Generation of hFra-expressing cell lines, BiTE constructs and retroviral constructs

Human FRα (hFRa) was PCR amplified from cDNA of SK-MEL-37 melanoma cell line using the following primers FWD: TGTCGTGAAAACTACCCCGCGGCCGCCACCATGGCTCAGCGGATGACAACACA and REV: TTCGTGGCTCCGGAGCCACTGCTGAGCAGCCACAGCAGCATT, where coding hFRa regions are underlined. The hFRa gene was genetically fused to the monomeric enhanced GFP (eGFP) reporter via SGSG-linker and a P2A translational skipping sequence and inserted into the pT2-EF sleeping beauty transposon plasmid [1] using NEBuilder® HIFI DNA assembly (New England Biolabs), with sequences confirmed by Sanger Sequencing at the Roswell Park Genomics Shared Resource. The pT2-EF-hFRa-GFP vector was co-electroporated with the CMV(CAT)T7-SB100 transposase vector (a gift from Zsuzsanna Izsvak (Addgene plasmid # 34879; http://n2t.net/addgene:34879 ; RRID:Addgene_34879) into IE9-mp1 and Pan02 cell lines using the Nucleofector 4D Instrument. Electroporated cells were cultured for 10-14 days prior to FACs sorting of GFP^{hi} cells using a BD FACSAria II cell sorter. Sorted cells were confirmed to express hFRa by flow cytometry. A hFRα-specific scFv with murine immunoglobulin kappa light chain was designed by fusing MOv19 kappa chain (Sequence ID: X99994.1) and heavy chain (Sequence ID: X99993.1) sequences via a 212 polypeptide-containing linker (GSTSGSGKSSEGKG) and was synthesized by Integrated DNA technologies gBlock. FR-B is a chimeric BiTE that binds human FRα and mouse CD3ε (via a previously described scFv derived from the 145-2C11 monoclonal antibody [2]. These scFvs are linked by a rigid and long G(EAAAK)3 linker sequence that resulted in optimal antigen binding and *in vivo* FR-B activity compared to a panel of tested linkers (data not shown). The BiTE leader sequence [2] and 145-2C11 derived scFv sequence were codon-optimized and synthesized by gBlock (Integrated DNA technologies), with the FR-B sequence designed to contain a 6x His Tag at the C terminus. The FR-B sequence was genetically fused to the monomeric enhanced GFP (eGFP) reporter via a SGSG-linker and P2A translational skipping sequence to allow monitoring of transduction efficiency (**Suppl. Fig. 6A**). The FR-Bh sequence was generated from the FR-B BiTE by exchanging the 145-2C11 derived mouse CD3ε binding scFv with the human CD3ε-specific UCHT1 scFv sequence (sequence kindly provided by Dr. Jonathan Bramson, McMaster University) ordered from Integrated DNA technologies as a gBlock containing the same BiTE leader sequence as above and the UCHT1 scFv). The DNA sequence corresponding to the G(EAAAK)3 linker, followed by Mov19 scFv, 6x His Tag, Furin cleavage peptide, SGSG linker + P2A translational skipping sequence, and monomeric enhanced GFP (eGFP) reporter (to monitor transduction efficiency) was PCR amplified from an existing plasmid to generate overlapping DNA fragments amenable to assembly using NEBuilder (**Suppl. Fig. 1A**). FR-B and FR-Bh sequences along with GFP reporter genes were inserted into the previously described retroviral vector [1] using NotI and PacI restriction sites, with DNA fragments assembled using NEBuilder HIFI DNA assembly (New England Biolabs). Plasmid sequences were confirmed by Sanger Sequencing, and retroviruses used to transduce human or mouse cells produced in PG13 or PLAT-E retroviral packaging cells lines, respectively. For murine studies, control T cells were either transduced with a retrovirus expressing a codon-optimized Luciferase (Luc2)-P2A-GFP gene and produced in PLAT-E cells or were Mock transduced. For human T cells, Control Engager secreting (Cont-ENG) T cells were generated by transducing human T cells with the FR-B retroviral vector (produced in PG13 cells), where secreted Engagers can bind FRα+ target cells, but not human T cells due to lack of cross-reactivity of the 145-2C11 scFv with human CD3 (confirmed by flow cytometry), thus preventing Engager-mediated T cell activation upon FRα binding. In all cases, cell culture supernatant from high-titer retrovirus producing clones was collected and used for viral transduction. For initial testing of BiTEs for antigen-specific binding, 293T

cells were retrovirally transduced, followed by collection of 293T cell culture supernatants. Supernatants were spun at 1500 rpm for 5mins to remove cells/debris, followed by 20-40x concentration using Corning Spin-X® UF20 30k MWCO columns according to the manufacturers recommended protocol. Concentrated supernatants were added to target cells and incubated for 90 mins at 4°C with supernatants from unmodified 239T cells used as controls. Following incubation, cells were washed and subsequently stained with an anti-His Tag antibody for detection of BiTE binding to target cells by flow cytometry as described separately in the methods section.

T cell activation and transduction

For human studies, peripheral blood mononuclear cells (PBMC) were isolated from the whole blood of healthy donors and received under an approved Biospecimen and Data Research (BDR) protocol. PBMC were activated using precoated plate-bound anti-human CD3ε antibody (OKT-3, 5µg/ml prepared in PBS, Bio X Cell) for 48hrs in cRPMI containing anti-human CD28 antibody (9.3, 2µg/ml, Bio X Cell), human IL-2 (50U/ml, Peprotech), and human IL-7 (10ng/ml, BioLegend). For murine studies, splenocytes were harvested from female C57BL/6J or T-Lux [3] mice, subjected to RBC lysis using ACK lysis buffer, and activated using precoated plate-bound anti-mouse CD3ε (145-2C11, 5µg/ml prepared in PBS, Bio X cell) for 48-72hrs in cRPMI containing anti-mouse CD28 antibody (37.51, 2µg/ml, Bio X Cell), human IL-2 (50U/ml, Peprotech), and either mouse IL-7 (10ng/ml, BioLegend) or mouse IL-15 (10ng/ml BioLegend). Following activation, T cells were harvested, counted, and loaded onto Retronectin (Takara) -coated non-tissue culture treated plates preloaded with retrovirus by spinning cleared cell supernatants from high-titer retrovirus-producing PG13 (human) or PLAT-E (mouse) cells at 3000 rpm for 1hr at 32°C (2 cycles of retrovirus preloading completed prior to T cell loading). T cell transduction was conducted on two consecutive days, followed by at least 24hr T cell expansion prior to assessment of T cell transduction efficiency (based on GFP+ cells, gated using GFP- mock transduced T cells) by flow cytometry. Following activation, T cells were maintained in cRPMI containing cytokine support (IL-2 + IL-7 or IL-15), which was replaced every 2-3 days.

Antibodies and Flow Cytometry Staining/Analysis

For mouse samples, Fc blocking was performed by using an anti-CD16/CD32 antibody (2.4G2, Bio X Cell, 15 min at 4°C) to inhibit non-specific antibody binding prior to surface staining. For intracellular staining, The BD Transcription Factor Buffer Set (BD Biosciences) was used according to the manufacturer's suggested protocol. In cases where fixation/permeabilization were performed, intracellular staining for GFP was additionally included to permit interrogation of FR-B T cells based on GFP in fixed cells. For human samples, blocking was performed using human FcR Block (Miltenyi Biotech) for 10 min at 4°C, followed by staining (30mins, 4°C) using antibodies prepared in either FACs Buffer or BD Horizon™ Brilliant Staining Buffer. For human studies involving addition of engineered T cells to OC patient specimens, CONT-ENG or FR-Bh T cells were harvested and pre-labeled with CellTrace Violet (Thermo Fisher) according to the recommended protocol prior to addition. For all direct *ex vivo* mouse studies and for studies using human OC specimens, Zombie UV or Zombie Near-IR fixable viability staining (BioLegend) was performed to ensure interrogation of only viable cells. Stained Samples were collected using BD LSR II or Fortessa Flow cytometers and downstream data analyzed using FlowJo V10 software (BD Biosciences).

In vitro co-culture studies

Human (SKOV-6) or mouse (IE9-mp1-hFRa or parental IE9-mp1) target cell lines were plated in 96 well (1-1.5 x 10^4 cells/well), 24 well (2-4 x 10^4 cells/well), or 6 well (10^5 cells/well) cell culture plates and co-cultured with T cells at the indicated Effector: Target cell ratios (E:T Range 8:1-1:1) for 24-72hrs as indicated in the Figures/Figure Legends. To assess target cell killing, T cells were gently washed from cultures using cold PBS and target cells were enumerated by counting a minimum of 4 randomly selected regions of interest (ROI's)/well using the Cytation 5 instrument (Biotek) or quantified using the CellTiter-Glo 2.0 Cell Viability Assay (Promega) to determine % target cell killing compared to control wells containing target cells alone. T cell activation (FR-B, Luc/GFP, or Mock transduced T cells) was assessed after 24hr co-culture with IE9-mp1-hFRa target cells using CD69 surface staining and flow cytometry. Culture supernatants from co-cultures were collected, spun down to remove debris, aliquoted and stored at -80^{o}C prior to analysis and were assessed for IFN-γ production using either the human or mouse IFN-γ ELISA MAX Deluxe set (BioLegend) according to the manufacturer's suggested protocol.

Transwell Assay to assess BiTE-mediated bystander T cell activity

2 x 10^5 untransduced (UTD) human T cells were plated with (2 x 10^4) SKOV-6 cells (E:T = 10:1) in 24 well tissue culture plates in 800µl cRPMI. Next, 0.4µm PETMembrane 24 well transwell inserts (Greiner Bio-One) were placed in the wells and 10^6 human CONT-ENG or FR-Bh T cells were added to the transwell in 200µl cRPMI. CONT-ENG or FR-Bh T cells added directly to the SKOV-6 target cells (no transwell) were used as negative and positive controls, respectively. Co-cultures were plated in technical duplicate or triplicate and incubated for 48hrs in the presence of human IL-2 (50U/ml), at which point T cells were gently washed from the lower chamber and target cells harvested and viable cells counted. Culture supernatants were collected and analyzed for human IFN-γ production by ELISA as described.

Measurement of BiTE secretion by FR-Bh T cells

As the FR-Bh BiTE sequence includes a 6x His Tag at the C terminus (**Suppl. Fig 1A**), BiTE secretion by FR-Bh T cells was measured using a His Tag ELISA Detection Kit (Genscript). Transduced FR-Bh T cells (GFP+) were FACs sorted using a BD FACSAria II cell sorter to ensure use of 100% BiTE-secreting T cells, with Untransduced (UTD) T cells used as a negative control. T cells were expanded *in vitro* prior to assay set up and maintained in cRPMI containing IL-2 (50U/ml, Peprotech) and IL-7 (10ng/ml, BioLegend) for the duration of the study. Sorted FR-Bh (or UTD T cells) were used to assess BiTE secretion under 3 conditions: At baseline (prior to antigen exposure), after acute antigen exposure, or after repeat antigen exposure. Acute antigen exposure was achieved by co-culturing FR-Bh and UTD T cells with 7.5 x 10^5 FRα+ SKOV-6 cells for 24hrs at a 4:1 E:T ratio in a T25 Flask. T cells were then collected, counted, and used to either set up a repeat antigen exposure (same experimental setup as above) or used to quantify BiTE secretion (acute antigen exposure). Repeat antigen-exposed T cells were collected after 24hrs, counted, and used to quantify BiTE secretion (repeat antigen exposure). BiTE secretion by FR-Bh T cells at baseline or following acute/repeat antigen exposure was measured by culturing harvested T cells (FR-Bh and UTD) at a density of ~10^6 T cells in 350µl of cRPMI + cytokines for 72hrs. The UCHT1 monoclonal antibody (anti-human CD3ε, corresponding to the same antibody clone used to generate the FR-Bh BiTE) was added to the cultures (10µg/ml, Bio X Cell) to limit the capacity of BiTEs to bind back to T cells following secretion and accumulate in the cell supernatant. After 72hrs, culture supernatants collected,

spun down to remove debris, and stored at -80°C prior to analysis. T cells were counted at the end of the culture period and used to determine BiTE secretion/10^6 T cells.

OC patient samples and targeting using FR-Bh T cells

Patient samples were assessed for the frequency of tumor cells (CD45-EpCAM+) that were FRα+ by flow cytometry. Following 48hr co-culture, culture supernatants were collected for downstream analysis and cells collected for flow cytometry as detailed. To allow enumeration of FRα+ and FRα- tumor cells following co-culture, CountBright Absolute Counting Beads (Thermo Fisher) were added prior to Flow cytometry analysis and cell numbers calculated using Lot # specific values as provided by the manufacturer. Culture supernatants were spun down to remove debris, aliquoted and stored at -80^{o}C prior to analysis and were assessed for IFN-γ production using the human IFN-γ ELISA MAX Deluxe set (BioLegend) according to the manufacturer's suggested protocol. Additionally, 4 responding patient culture supernatants were selected to assess broad immunological effects between OC patient samples containing either CONT-ENG or FR-Bh T cells using the Isoplexis Human Adaptive Immune Codeplex Secretome to measure 22 inflammatory parameters using the Isoplexis Isolight system according to the manufacturer's recommended protocol.

Preclinical mouse models and therapeutic delivery of FR-B T cells

For evaluation of FR-Bh T cell therapeutic activity using a human xenograft model, 3 x 10^6 SKOV-6 cells (prepared in 100µl PBS) were implanted subcutaneously in the flanks of female 6–8-week-old female NSG mice bred in the Roswell Park Laboratory Animal Shared Resource (LASR). Tumor volumes were calculated as 0.5 x (Length x Width^2) and when tumors reached ~150mm^3, mice were stratified into groups to remain untreated or receive 3 x 10^6 FR-Bh or Cont-ENG T cells (delivered as split dose between IV and intratumoral routes, prepared in 200µl PBS). Mice received 3 daily doses of 2 x 10^4 U of IL-2 (IP injection, 200µl PBS) beginning on the day of T cell infusion. Tumor measurements (length and width) were obtained using digital calipers and tumor volume calculated as 0.5 x (Length x Width^2). Changes in tumor volume were determined twice/week for the duration of study. In studies involving depletion of endogenous lymphocytes in immunocompetent mice, mice were treated with 5 Gy Total body irradiation immediately prior to tumor implantation and lymphodepletion confirmed by flow cytometry prior to adoptive T cell transfer. IP tumor progression was monitored based on increased abdominal distension (measured as changes in circumference) due to accumulation of peritoneal ascites, which closely correlates with solid tumor growth in this model [4 5], with mice considered endpoint and euthanized when abdominal circumference reached 10cm (or at earlier measurements if mice developed decreasing health status due to peritoneal disease progression). For subcutaneously implanted Pan02-hFRa, length and width measurements were collected using digital calipers and tumor volume was again calculated at 0.5 x (Length x Width^2), with mice considered endpoint and euthanized when tumor dimensions exceed 10mm in both directions. All performed experiments and procedures were reviewed and approved by the Roswell Park IACUC (Protocol #'s 1430M, 1458M, and 1346M) prior to conducting experiments.

Monitoring of FR-B T cell responses in treated mice

Following infusion of FR-B 2/7 or 2/15 T cells into IE9-mp1-hFRa tumor-bearing mice (8.4 x 10^5 FR-B T cells/mouse, IP injection), blood was collected by retro-orbital blood draw, peritoneal lavage collected

following IP injection of PBS, and solid tumors excised from the omental region of animals at the indicated time points post ACT. RBC lysis was performed on blood and peritoneal wash samples using ACK lysis buffer and solid tumors were processed using the gentleMACs Dissociator (Miltenyi Biotec), followed by passage through 70µm cell strainers. Samples were subsequently stained for flow cytometry analysis as outlined.

Metabolomic Assessment of FR-B T cells using Seahorse

FR-B 2/7 or FR-B 2/15 T cells were generated for comparison of metabolic function using the Mitochondrial Stress Test conducted using the Seahorse XFe96 Analyzer. Briefly, the Mitochondrial Stress Test was performed in XF DMEM Base Media with no Phenol red containing 10mM glucose, 1mM sodium pyruvate, and 2mM L-glutamine and the following inhibitors were added at the following final concentrations: Oligomycin (2µM), Carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone (FCCP) (1.0 µM), Rotenone/Antimycin A (0.5µM each). Prepared T cells were plated on cell-tak (Corning) coated Seahorse XF96 cell culture microplates at a density of 2.5 $X10^5$ cells per well and in replicates of 8 wells per cell type. The assay plates were allowed to rest at RT for 45mins and then spun for 5minutes at 1,000rpm. The plates were then incubated at 37°C without CO_2 for 10mins prior to performing the assay on the Seahorse XFe96 Analyzer. Assay was set up and ran according to manufacturer's recommended method and post run data was obtained through Seahorse Wave Desktop Software, with additional statistical analysis completed using GraphPad Prism.

FACs sorting of CD8+ FR-B TALs, RNA sequencing, and Transcriptomic Analysis

FR-B 2/7 or FR-B 2/15 T cells (8.4 x 10^5/mouse, prepared in 200µl PBS) were adoptively transferred by IP injection into IE9-mp1-hFRa tumor-bearing mice and peritoneal washes collected from mice 5 days later following IP injection of 5ml PBS. Collected cells were washed and immediately stained for cell viability (Zombie UV, prepared in PBS) followed by Fc blocking and surface phenotyping using antibodies prepared in BD FACs Pre-Sort Buffer (BD Biosciences). Live CD45+$CD11b^-CD19^-CD4^-$ (Gated out using a PE-Cy7 Dump Channel) CD8+ GFP+ FR-B TALs were sorted using a BD FACSAria II Cell Sorter and collected directly into PCR tubes containing 2µl of Takara Plain Sorting Solution with a target collection of 500 cells/sample. Sufficient cell input (359-500 cells) was achieved for downstream analysis using 5 unique biological samples (n=2 for FR-B 2/7 CD8+ TALs, n=3 for FR-B 2/15 CD8+ TALs). Sorted cells were kept on ice and immediately brought to the Roswell Park Genomics Shared Resource for further processing and RNAseq analysis. RNAseq analysis of sorted CD8+ FR-B TALs was conducted using the Takara Bio USA, Inc. SMART-Seq® v4 PLUS Kit. Final libraries were sequenced on an Illumina NovaSeq 6000 using 2X100 sequencing and an average of 50 million paired reads/sample were generated. Following sequencing, samples were passed through Illumina bcl2fastq v2.20 to generate fastq files for downstream analysis. Bioinformatics pre-processing and quality control (QC) steps were carried out by the Roswell Park Bioinformatics Shared Resource, using an established pipeline following commonly adopted practices for RNA-seq data analysis. Raw reads that passed the Illumina RTA quality filter were demultiplexed and pre-processed using FastQC for sequencing base quality control. Raw reads that passed the Illumina RTA quality filter were demultiplexed and pre-processed using FastQC for sequencing base quality control. Reads were then mapped to the mouse reference genome (GRCm39) and reference transcriptome GENCODE (vM28) using STAR [6]. Raw feature counts were normalized and differential expression analysis was carried out using DESeq2 [7]. Differential expression rank order was used for subsequent gene set enrichment analysis (GSEA) [8], performed using the cluster profile package in R. Gene sets queried included the Hallmark, Canonical pathways, and GO Biological Processes Ontology collections available through the Molecular Signatures Database (MSigDB) [9]. The metabolic

pipeline [10] utilizes DEG output, with scores for each gene produced by multiplying the -log(adjusted p value)*logFC on a gene level. Absolute value scores were produced by taking the absolute value of the scores for each gene. The pipeline was then used to assess transcriptional metabolic pathway dysregulation. Pathways were subsequently constructed using the WikiPathways application in Cytoscape network builder.

Supplemental References:

1. Tsuji T, Yoneda A, Matsuzaki J, et al. Rapid Construction of Antitumor T-cell Receptor Vectors from Frozen Tumors for Engineered T-cell Therapy. *Cancer Immunol Res* 2018;6(5):594-604. doi: 10.1158/2326-6066.Cir-17-0434 [published Online First: 2018/03/29]
2. Gilliland LK, Norris NA, Marquardt H, et al. Rapid and reliable cloning of antibody variable regions and generation of recombinant single chain antibody fragments. *Tissue Antigens* 1996;47(1):1-20. doi: 10.1111/j.1399-0039.1996.tb02509.x [published Online First: 1996/01/01]
3. Chewning JH, Dugger KJ, Chaudhuri TR, et al. Bioluminescence-based visualization of CD4 T cell dynamics using a T lineage-specific luciferase transgenic model. *BMC Immunol* 2009;10:44. doi: 10.1186/1471-2172-10-44 [published Online First: 2009/08/05]
4. McGray AJR, Eppolito C, Miliotto A, et al. A prime/boost vaccine platform efficiently identifies CD27 agonism and depletion of myeloid-derived suppressor cells as therapies that rationally combine with checkpoint blockade in ovarian cancer. *Cancer Immunol Immunother* 2021;70(12):3451-60. doi: 10.1007/s00262-021-02936-1 [published Online First: 2021/04/22]
5. McGray AJR, Huang RY, Battaglia S, et al. Oncolytic Maraba virus armed with tumor antigen boosts vaccine priming and reveals diverse therapeutic response patterns when combined with checkpoint blockade in ovarian cancer. *J Immunother Cancer* 2019;7(1):189. doi: 10.1186/s40425-019-0641-x [published Online First: 2019/07/19]
6. Dobin A, Davis CA, Schlesinger F, et al. STAR: ultrafast universal RNA-seq aligner. *Bioinformatics* 2013;29(1):15-21. doi: 10.1093/bioinformatics/bts635 [published Online First: 2012/10/30]
7. Love MI, Huber W, Anders S. Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. *Genome Biol* 2014;15(12):550. doi: 10.1186/s13059-014-0550-8 [published Online First: 2014/12/18]
8. Reimand J, Isserlin R, Voisin V, et al. Pathway enrichment analysis and visualization of omics data using g:Profiler, GSEA, Cytoscape and EnrichmentMap. *Nat Protoc* 2019;14(2):482-517. doi: 10.1038/s41596-018-0103-9 [published Online First: 2019/01/22]
9. Liberzon A, Subramanian A, Pinchback R, et al. Molecular signatures database (MSigDB) 3.0. *Bioinformatics* 2011;27(12):1739-40. doi: 10.1093/bioinformatics/btr260 [published Online First: 2011/05/07]
10. Rosario SR, Long MD, Affronti HC, et al. Pan-cancer analysis of transcriptional metabolic dysregulation using The Cancer Genome Atlas. *Nat Commun* 2018;9(1):5330. doi: 10.1038/s41467-018-07232-8 [published Online First: 2018/12/16]