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In vivo persistence, tumor localization and anti-tumor 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 (FR α) have shown robust anti-tumor activity against epithelial cancers *in vitro* but not in the clinic due to their inability to persist and home to tumor *in vivo*. In this study, CARs were constructed containing a FR α -specific scFv (MOv19) coupled to the T cell receptor CD3 ζ chain signaling module alone (MOv19- ζ) or in combination with the CD137 (4-1BB) costimulatory motif in tandem (MOv19-BB ζ). Primary human T cells transduced to express conventional MOv19- ζ or costimulated MOv19-BB ζ CARs secreted various proinflammatory cytokines and exerted cytotoxic function when cocultured with FR α + tumor cells *in vitro*. However, only transfer of human T cells expressing the costimulated MOv19-BB ζ CAR mediated tumor regression in immunodeficient mice bearing large, established FR α + human cancer. MOv19-BB ζ 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-BB ζ CAR T cells. T cell persistence and anti-tumor activity were primarily antigen-driven; however, antigen-independent CD137 signaling by CAR did improve T cell persistence but not anti-tumor activity *in vivo*. Our results show that anti-FR α CAR outfitted with CD137 costimulatory signaling in tandem overcome issues of T cell persistence and tumor localization that limit the conventional FR α T cell targeting strategy to provide potent antitumor activity *in vivo*.

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Introduction

Immune targeting of tumor antigens that are over-expressed by cancer cells in numerous cancer types with limited expression in normal tissues holds significant promise for widespread clinical application. Folate receptor-alpha (FR α) 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). FR α overexpression is associated with high-grade, tumor progression and poor prognosis in ovarian cancer (9) and poor survival in breast cancer (10), and therefore represents an attractive candidate for targeted biologic therapy of epithelial-derived cancers, particularly for epithelial ovarian carcinomas (EOC) where nearly 90% of cancers express FR α .

Chimeric antigen receptors (CAR) or “T-bodies” couple the high affinity binding of antibodies with the signaling domains of the T cell receptor (TCR) CD3 ζ 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 re-directed with CAR to FR α 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 anti-tumor response *in vivo*. The addition of costimulatory domains, including the intracellular domain of CD28 and tumor necrosis factor 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-FR α CAR therapy.

Here, we addressed the issue of limited FR α -specific T cell persistence and tumor activity *in vivo* through the introduction of the CD137 co-stimulatory signaling domain into a FR α -specific CAR and studied the role of CD137 signaling in FR α -directed CAR T cell therapy of human cancer. Compared to “first generation” CAR that provide CD3 ζ signaling to T cells but lack cis co-stimulatory signaling capacity, T cells expressing FR α -specific CAR with a CD137 signaling domain in tandem showed minimally improved anti-tumor activity *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 *in vivo*. Tumor regression and T cell persistence were both attainable by various routes of T cell infusion, and intravenous cell infusion mediates the regression of human cancer in xenograft models of advanced intraperitoneal, subcutaneous, and lung-involved metastatic disease. T cell persistence and tumor activity *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 anti-tumor activity *in vivo*. Incorporation of the CD137 signaling domain in FR α -specific CARs thus overcomes the limitation of past CAR approaches by improving the persistence of transferred T cells *in vivo*, and bolstering their accumulation in tumor and antitumor potency.

Materials and Methods

Anti-FR α chimeric immune receptor construction

The MOv19 scFv-based (2;25;26) chimeric immune receptor was constructed 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 University Institutional Review Board-approved protocol, and written informed consent was obtained from each donor. T cells were cultured in complete media (RPMI 1640 supplemented with 10% heat inactivated fetal bovine serum (FBS), 100 U/ml penicillin, 100 ug/ml streptomycin sulfate, 10-mM HEPES), and stimulated with anti-CD3 and anti-CD28 mAbs coated beads (Invitrogen) as described (27). 12-24hr after activation, T cells were transduced with lentiviral vectors at MOI of ~5-10. CD4⁺ and CD8⁺ T cells used for *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 0.5-1 $\times$ 10⁶ cells/ml was maintained. Once T cells appeared to rest down, as determined by both decreased growth kinetics and cell sizing 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 performed using an interferon-gamma (IFN- γ) ELISA Kit (Biolegend) or using 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 cytotoxicity were performed as previously described (28;29).

Xenograft model of ovarian cancer

Mouse studies were performed 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 performed using two-way repeated measures ANOVA for the tumor burden (tumor volume, photon counts). Student's *t* test was used to evaluate differences in absolute numbers of transferred T cells, cytokine secretion and specific cytotoxicity. Kaplan-Meier survival curves were compared 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 FR α -specific scFv MOv19 was selected based upon its high binding affinity for FR α (10^8 - 10^9 M⁻¹) (2;25;26). FR α CAR constructs were comprised of the MOv19 scFv linked to a CD8 α hinge and transmembrane region, followed by a CD3 ζ signaling moiety alone (MOv19- ζ) or in tandem with the CD137 intracellular signaling motif (MOv19-BB ζ ; Fig. 1A). A signaling deficient FR α -specific CAR containing a truncated CD3 ζ intracellular domain (MOv19- ζ) was designed to assess the contribution of CD3 ζ signaling. An anti-CD19 CAR containing CD3 ζ and CD137 signaling motifs in tandem (anti-CD19-BB ζ) was used as an antigen specificity control (30). CAR constructs were subcloned into the pCLPS lentiviral vector where transgene expression is driven off the CMV promoter. Using gene transfer technology established for clinical application, lentiviral vectors efficiently transduced primary human T cells to express the anti-FR α CAR (Fig. 1B). T-cell transduction efficiency, as assessed by flow cytometry, was equilibrated for all constructs at ~50% in all assays.

Primary human FR α CAR T cells exert antigen specific function *in vitro*

Since ovarian cancer frequently express FR α (2), a panel of established human ovarian cancer cell lines that express surface FR α at varying levels (SKOV3, A1847 and OVCAR3) was selected for assays (Fig. 1C). Two ovarian cancer lines, C30 and PEO-1, were negative for FR α . Transduced T cells expressing MOv19-BB ζ or MOv19- ζ CARs recognized FR α + tumor lines and secreted high levels of IFN- γ , but not when stimulated with FR α -negative lines (Fig. 1D). FR α -specific CAR T cells also secreted high levels of IL-2 and TNF- α when stimulated with FR α + 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-BB ζ T cells secreted more IFN- γ than MOv19- ζ T cells after specific stimulation. CD19-BB ζ 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- ζ CAR did not secrete cytokine when stimulated with FR α + cancer cells (Fig. 1D), demonstrating that antigen specificity and CD3 ζ signaling are required for CAR activity in T cells.

To interrogate antigen-specific cytolytic potential, anti-FR α CAR CD8+ T cells were cocultured with FR α -negative AE17 (31), a mouse malignant mesothelioma cell line, or AE17.FR α (an AE17 line derivative transduced to express high surface levels of human FR α). In standard 4hr chromium release and 24hr bioluminescence assays, FR α -specific

CAR T cells (MOv19- ζ and MOv19-BB ζ) specifically lysed AE17.FR α cells but not the parental AE17 line (Supplementary Fig. S2). T cells expressing anti-CD19-BB ζ , MOv19- ζ or gfp did not lyse AE17.FR α or AE17 cells. Consistent with cytokine production results, primary human CD8 $^{+}$ T cells expressing MOv19- ζ or MOv19-BB ζ CAR directly and efficiently lysed FR α + human ovarian cancer cell lines SKOV3 and A1847, but not FR α -negative lines C30 or 624mel, a melanoma cell line (Fig. 1E). MOv19-BB ζ CAR T cells exhibited increased cytotoxicity compared to MOv19- ζ CAR T cells, but not at a level of statistical significance. Thus, human T cells transduced with FR α -specific CAR specifically recognize FR α + human and mouse cancer cells and exert MHC-unrestricted cytotoxic activity *in vitro*.

Antitumor activity of primary human FR α CAR T cells *in vivo*

CAR functional activity *in vitro* cannot adequately predict the antitumor potential of transduced human T cells *in vivo*. The antitumor efficacy of FR α CAR constructs were evaluated in a xenograft model of large, established cancer. Immunodeficient NOD/SCID/IL-2R γ ^{null} (NSG) mice were inoculated s.c. with firefly luciferase (fLuc) transfected FR α + SKOV3 human ovarian cancer cells on the flank and received intratumoral injections of CAR+ T cells on day 40 and 45 post-tumor inoculation (p.i.), when tumors were 250 mm³ in size. Tumors in mice receiving saline, MOv19- ζ 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 2. A, B). Tumor growth was modestly delayed in mice receiving MOv19- ζ T cells ($P=0.027$), compared to all three control groups at the latest evaluated time point (38 days after first T cell dose). In contrast, mice receiving intratumoral injection of MOv19-BB ζ 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 anti-tumor activity *in vivo*. Tumor-bearing mice treated with MOv19-BB ζ transduced T cells delivered via intravenous (i.v.), intraperitoneal (i.p.) injection or intratumoral (i.t.) routes experienced tumor regression (Fig. 2C). Following i.v. or i.p. infusion of MOv19-BB ζ T cells, anti-tumor activity was again observed, though delayed in regression by ~7 days relative to i.t. delivery, indicating that, while 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 FR α 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 above, peripheral blood was collected from tumor-bearing mice three 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-BB ζ CAR T cells, whether delivered by i.t., i.p. or i.v. routes of administration, compared to gfp, MOv19- ζ and MOv19- ζ treatment groups. Notably, human T cell counts in mice receiving MOv19-BB ζ CAR T cells by i.v. injection was significantly higher than 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-BB ζ 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 since circulating MOv19- ζ CAR CD8⁺ T cells did persist at greater numbers than MOv19- ζ CAR ($P = 0.026$) or gfp ($P = 0.013$) cells. Four weeks after last MOv19-BB ζ 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-BB ζ CAR T cells, compared to MOv19- ζ , appeared to be attributed in part to an increased upregulation of anti-apoptotic Bcl-X_L 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-BB ζ CAR anti-tumor activity is antigen specific, comparative study with an anti-CD19-specific CAR also containing the CD137 signaling domain (30) was performed. NSG mice with established s.c. SKOV3 fLuc+tumor receiving two i.t. T cell injections experienced rapid tumor regression, while tumor grew progressively in mice treated with T cells expressing gfp or CD19-BB ζ CAR (Fig. 3A), excluding alloreactivity as a mechanism of tumor regression. Mice receiving MOv19-BB ζ 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-BB ζ CAR T cells compared to 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-BB ζ CAR and gfp groups ($P = 0.065$) even at the latest time point studied (day 73), demonstrating 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-BB ζ T cells was higher than that observed in mice receiving CD19-BB ζ 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 one month after infusion was significantly highest in mice receiving MOv19-BB ζ T cells (76 ± 13 cells/ul; $P = 0.013$); mice in CD19-BB ζ CAR and gfp groups had little to no detectable persistence of CAR⁺ T cells with counts of 12 ± 4 cells/ul and 0 ± 0 cells/ul, respectively (Fig. 3D). Consistent with the increased persistence of MOv19-BB ζ 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-BB ζ 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 intraperitoneal metastatic cancer was established to evaluate the functional activity of FR α -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-BB ζ or control anti-CD19-BB ζ CAR T cell therapy was administered (Fig. 5A). Within three weeks of T cell transfer, all mice that received control anti-CD19-BB ζ CAR T cells developed distended abdomens, marked bloody ascites of ~5-8ml volume and multiple nodular peritoneal tumors, and had to be euthanized due to tumor-associated, abdominal distention (Fig. 5B,C). By comparison, mice treated with MOv19-BB ζ CAR T cells did not develop distended abdomens or ascites, and exhibited a profound enhancement in tumor-related survival ($p = 0.0002$) with no cases of tumor-related mortality in the MOv19-BB ζ CAR group (Fig. 5C). At the time of euthanasia of mice treated with MOv19-BB ζ , tumor burden was minimal to none, but mice required euthanizing due to signs of distress compatible with graft-versus-host disease that develops in NSG mice following xenogeneic transfer of activated human lymphocytes (32). Still, median survival times of 52 days after last T cell infusion by i.v. injection and 68 days by the i.p. route were observed in MOv19-BB ζ CAR treated mice, compared to 9 and 12 days in the anti-CD19-BB ζ CAR T cell groups, respectively (MOv19-BB ζ i.p. vs anti-CD19-BB ζ i.p., $P = 0.0023$; MOv19-BB ζ i.v. vs anti-CD19-BB ζ i.v., $P = 0.0025$; Fig. 5C). Two months after treatment with MOv19-BB ζ CAR cells via i.p. or i.v. routes, 60% (3/5) and 40% (2/5) 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 via tail-vein injection resulting in progressive lung metastases in 100% of mice 3 days p.i. (Fig. 6). Two i.v. injections of MOv19-BB ζ T cells resulted in rapid regression of lung metastasis in all treated animals 14 days p.i. and 80% (4/5) of mice had no evidence of recurrence after one month. By contrast, disease progression occurred in all mice receiving anti-CD19-BB ζ T cells.

Discussion

Chimeric antigen receptors (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 (TAAs) 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) co-stimulatory 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 based on its demonstrated capacity to support of CD8 T cell expansion(36), and upregulate important anti-apoptotic protein Bcl-X_L 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 prior study which demonstrated 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 anti-tumor activity *in vitro*. Here, IFN-γ cytokine production levels generally were associated with the level of FRα expressed by tumor cell targets and cytolysis of tumor cells by Mov19-ζ CAR and Mov19-BBζ CAR T cells was efficient even at a 3:1 E:T ratio *in vitro*. In all *in vitro* anti-tumor 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, while tumor progression was nearly unabated with Mov19-ζ CAR T cells. Transfer of 16×10^6 total Mov19-BBζ CAR T cells eliminated an estimated 2.5×10^8 tumor cells (assuming that a 250 mm³ tumor mass contains about 2.5×10^8 cells); in effect, a ~1:15 effector to target cell 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 appeared to be attributed in part to upregulated expression of Bcl-X_L following stimulation with tumor. Tumor regression was antigen-specific as transfer of anti-CD19BBζ T cells had no impact on tumor progression. Tumor regression and T cell persistence were attainable via systemic or local T cell delivery, demonstrating the capacity of transferred T cells to circulate, home to tumor and perform anti-tumor functions. While intravenous injections are favorable in clinical application due to the ease of administration and effective in our model, our data suggests that local administration of T cells may provide optimal therapeutic effect, which may be due in part to increased T cell trafficking to tumor and provision of favorable effector to target cell ratios. However, such delivery may not be applicable for tumors with multiple gross metastatic sites or micrometastases.

While Mov19-BB ζ and anti-CD19-BB ζ T cells could be detected in the peripheral blood 3 weeks after T cell infusion, the accumulation of Mov19-BB ζ , but not anti-CD19-BB ζ T cells, in FR α + 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 prior 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 anti-tumor 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-BB ζ T cells as specificity control in our assays however shows that provision of CD137 signaling by CAR permitted improved T cell persistence but not anti-tumor 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 non-specific 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 FR α 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 pre-activated T cells with a bispecific mouse monoclonal antibody (MAb) OC/TR, directed to the CD3 molecule on T lymphocytes and to FR α on ovarian carcinoma cells (43). Administration of FR α -redirected T cells to women with minimal residual ovarian cancer resulted in anti-tumor responses in 27% of patients with mild to moderate immunotherapy-related toxicities, however therapy was limited by the inability to generate stable anti-FR α -specific T cell memory and the induction of human anti-mouse antibodies (HAMA) against the bispecific MAb in nearly 90% of treated patients (44). In a phase I study of anti-FR α CAR therapy for cancer, Kershaw et al. (16) transferred T cells that were retargeted to FR α by a first generation MOv18 scFv-based CAR to immunocompetent patients with advanced ovarian cancer. The parental MOv18 antibody has a similar affinity for FR α (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 *in vivo* study (16). Our studies, combined with recent clinical strategies, address these issues. Similar to the Kershaw study (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-FR α CAR T cells, which is facilitated principally by engagement of their CAR with tumor antigen. Moreover, transfer of MOv19-BB ζ T cells leads to increased accumulation of human T cells in regressing ovarian cancer lesions. While the mouse anti-human MOv19

scFv used in the construction of the MOv19-BB ζ 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), non-myeloablative immunosuppressive pre-conditioning 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-FR α 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 FR α -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 FR α , 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-BB ζ CAR T cell therapy in combination with lymphodepleting pre-conditioning regimens for the treatment of a wide spectrum of FR α -expressing epithelial malignancies.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Generation and specific immune recognition by FRα CAR-transduced human T cells in vitro

A. Schematic representation of MOv19 based Chimeric Antigen Receptor (CAR) constructs containing the CD3ζ cytosolic domain alone (MOv19-ζ) or in combination with CD137 costimulatory module (MOv19-BBζ). FRα-specific CAR with a truncated CD3ζ domain (MOv19-Δζ) and anti-CD19-BBζ CAR are shown. MOv19, anti-FRα scFv; VL, variable L chain; L, Linker; VH, variable H chain; TM, transmembrane region. B. MOv19 CAR expression (solid black line) on human CD3-gated cells after transduction with lentivirus compared to parallel untransduced T cells (filled gray histograms). Percent transduction is indicated. C. Surface FRα expression (solid black line) by various human ovarian cancer cell lines by flow cytometry; isotype antibody control (filled gray histograms). D. Antigen-specific IFN-γ secretion by MOv19-ζ and MOv19-BBζ CAR-transduced T cells but not MOv19-Δζ anti-CD19-BBζ T cells, following overnight incubation with FRα+ cancer cell lines. Mean IFN-γ concentration ± SEM (pg/ml) from triplicate cultures is shown. E. Antigen-specific killing of FRα+ tumor cells by FRα CAR+ CD8+ T cells in 18hr bioluminescence assay at the indicated effector to target (E/T) ratio. Untransduced T cells (UNT) or gfp transduced human CD8+ T cells served as controls.

Figure 2. Human MOv19-BB ζ CAR T cells eradicate large pre-established tumors *in vivo*: effect of CD137 costimulatory signaling domains and route of administration

A. NSG mice bearing established s.c. tumor were treated with i.t. injections of 8×10^6 CAR + T cells on day 0 and 5 and imaged every two weeks. Tumor growth was assessed by caliper measurement ($V = 1/2(\text{length} \times \text{width}^2)$). B. SKOV3 fLuc+ bioluminescence signal was decreased in MOv19-BB ζ CAR treated mice compared with the MOv19- ζ and the control treatment groups 2 weeks and 4 weeks after last T cell dose. C. SKOV3 fLuc-bearing NSG mice were treated with 8×10^6 MOv19-BB ζ T cells via i.t., i.p., or i.v. routes. Tumor growth was assessed by caliper measurement. D. CD137 signaling enhances the survival of human CD4+ and CD8+ T cells *in vivo* on day 73 (4 weeks following last T cell dose) in the peripheral blood. CD4 and CD8 T cells were quantitated from blood using the TruCount method. Mean cell concentration (cells/ul) $\pm$ SD for all evaluable mice in each treatment group is shown.

Figure 3. Tumor eradication by CAR T cells is antigen specific

A. NSG mice with subcutaneous SKOV3 fLuc⁺ tumor were treated with 8×10^6 T cells (40% transduction efficiency) expressing MOv19-BB ζ , anti-CD19-BB ζ or gfp via i.t. infusion on days 0 and 5 and measured for tumor volume by calipers every 2-3 days. B. Peripheral blood was collected 3 weeks following last T cell infusion and quantified for the absolute number of human CD4⁺ and CD8⁺ T cells/ μ l of blood. Mean cell count $\pm$ SD is shown. C. FR α - and CD19-specific CAR expression on human CD3⁺ T cells from peripheral blood of treated mice measured by flow cytometry using goat anti-mouse IgG F(ab')₂. Mean CAR⁺ expression frequency $\pm$ SD per group is shown. D. Absolute CAR⁺ T-cell count was calculated as number of human CD3⁺ T cells/ μ l of blood times percent CAR⁺. Mean count $\pm$ SD was determined.

Figure 4. CAR T cell localization to tumor in vivo is antigen-specific

NSG mice with subcutaneous SKOV3 fLuc⁺ tumors were treated with intravenous injections of 8×10^6 T cells expressing MOv19-BB ζ (upper), anti-CD19-BB ζ (middle) or gfp (lower) on day 0 and 5. SKOV3 tumors grown for ~40 additional days were collected from euthanized mice and stained for human CD3 expression (brown). Representative sections are shown at 100 $\times$ magnifications.

Figure 5. Mov19-BB ζ T cells inhibit tumor growth and ascites formation in SKOV3 murine model of peritoneal carcinomatosis

A. NSG mice received intraperitoneal injection of 5×10^6 SKOV3 fLuc+ tumor cells and were randomized into four groups before beginning therapy with 9×10^6 T cells expressing MOV19-BB ζ or anti-CD19-BB ζ via i.p. or i.v. infusion on day 30 and 35 after tumor inoculation. B. Representative NSG mice treated with MOV19-BB ζ T cells (left) via i.v. (upper) or i.p (lower) infusion; Mice treated with anti-CD19-BB ζ T cells (right) developed ascites as evidenced by a distended abdomen (middle). Postmortem visualization of the peritoneum shows nodular tumor masses (arrows; far right). C. Kaplan-Meier survival curve of tumor-bearing NSG mice treated with either MOV19-BB ζ or anti-CD19-BB ζ T cells via i.v. or i.p. injection.

Figure 6. Adoptive transfer of FR α -specific T cells induces regression of ovarian cancer lung metastasis

A. NSG mice with 3 day established SKOV3 fLuc+ tumor in the lungs received tail-vein injections of 6×10^6 T cells expressing either MOv19-BB ζ or anti-CD19-BB ζ on day 3 and day 8 and were monitored by bioluminescence imaging. **B.** Photon emission from fLuc+ tumor cells was quantified and the mean $\pm$ SD bioluminescence signal determined.