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Supplemental Information

Adoptive Transfer of IL13Rα2-Specific Chimeric Antigen Receptor T Cells Creates a Pro-inflammatory Environment in Glioblastoma

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SUPPLEMENTAL METHODS

Cell culture

293T (ATCC), murine parental GL261 (NIH), and SMA560 glioma cell lines (a generous gift from Dr. John Sampson, Duke University), and their modified sublines, GL261-hIL13Rα2, GL261-hIL13Rα2-OVA and SMA560-hIL13Rα2, were maintained in complete Dulbecco's Modified Eagle Medium (DMEM; Invitrogen, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS; GE Healthcare Life Sciences, Pittsburgh, PA), streptomycin (100 mg/ml), and penicillin (100 U/ml) (Invitrogen, Grand Island, NY). T cells were maintained in RPMI 1640 (Invitrogen, Grand Island, NY), supplemented with 10% FBS, 0.025% GlutaMax (Invitrogen, Grand Island, NY), 0.001% penicillin, 0.002% streptomycin, and 50 μg/mL of mIL-2 (Peprotech, Rocky Hill, NJ), supplemented with 2-mercaptoethanol (Sigma-Aldrich, St. Louis, MO) to a final concentration of 45 μM. All cell cultures were maintained in a standard humidified incubator at 37°C in 5% CO_2 atmosphere.

Generation of glioblastoma cell lines expressing human IL13Rα2 and ovalbumin

GL261-hIL13Rα2 and SMA560-hIL13Rα2 were produced by transducing parental GL261 and SMA560 cell lines with a previously developed pEF6myc,his vector encoding human IL13Rα2[1] using Lipofectamine 2000 Transfection Reagent (Invitrogen, Grand Island, NY) according to the manufacturer's specifications. Transfected cells were selected with 5μg/mL of blasticidin S HCl (Invitrogen, Grand Island, NY) for 7 days. The expression of hIL13Rα2 was validated by staining with anti-human IL13Rα2 mAbs (clone 47) conjugated to PE and isotype control IgG1-PE (BioLegend, San Diego, CA). Cells were sorted using a BD FACSAria II cell sorter to enrich the

positive cell population. Sorted cells were routinely maintained in the presence of blasticidin S HCl at a concentration of 2 μg/mL with periodic validation of hIL13Rα2 expression on the surface of the modified cells by flow cytometry using a BD LSRFortessa Analyzer. GL261-hIL13Rα2 expressing ovalbumin (GL261-hIL13Rα2-OVA) were produced by transducing GL261-hIL13Rα2 cells with pAc-Neo-OVA (Addgene, Cambridge, MA) using Lipofectamine 2000 Transfection Reagent. Transfected cells were selected with 400 μg/mL and maintained in 200 μg/mL of geneticin (G418, Invitrogen, Grand Island, NY) in complete culture medium.

Generation of retroviral chimeric antigen receptor constructs

The codon-optimized cDNA encoding for CARs was synthesized by Thermo Fisher Scientific (Waltham, MA) and subcloned into a multiple cloning site (MCS) of the pRV2011(M) vector. pRV2011 (M) was derived from pRV2011 oFL,[2] which encodes an MSCV-based retroviral vector optimized for expression in murine T cells, the firefly luciferase gene, an internal ribosomal entry site (IRES), and Thy1.1, by replacing the firefly luciferase gene with an MCS. The IL13Rα2.CAR.CD28.ζ CARs consisted of 1) a murine IgG leader peptide, 2) the IL13Rα2-specific scFv47;[1,3] 3) the hinge region of murine IgG1, 4) the CD28 transmembrane domain, and 5) the CD28 and CD3ζ signaling domains. IL13Rα2.CAR.Δ was generated by deleting the CD28.ζ signaling domain of IL13Rα2.CAR.CD28.ζ. The sequences of all final CAR constructs were verified by sequencing.

Generation of CAR T cells

$CD3^+$ T cells were isolated from the spleens of 6-8-week old C57BL/6 (syngeneic for GL261) or VM/Dk (syngeneic for SMA560) male and female mice using a Negative Selection Mouse T Cell

Isolation Kit (eBioscience Affymetrix, San Diego, CA) according to the manufacturer's directions. Pure $CD3^+$T cells were activated in plates covered with anti–mouse CD3 at 5 μg/mL (eBioscience, Affymetrix, San Diego, CA) and anti–mouse CD28 antibodies at 2 μg/mL (eBioscience, Affymetrix, San Diego, CA) in RPMI 1640 media containing 10% FBS, antibiotics, and 50 ng/mL mouse recombinant IL2. T cell cultures were transduced with replication-deficient retrovirus encoding for CAR constructs after 24 hours of stimulation. Retroviral supernatants were prepared by co-transfection of 293T cells with plasmids encoding for CAR constructs, VSV-G, and Peg-Pam-e plasmid encoding MoMLV gag-pol genes using a GeneJuice transfection reagent (EMD Biosciences, San Diego, CA), as specified by the manufacturer. The supernatant was collected after 48 and 72 hours, filtered through 0.22 μm-pore filters (Invitrogen, Grand Island, NY), and used to spinoculate T cells for 3 consecutive days starting 24 hours after T cell stimulation. Spinoculation was carried out with 1 mL of viral supernatant, supplemented with 50 ng/mL of IL-2 in 24 well plates pre-coated with 10 μg/mL RetroNectin (Takara, Mountain View, CA). T cells were added to viral supernatant, spun at 2,000*g*, 21°C for 90 minutes, and recovered at 37°C in 5% CO_2 atmosphere each time. Additional freshly prepared 1 mL aliquots of RPMI-1640 medium containing 10% FBS, antibiotics, and IL2 at 50 ng/mL were added to each well 4 hours later. CAR and Thy1.1 tag protein expression was assessed on day 5 or 6 using flow cytometry.

Intracranial implantation of murine glioma cells and treatment with CAR T cells

CD45.1 and CD45.2 C57BL/6 mice were obtained from Jackson Laboratory. VM/Dk mice were bred in-house in accordance with a study-specific animal protocol approved by the Northwestern University Institutional Animal Care and Use Committee (IACUC). We utilized mixed gender animals, 6-12 weeks of age for glioma implantation. Mice were anesthetized with a

ketamine/xylazine mixture at 115/17 mg/kg, hydrated with 0.5 mL of lactated Ringer's electrolyte solution and prophylactically treated with meloxicam (0.2 mg/kg every 24 hours) and buprenorphine (0.05 mg/kg every 8 hours) for 3 days post-procedure.

Glioma cells were harvested using 0.5% trypsin-EDTA, neutralized by serum-containing medium, and washed twice with Dulbecco's phosphate-buffered saline (DPBS). We assessed cell viability using trypan blue dye exclusion and resuspension in DPBS (GL261) or 1.5% methylcellulose in Zinc Option Modified Eagle's medium (MEM; Invitrogen, Grand Island, NY) (SMA560). Glioma cells were injected into mice at $4x10^5$ GL261, GL261-hIL13Rα2, or GL261-hIL13Rα2-OVA cells or $7.5x10^4$ SMA560 or SMA560-hIL13Rα2 cells per animal in 2.5μL of sterile saline using a 25 μL syringe equipped with a 25-gauge needle (Hamilton, Reno, NV). The following coordinates were used: 2 mm from bregma, 3 mm right of the cranial midline suture, and 3.5 mm depth below the dura. On day 7 after implantation of glioma cells, animals received an intratumoral (i.t) injection of $1.5x10^6$ CAR T cells. Treated animals were randomly assigned to housing cages, separated by gender. *In vivo* survival experiments were repeated at least twice. Animals were monitored for survival according to Northwestern University IACUC-approved protocols.

Flow Cytometry

Immunophenotypic analyses of immune cells in tumor-bearing animals were done on days 3 and 7 after i.t. injection with IL13Rα2.CAR.Δ or IL13Rα2.CAR.CD28.ζ. CAR T cells. Brains, cervical draining lymph nodes (dLNs), and spleens were dissected from sacrificed mice and processed in a single cell suspension by pulverization through a sterile 70 μm cell strainer (Invitrogen, Grand Island, NY) using a 3 mL syringe plunger (BD, Franklin Lakes, NJ) into ice-cold PBS. Cell pellets

were treated with Ammonium-Chloride-Potassium buffer (ACK; Lonza, Walkersville, MD) to remove red blood cells. Brain cell pellets were mixed in a PBS/30% percoll (GE Healthcare Life Sciences, Pittsburgh, PA) solution and placed over a 70% percoll gradient. Samples were centrifuged at 1200*g* for 30 minutes without brakes. The top layer was aspirated, and the leukocyte interphase was collected into 20 mL of ice-cold PBS and washed twice. Cells were stained with antibodies according to the assay's protocol. Flow cytometric analysis was carried out using the BD Fortessa flow cytometer (Becton Dickinson, Franklin Lakes, NJ) at the Robert H. Lurie Comprehensive Cancer Center Flow Cytometry Core Facility. The general protocol used for surface staining is as follows: single cell isolates were incubated in PBS supplemented with 2% FBS and blocked with mouse CD16/32 blocking reagent (BioLegend, San Diego, CA). Following the Fc blockade, flourochrome-conjugated antibodies (all from BioLegend, San Diego, CA unless specified) were added and incubated on ice for 20 minutes. Cells were then washed and analyzed for surface staining. The surface staining antibody panels are detailed as follows:

Surface T-cell panel for ICCS: CD3-Alexa 700, CD4- Percp-Cy5.5, CD8-BV605, Thy1.1-APC-Cy7, CD45.1 PE-Cy7.

Exhaustion analysis: CD3-Alexa 700, CD4-Percp-Cy5.5, CD8-BV605, Thy1.1-APC-Cy7, CD45.1-PE-Cy7, Tim3-PE, Lag3-APC, PD1-FITC. This keeps the 450 laser open for *in-vivo* Cell Trace Violet experiments and Regulatory T-cell determination using Foxp3 Efluor-450.

Myeloid analysis: CD3-PE, CD11b-Efluor 450, CD11c-APC, CD8α-BV605, Ly6G-Percp-Cy5.5, Ly6C-Alexa-700.

Surface Panel for Tetramer Analysis: CD3-PE-Cy7, CD4-APC-Cy7, CD8-PE (Clone KT15 from MBL Life Science, Woburn, MA), CD44-Percp-Cy5.5, MHC-Class I Tetramer H2-

k(b) (produced by the NIH Tetramer core facility, Atlanta, GA). Tetramer staining was performed for 1 hour at 37°C before surface staining for respective CD3, CD4, and CD8 antigens.

In order to determine Foxp3 expression, cells were permeabilized, fixed overnight at 4°C, and subsequently stained using anti-Foxp3 Efluor450 utilizing the Foxp3 / transcription factor staining buffer set (eBioscience, Affymetrix, San Diego, CA).

Cell surface expression of CARs was determined using 10 μg/mL of hrIL13Rα2Fc protein (R&D Systems, Minneapolis, MN) with subsequent detection by fluorescein isothiocyanate (FITC)-conjugated anti-human Fc secondary antibody (Jackson ImmunoResearch Laboratories, West Grove, PA). Intracellular cytokine staining was done on single-cell isolates incubated for one hour in complete RPMI medium before the addition of a protein transport inhibitor cocktail (eBioscience, Affymetrix, San Diego, CA). Thirty minutes later, a cell stimulation cocktail was added and incubated for 6-8 more hours. Cells were subsequently surface stained and fixed with 2% paraformaldehyde (Sigma-Aldrich, St. Louis, MO). Intracellular staining was then performed using flourochrome-conjugated anti-interferon gamma (IFNγ)-PE and anti-tumor necrosis factor alpha (TNFα)-BV421 antibodies (BioLegend, San Diego, CA). Cellular frequency and mean fluorescent intensity values were determined using FlowJo analysis software (TreeStar, Cupertino, CA).

The leukocytes were obtained from a Percoll gradient centrifugation of brain tissue. The cell number in each sample was determined using the Bio-Rad Cell Counter. These values were used to calculate the number of analyzed cells present within the brains.

***In vitro* functional assays**

The cytotoxicity of CAR T cells against control and IL13Rα2-expressing glioma cells was determined in a standard ^{51}Cr release assay with increasing ratios of the effector (CAR T cells) to target (glioma) cells. First, $1x10^6$ glioblastoma cells were labeled with 50 µCi ^{51}Cr, as we previously described[4] and mixed with CAR T cells at 1:5, 1:10, 1:20, and 1:30 target to effector (T:E) ratios. Maximum ^{51}Cr release was measured on cells treated with 1% Triton X-100 (Sigma-Aldrich, St. Louis, MO). Spontaneous ^{51}Cr release was determined in cells incubated in complete medium without CAR T cells. After 4 hours of co-culture at 37°C in a 5% CO_2 atmosphere, 20 µl of supernatant was placed in a 96-well LumaPlate (PerkinElmer, Akron, OH) and dried for 24 hours. Plates were then read using a Packard TopCount NXT Gamma Counter (PerkinElmer, Akron, OH). Specific lysis was determined using the following formula: (experimental release–spontaneous release)/(maximal release–spontaneous release) × 100. CAR T cells were stimulated with IL13Rα2-expressing cells for 24 hours. The analysis of IFNγ and TNFα production by CAR T cells was analyzed by ELISA per manufacturer's recommendations (R&D Systems, Minneapolis, MN).

***In vivo* functional assays**

In order to determine if CAR T cells proliferate in the tumor environment *in vivo*, CAR T cells were labeled with CellTrace™ Violet (Invitrogen, Grand Island, NY) prior to intratumoral injection according to the manufacturer's specifications. At days 3 and 7 after implantation, immune cells were extracted from brains, dLNs, and spleens and analyzed for persistence and proliferation by flow cytometry. A degranulation assay of CAR T cells for the intracellular detection of cytokines by flow cytometry were carried out after 12 hours in culture. In the last 6 hours of the culture, cells were stimulated with a stimulation cocktail, followed by treatment with

protein transport inhibitors (eBioscience, Affymetrix, San Diego, CA). CAR T cells were then removed from cultures and processed for flow cytometric analysis of IFNγ and TNFα expression and lineage markers using specific antibodies.

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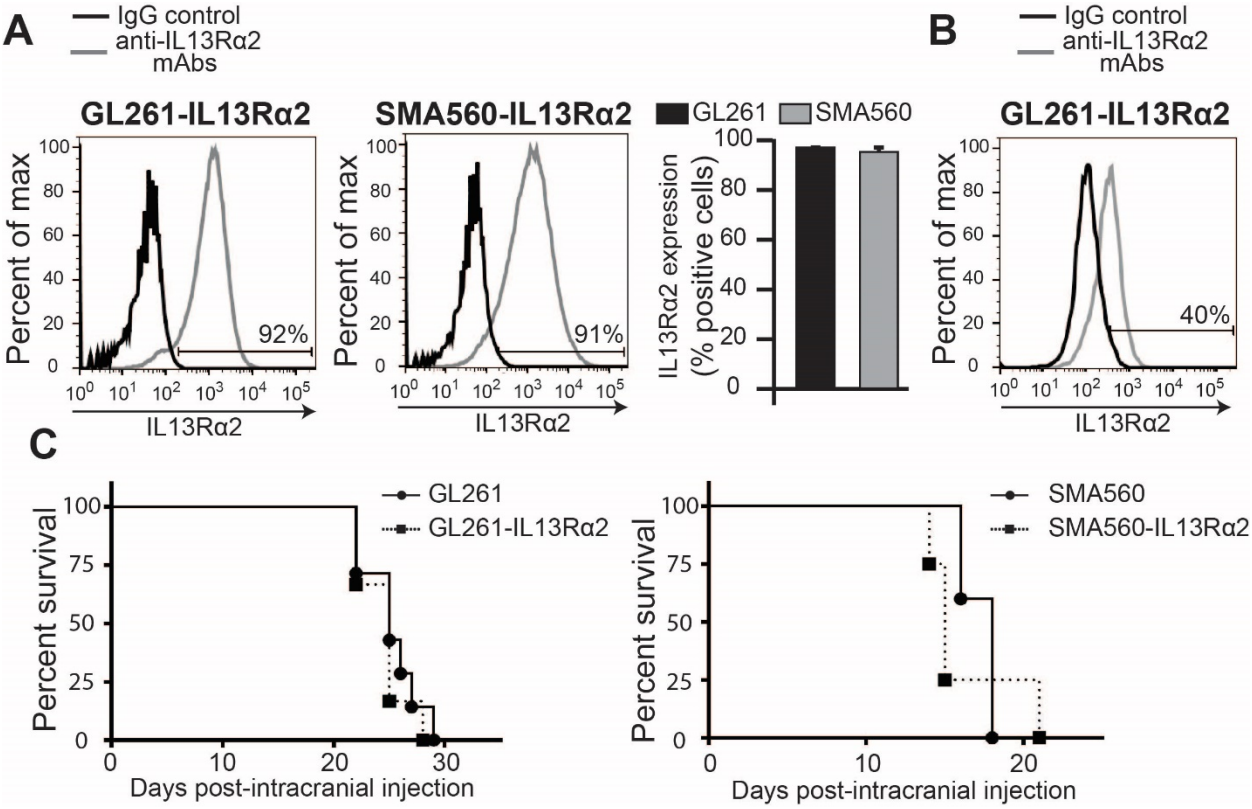
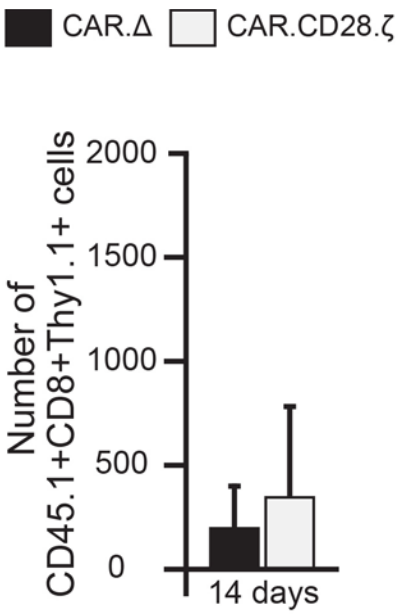
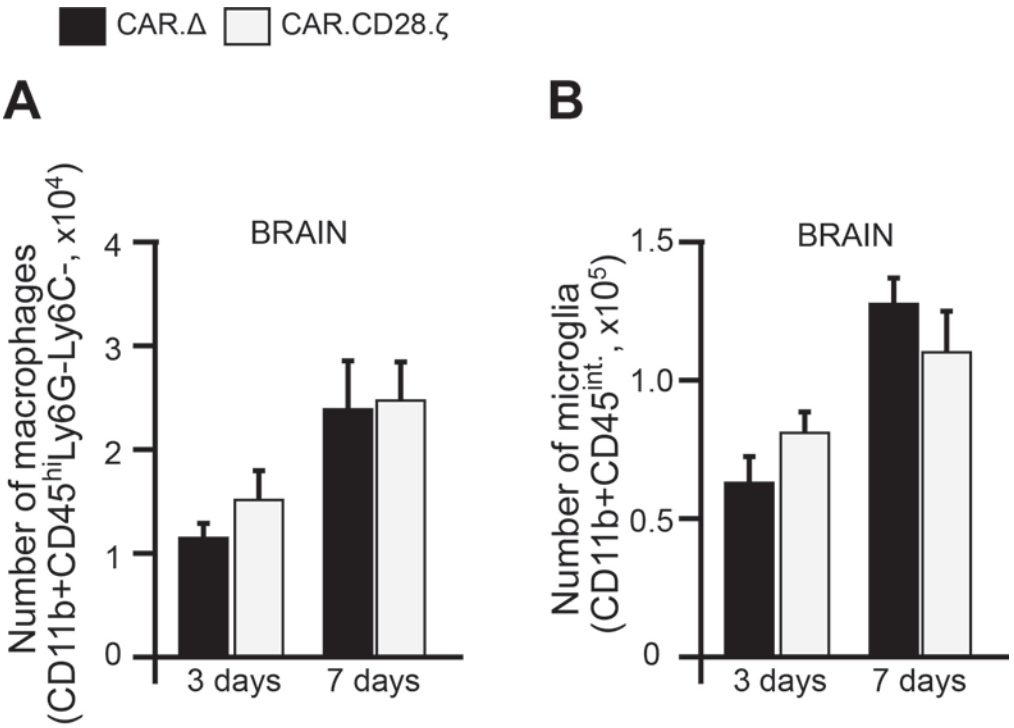


Supplemental Figure 1. Expressing human IL13Rα2 in murine glioma cells does not alter their tumorigenicity *in vivo*. A) Example of flow cytometry histograms of mouse glioma cell lines GL261 and SMA560 modified to express human IL13Rα2. Staining with IgG1 isotype control is shown in black, and staining with anti-IL13Rα2 mAb (clone 47) is shown in gray. The expression levels of IL13Rα2 were 92±0.3% for GL261 and 91±0.9% for SMA560 (n≥3). **B)** Example of a flow cytometry histogram showing that 40% of cells express hIL13Rα2 in GL261-IL13Rα2 tumors, excised from glioma-bearing animals 7 days post-intracranial injection. **C)** Kaplan-Meier survival plots of C57BL/6 and VM/Dk mice. GL261 or GL261-IL13Rα2 cells were intracranially injected to congenic C57BL/6 mice. Median survival of animals was 25 days in both groups (n≥8). SMA560 or SMA560-IL13Rα2 cells were intracranially injected to congenic Vm/DK mice. Median survival of animals was 15 days for SMA560 and 18 days for the SMA560-IL13Rα2 group (n≥5). Data are presented as mean±SEM.



Supplemental Figure 2. The persistence of CD8+ IL13Rα2-CAR.CD28.ζ T cells in the brains at the end point of glioma-bearing mice. CD3+ T cells from CD45.1 mice were utilized to generate IL13Rα2-CAR.CD28.ζ and control IL13Rα2-CAR.Δ T cells and i.t.-injected into CD45.2 mice bearing GL261-IL13Rα2 glioma. Quantitative analysis of CD45.1+Thy1.1+ T cells' populations was performed on day 14, which represented the end point of glioma-bearing mice treated with control IL13Rα2-CAR.Δ T cells. Data are presented as mean±SEM.



Supplemental Figure 3. Figure 5. The immune landscape of glioma-bearing mice after IL13Rα2-CAR.CD28.ζ T cell treatment. Analyses of macrophages **A)** and microglia **B)** in the brains of glioma-bearing mice was performed at 3 and 7 days after i.t. injection of CAR T cells. Data are presented as mean±SEM. Hi-high; int-intermediate.