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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Supplementary Methods

Anti-FRα chimeric immune receptor construction. The chimeric immune receptor backbone constructs were generated as previously described (1). The anti-FRα scFv sequence was derived from MOv19 (2;3), a monoclonal antibody directed against FRα. The MOv19 scFv has been fully characterized (4;5) and was amplified using the following primers: 5'-GCGGGATCCTCTAGAGCGGCCCAGCCGGCCATGGCCCAGGTG -3' (Bam-HI is underlined) and 5'-GCGGCTAGCGGCCGCCCGTTTTATTTCCAACTTTGTCCCCCC -3' (Nhe-I is underlined) and then cloned into the CAR backbone vector. The scFv PCR product was digested with BamHI and NheI endonucleases and gel purified before ligation into the pCLPS vector, a third generation self-inactivating CMV promoter based lentiviral expression vector based on pRRL-SIN-CMV-eGFP-WPRE (6). The anti-CD19-BBζ CAR construct was provided by Michael Milone at University of Pennsylvania (7). High-titer lentiviral vectors were produced and concentrated 10-fold by ultracentrifugation for 3 h at 26,000 rpm as previously described (8).

Cell lines. Lentivirus packaging was performed in the immortalized normal fetal renal 293T cell line purchased from ATCC. Human cell lines used in immune based assays include the established human ovarian cancer cell lines SKOV3, A1847, OVCAR3, C30, and PEO-1. For bioluminescence assays, target cancer cell lines were transfected to express firefly luciferase (fLuc), enriched by antibiotic selection positive expression by bioluminescence imaging. For specificity controls, the mouse malignant mesothelioma cell line, AE17 (kindly provided by Steven Albelda, University of Pennsylvania), was transduced with lentivirus to express FRα (AE17.FRα). CD19-expressing K562 (CD19+K562) cells, a human erythroleukemic cell line, were kindly provided by Michael Milone (University of Pennsylvania). 293T cells and tumor cell lines were maintained in RPMI-1640 (Invitrogen) supplemented with 10% (v/v) heat-inactivated FBS, 2 mM L-glutamine, 100μg/mL penicillin and 100U/mL streptomycin. All cell lines were

routinely tested for mycoplasma contamination.

Flow cytometric analysis. The following MAbs were used for phenotypic analysis: APC-Cy7 Mouse Anti-Human CD3; FITC anti-human CD4; APC anti-human CD8; PE-anti human CD45. All mAbs were purchased from BD Biosciences PharMingen. In T cell transfer experiments, peripheral blood was obtained via retro-orbital bleeding and stained for the presence of human CD45, CD4, and CD8 T cells. After gating on the human CD45+ population, the CD4+ and CD8+ subsets were quantified using TruCount tubes (BD Biosciences) with known numbers of fluorescent beads as described in the manufacturer's instructions. Tumor cell surface expression of FRα was detected by Mov18/ZEL antibody (Enzo Life Sciences). FRα specific CAR expression was detected by PE conjugated goat anti-mouse IgG $F(ab')_2$ (specific for scFvs of murine origin) that was purchased from Jackson ImmunoResearch. For intracellular staining, cells were fixed,permeabilized, and stained with PE-conjugated anti-Bcl-X_L antibody (Southern Biotech). Isotype matched control Abs were used in all analyses. Flow cytometric data were analyzed by FlowJo software.

Cytokine release assays. Cytokine release assays were performed by coculture of $1x10^5$ T cells with $1x10^5$ target cells per well in triplicate in 96-well round bottom plates in a final volume of 200ul of T cell media. After 20~24 hr, co-culture supernatants were assayed for presence of IFN-g using an ELISA Kit, according to manufacturer's instructions (Biolegend). Values represent the mean of triplicate wells. IL-2, IL-4, IL-10, TNF-α cytokines were measured by flow cytometry using Cytokine Bead Array, according to manufacturer's instructions (BD Biosciences).

Cytotoxicity Assays. For the cell based bioluminescence assay, $5x10^4$ firefly Luciferase expressing (fLuc+) tumor cells were cultured with complete media in the presence of different ratios of transduced T cells using a 96-well Microplate (BD Biosciences). After incubation for 18-20 hours at 37°C, each well was filled with 50 ul DPBS resuspended with 1ul D-luciferin (0.015 g/ml) and imaged using a Xenogen IVIS Spectrum. Percent tumor cell viability was calculated as the mean luminescence of the experimental sample minus background divided by the mean luminescence of the input number of target cells used in the assay minus background times 100. All data are represented as a mean of triplicate wells. ^{51}Cr release assays were

performed as described(9). Target cells were labeled with 100uCi 100μCi ^{51}Cr at 37°C for 1.5 hours. Target cells were washed three times in PBS, resuspended in CM at 10^5 viable cells/mL and 100uL added per well of a 96-well V-bottom plate. Effector cells were washed twice in CM and added to wells at the given ratios. Plates were quickly centrifuged to settle cells, and incubated at 37°C in a 5% CO_2 incubator for 4 or 8 hours after which time the supernatants were harvested and counted using a 1450 Microbeta Liquid Scintillation Counter (Perkin-Elmer). Percent specific lysis was calculated as (experimental - spontaneous lysis / maximal - spontaneous lysis) times 100. For gfp target cell lysis assays, transduced T cells were co-cultured at various effector to target ratios for 24hrs with 5 x 10^4 gfp expressing AE17 or AE17.FRα cells and photographed under fluorescent microscopy. Target cell lysis was indicated by imaging reduction in gfp-labeled adherent tumor cells.

Xenograft model of ovarian cancer. All animals were obtained from the Stem Cell and Xenograft Core of the Abramson Cancer Center, University of Pennsylvania. Eight to 12-week-old NOD/SCID/γ-chain-/- (NSG) mice were bred, treated and maintained under pathogen-free conditions in-house under University of Pennsylvania IACUC approved protocols. For an established ovarian cancer model, 6 to 12-week-old female NSG mice were inoculated s.c. with 3×10^6 SKOV3 fLuc+ cells on the flank on day 0. After tumors become palpable at about 1 month, human primary T cell (CD4+ and CD8+T cells used were mixed at 1:1 ratio) were activated, and transduced as described above. After 2 weeks T cell expansion, when the tumor burden was ~200-300 mm^3, mice were treated with T cells. The route, dose, and timing of T-cell injections is indicated in the individual figure legends. Tumor dimensions were measured with calipers, and tumor volumes calculated using the formula $V = 1/2(\text{length} \times \text{width}^2)$, where length is greatest longitudinal diameter and width is greatest transverse diameter. Animals were imaged prior to T cell transfer and about every week thereafter to evaluate tumor growth. Photon emission from fLuc+ cells was quantified using the "Living Image" software (Xenogen) for all *in vivo* experiments. Tumors were resected immediately after euthanasia approximately 40 days after first T cell dose for size measurement and immunohistochemistry. For the intraperitoneal model of ovarian cancer, 8 to 12-week-old NSG mice were injected i.p. with 5×10^6 SKOV3 fLuc+ cells. Thirty days after peritoneal inoculation, mice bearing well-established SKOV3 tumors were divided into groups and treated. Mice were sacrificed and necropsied when the mice

became distressed and moribund. Lung metastases were established by injecting 2×10^6 SKOV3 fLuc+ cells into the tail vein of female NSG mice. After evidence of tumor establishment in the lungs on day3, animals were treated with tail-vein injections of engineered T cells on day 3 and day 8. To monitor the extent of tumor progression, the mice were imaged weekly or biweekly and body weights of the mice were measured. In all models, 4-5 mice were randomized per group prior to treatment.

Bioluminescence imaging. Tumor growth was also monitored by Bioluminescent imaging (BLI). BLI was done using Xenogen IVIS imaging system and the photons emitted from fLuc-expressing cells within the animal body were quantified using Living Image software (Xenogen). Briefly, mice bearing SKOV3 fLuc+ tumor cells were injected intraperitoneally with D-luciferin (150 mg/kg stock, 100 μL of D-luciferin per 10 grams of mouse body weight) suspended in PBS and imaged under isoflurane anesthesia after 5~10 minutes. A pseudocolor image representing light intensity (blue, least intense; red, most intense) was generated using Living Image. BLI findings were confirmed at necropsy.

Immunohistochemistry. Mice were euthanized by CO_2 inhalation and tumors were collected in Tissue-Tek O.C.T. Compound, and frozen at -80^{o}C. A standard Strept-avidin horseradish immunoperoxidase method was used for human CD3 staining. Primary and secondary antibodies were diluted in buffer containing 10% normal goat serum. 7μm cryosections were fixed in cold acetone for 5 min at 4^{o}C and blocked with Dako's (Carpentaria, CA) peroxidase blocking system for 10 minutes. Sequential incubations included the following: 10% normal goat serum (30 min at RT); primary rabbit anti-human CD3 monoclonal antibody (Thermo Scientific RM-9107) at 1:100 dilution (45 min. at RT); secondary biotinylated goat anti-rabbit antibody at 1:200 dilution (30 min at RT); strept-avidin-biotinylated horseradish peroxidase complex reagent (Dako) (30 min at RT); and three 5 minute washes in buffer after each incubations. Sections were then exposed to the chromagen DAB plus from Dako for 5 min at RT and counterstained with hematoxylin, dehydrated, cleared and mounted.

Reference

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