAR T cells facilitated superior regression of large vascularized tumors in an established human ovarian cancer xenograft model, whereas tumor progression was almost unabated with Mov19-ζ CAR T cells. Transfer of 16 × 10⁶ total Mov19-BBζ CAR T cells eliminated an estimated 2.5 × 10⁸ tumor cells (assuming that a 250 mm³ tumor mass contains approximately 2.5 × 10⁸ cells); in effect, an approximately 1:15 E/T ratio. Consistent with previous clinical observations (18, 39), tumor response was associated with enhanced T-cell persistence and tumor localization of Mov19-BBζ CAR T cells in vivo, which seemed to be attributed in part to upregulated expression of Bel-XL following stimulation with tumor. Tumor regression was antigen-specific, as transfer of anti–CD19-BBζ T cells had no impact on tumor progression. Tumor regression and T-cell persistence were attainable via systemic or local T-cell delivery, showing the capacity of transferred T cells to circulate, home to tumor and perform antitumor functions. Although i.v. injections are favorable in clinical application due to the ease of administration and effective in our model, our data suggest that local administration of T cells may provide optimal therapeutic effect, which may be in part due to increased T-cell trafficking to tumor and provision of favorable E/T ratios. However, such delivery may not be applicable for tumors with multiple gross metastatic sites or micrometastases.

Although Mov19-BBζ and anti–CD19-BBζ T cells could be detected in the peripheral blood 3 weeks after T-cell infusion,
the accumulation of Mov19-BBζ, but not anti–CD19-BBζ T cells, in FRα+ tumor lesions suggests that antigen-selective retention of CAR bearing T cells in tumor occurs and may be requisite in part for tumor regression (40). In a previous study, transferred TCR transgenic T cells migrated indiscriminately early after adoptive transfer but experienced antigen-dependent activation exclusively in antigen-positive tumor resulting in tumor destruction (41). Transfer of chemokine receptor expressing CAR T cells can enforce preferential migration to tumor sites to boost antitumor activity in vivo (42). Our results support the hypothesis that T-cell persistence, localization, and tumor activity in vivo are largely antigen-dependent, likely linked, processes. Notably, the use of anti–CD19-BBζ T cells as specificity control in our assays, however, shows that provision of CD137 signaling by CAR permitted improved T-cell persistence but not antitumor activity in vivo through a mechanism that is independent of scFv engagement with antigen, suggestive of low-level constitutive activity by the CD137 module, consistent with previous data (30). In this scenario, it remains possible that persistence of nonspecific CD137-costimulated human T cells was driven by low-level TCR recognition of xenoantigens in mice combined with constitutive CD137 signaling by CAR, as shown by the occurrence of graft-versus-host manifestations, which is an inherent limitation of the xenogeneic NSG mouse model used.

T-cell–based targeting of FRα has been tested in patients with advanced ovarian cancer with promising results. In an earlier clinical study, retargeted T cells were generated for therapy by loading preactivated T cells with a bispecific mouse mAb OC/TR, directed to the CD3 molecule on T lymphocytes and to FRα on EOC cells (43). Administration of FRα-redirected T cells to women with minimal residual ovarian cancer...
resulted in antitumor responses in 27% of patients with mild to moderate immunotherapy-related toxicities; however, therapy was limited by the inability to generate stable anti-FRα-specific T-cell memory and the induction of human anti-mouse antibodies against the bispecific mAb in approximately 90% of treated patients (44). In a phase I study of anti-FRα CAR therapy for cancer, Kershaw and colleagues (16) transferred T cells that were retargeted to FRα by a first-generation MOv18 scFv-based CAR to immunocompetent patients with advanced ovarian cancer. The parental MOv18 antibody has a similar affinity for FRα (10^8–10^9 M^-1) as MOv19 used in our CAR construct (2, 26) though the relative affinities of their scFv products in CARs is not known. MOv18 and MOv19 also bind non–cross-reactive epitopes (2), which may influence their relative ability to access surface antigen. Therapy using MOv18-ζ CAR was safe and feasible; however, no patient experienced a tumor response which was attributed to a lack of transferred T-cells persistence after infusion, poor tumor localization, and the development of a serum inhibitory factor that reduced CAR T-cell activity in vivo study (16). Our studies, combined with recent clinical strategies, address these issues. Similar to the study of Kershaw and colleagues (16), first-generation MOv19-ζ CAR, which redirected T-cell cytotoxicity in vitro, only delayed tumor progression in vivo and CARs did not persist long-term in vivo. We show that tumor response and T-cell persistence can be evoked by provision of CD137 costimulatory signals to anti-FRα CAR T cells, which is facilitated principally by engagement of their CAR with tumor antigen. Moreover, transfer of MOv19-BBζ T cells leads to increased accumulation of human T cells in regressing ovarian cancer lesions. Although the mouse anti-human MOv19 scFv used in the construction of the MOv19-BBζ CAR is likely to elicit anti-mouse humoral responses in immunocompetent recipients, as seen in past CAR studies and trials using MOv18 scFv (16, 44, 45), nonmyeloablative immunosuppressive preconditioning can disable host endogenous immunity to promote the in vivo persistence of T cells expressing CARs and TCRs of mouse origin, facilitating tumor regression (14, 46, 47). The use of immunodeficient NSG mice models T-cell transfer in the setting of host lymphodepletion, albeit in the absence of human derivatives and endogenous immune reconstitution. Still, the use of fully human anti-FRα scFv candidates for the next generation of CAR-redirected therapy is worthy of investigation (26, 48). Our preclinical results support the notion that incorporation of the CD137 signaling domain in FRα-specific CARs overcomes the limitations of past CAR approaches by improving the persistence of transferred T cells in vivo, thereby increasing their retention in tumor and bolstering antitumor potency. Careful considerations must be made when targeting of self/tumor antigens with CARs or exogenous TCRs, which hold the potential for mediating serious adverse events (47, 49); however, FRα, which is present on normal tissues, is localized primarily to the apical surfaces of polarized epithelia, where it may be inaccessible to parenterally administered folate conjugates and redirected T cells (50). Our results provide the rationale for the clinical investigation of MOv19-BBζ CAR T-cell therapy in combination with lymphodepleting preconditioning.
regimens for the treatment of a wide spectrum of FRα-expressing epithelial malignancies.

**Disclosure of Potential Conflict of Interest**

No potential conflicts of interest were disclosed.

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