

Chimeric antigen receptor constructs and uses thereof

The field of the invention

The present invention relates to a polynucleotide molecule comprising a nucleotide sequence
5 encoding a chimeric antigen receptor (CAR) or a fragment thereof i) comprising an
extracellular domain comprising an antigen-binding region which binds to epidermal growth
factor receptor variant III (EGFRvIII); or ii) comprising an extracellular domain comprising
an antigen-binding region which binds to CD19; wherein the nucleotide sequence encoding
the CAR is operably linked to a promoter and wherein the polynucleotide molecule further
10 comprises a nucleotide sequence encoding a heterologous signal peptide fused to a signal
regulatory protein gamma (SIRP γ)-related protein (SGRP). The present invention relates
further to a chimeric antigen receptor (CAR)-T cell expressing the polynucleotide molecule
and methods of treating cancer using the polynucleotide molecule and/or the chimeric antigen
receptor (CAR)-T cell expressing the polynucleotide molecule.

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Background of the invention

Glioblastoma (GBM) is an aggressive malignant primary brain tumor resistant to the current
standard of care (SOC). The hard-to-access localization and infiltrative nature of GBM often
make complete surgical resection unachievable. Moreover, chemo- and radiotherapy regimens
20 are not curative, invariably leading to recurrent disease. The survival time for patients with
GBM is around 15 months, underscoring the need for a significant breakthrough in effective
medical treatment¹⁻³.

Chimeric antigen receptor (CAR) T cell-based immunotherapies have had remarkable outcomes
in the clinical treatment of hematological malignancies, yet the development of effective CAR
25 T cell therapies against solid tumors remains challenging^{4,5}. A critical limitation of CAR T cells
is the scarcity of known tumor-specific surface antigens and their heterogeneous expression
profiles in GBM⁶. One of the most well-studied target antigens in GBM is EGFRvIII, a tumor-
specific, mutated form of EGFR expressed in approximately 40% of GBM cases^{7,8}. Although
strictly expressed on tumor cells, EGFRvIII mutations arise with concomitant EGFR
30 amplification during clonal evolution events in GBM development, resulting in EGFRvIII-
mosaic tumors^{8,9}. A clinical trial of anti-EGFRvIII CAR T cells against recurrent GBM
(NCT01454596) showed safety and transient efficacy but failed to produce long-lasting
treatment responses due to adaptive resistance and antigen escape¹⁰. Conversely, targeting more

homogeneously-expressed GBM-associated antigens is controversial due to the potential risk of on-target/off-tumor cross-reactions leading to toxicity^{11,12}.

Immune checkpoint inhibitors have recently shown promising responses against solid tumors¹³.

However, the highly immunosuppressive tumor microenvironment (iTME) of GBM severely limits the efficacy of immune checkpoint blockade (ICB)¹⁴. Thus, understanding the complex context-dependent interactions of GBM with the surrounding iTME is crucial for the effective targeting of these tumors by immunotherapeutic approaches¹⁵.

The most important and numerous immune cells that populate GBM are pro-inflammatory and proliferative brain-resident microglia, peripheral monocyte-derived macrophages, polymorphonuclear myeloid-derived suppressor cells and T_{regs}^{16,17}. As a major immune cell population in the GBM-iTME, glioma-associated macrophages and microglia (GAMs) substantially contribute to GBM progression¹¹. Microglia are professional phagocytes of the brain. They play an important role in the brain's innate immune surveillance and strongly influence the outcome and response to pathological states through the release of cytokines, chemokines, and growth factors^{18,19}. The phagocytic activity of both microglia and macrophages is regulated through, among others, the CD47-SIRP α phagocytosis axis, whereby SIRP α , expressed on the surface of microglia and macrophages, interacts with the ubiquitously-expressed CD47 transmembrane protein, thereby inhibiting phagocytosis^{20,21}. Therefore, CD47 is an innate immune checkpoint co-opted by tumor cells as a 'don't eat me' signal, which results in immune evasion by tumor cells through reduced recognition by phagocytic cells^{11,22}. Blockade of CD47 has been shown to rescue GAM phagocytic function in GBM-bearing mice leading to a strong antitumoral response *in vivo*²³⁻²⁵. However, clinical studies of systemic monotherapy with CD47 blockade have only recently begun to assess efficacy against solid tumors, showing promising clinical activity^{26,27}. The published data from these trials suggest overall safety and considerable activity but also report low bioavailability within the tumor as well as treatment-associated toxicity²⁸.

Hence the current treatment options for GBM are far from being satisfactory and there is still a high medical need to provide efficient treatments to patients.

Summary of the invention

The present invention relates to a polynucleotide molecule comprising a nucleotide sequence encoding a chimeric antigen receptor (CAR) or a fragment thereof i) comprising an extracellular domain comprising an antigen-binding region which binds to epidermal growth

factor receptor variant III (EGFRvIII); or ii) comprising an extracellular domain comprising an antigen-binding region which binds to CD19; wherein the nucleotide sequence encoding the CAR is operably linked to a promoter and wherein the polynucleotide molecule further comprises a nucleotide sequence encoding a heterologous signal peptide fused to a signal regulatory protein gamma (SIRP γ)-related protein (SGRP).

The inventors of the present invention have developed a new combinatorial approach of intratumoral (i.t.) CAR T cell therapy and GAM modulation for an additive elimination of GBM with a fourth-generation CAR design, whereby anti-EGFRvIII CAR T cells constitutively release a soluble SIRP γ -related protein (SGRP) with high affinity to CD47²⁹. It has been found by the inventors of the present invention that the combination of tumor-targeting CAR T cells with SGRP dramatically improved the elimination of EGFRvIII-mosaic GBM in vivo in orthotopic GBM and peripheral lymphoma xenograft mouse models.

The inventors herewith provide the present invention in its following aspects.

In one aspect, the present invention provides a polynucleotide molecule comprising a nucleotide sequence encoding a chimeric antigen receptor (CAR) or a fragment thereof i) comprising an extracellular domain comprising an antigen-binding region which binds to epidermal growth factor receptor variant III (EGFRvIII); or ii) comprising an extracellular domain comprising an antigen-binding region which binds to CD19; wherein the nucleotide sequence encoding the CAR is operably linked to a promoter and wherein the polynucleotide molecule further comprises a nucleotide sequence encoding a heterologous signal peptide fused to a signal regulatory protein gamma (SIRP γ)-related protein (SGRP).

In a further aspect, the present invention provides an amino sequence comprising a chimeric antigen receptor (CAR) or a fragment thereof comprising an extracellular domain comprising an antigen-binding region which binds to epidermal growth factor receptor variant III (EGFRvIII); wherein the nucleotide sequence encoding the chimeric antigen receptor (CAR) is operably linked to a promoter and wherein the polynucleotide molecule further comprises a nucleotide sequence encoding a heterologous signal peptide fused to a signal regulatory protein gamma (SIRP γ)-related protein (SGRP).

In a further aspect, the present invention provides an amino sequence comprising a chimeric antigen receptor (CAR) or a fragment thereof comprising an extracellular domain comprising an antigen-binding region which binds to CD19; wherein the nucleotide sequence encoding the CAR is operably linked to a promoter and wherein the polynucleotide molecule further
5 comprises a nucleotide sequence encoding a heterologous signal peptide fused to a signal regulatory protein gamma (SIRP γ)-related protein (SGRP).

In a further aspect, the present invention provides a construct comprising the polynucleotide molecule described herein.

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In a further aspect, the present invention provides a chimeric antigen receptor (CAR)-T cell comprising T cells expressing the polynucleotide molecule and/ or the construct described herein.

15 In a further aspect, the present invention provides a method of treating cancer in a subject suffering from an epidermal growth factor receptor (EGFR)-associated cancer or suffering from a CD19-associated cancer, the method comprising administering to the subject a therapeutically effective amount of a chimeric antigen receptor (CAR)-T cell described herein.

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In a further aspect, the present invention provides a method of treating a solid cancer in a subject, the method comprising administering to the subject a therapeutically effective amount of a chimeric antigen receptor (CAR)-T cell described herein.

25 In a further aspect, the present invention provides a method of treating cancer in a subject, wherein the cancer is located in the central nervous system and wherein the method comprises administering to the subject a therapeutically effective amount of a chimeric antigen receptor (CAR)-T cell described herein, wherein the CAR or a fragment thereof of the CAR-T cell comprises a CAR or a fragment thereof comprising an extracellular domain comprising an
30 antigen binding region which binds to epidermal growth factor receptor variant III (EGFRvIII).

In a further aspect, the present invention provides a method of treating cancer in a subject,

wherein the cancer is selected from the group consisting of a breast cancer, lung cancer, melanoma, lymphoma, acute lymphocytic leukemia (ALL), and non-Hodgkin's lymphoma (NHL), preferably lymphoma, and wherein the method comprises administering to the subject a therapeutically effective amount of a chimeric antigen receptor (CAR)-T cell described
5 herein, wherein the CAR or a fragment thereof of the CAR-T cell comprises a CAR or a fragment thereof comprising an extracellular domain comprising an antigen binding region which binds to CD19.

In a further aspect, the present invention provides a method of preparing a population of
10 activated T cells comprising a polynucleotide molecule described herein or a construct described herein, the method comprising: (i) contacting in vitro one or more T cells that have been modified to comprise a polynucleotide molecule described herein or a construct described herein with a stimulus that induces expansion of the T cells to provide an expanded T cell population; and (ii) activating in vitro the T cells to produce an activated T cell
15 population.

In a further aspect, the present invention provides a nucleotide sequence encoding a signal regulatory protein gamma (SIRP γ)-related protein (SGRP) comprising the sequence as shown in SEQ ID NO: 5.
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In a further aspect, the present invention provides an amino sequence comprising a signal regulatory protein gamma (SIRP γ)-related protein (SGRP) comprising the sequence as shown in SEQ ID NO: 6.

25 Brief description of the figures

Fig. 1: Proposed mechanism of action of conventional aEGFRvIII CAR T cell monotherapy (left panel) and aEGFRvIII-SGRP CAR T cell combination therapy whereby SGRP-mediated CD47 blockade induces phagocytic modulation of GAMs (right panel) in the context of EGFRvIII-heterogenous GBM and its iTME.

30 **Fig. 2:** Outline of the SGRP engineering strategy including specific AA substitutions to the endogenous human SIRP γ -V1 sequence and addition of an N-terminal IL-2 signal sequence (IL2sig) leading to constitutive SGRP secretion. Full CAR sequences are listed in Table 2.

Fig. 3: AlphaFold^{30,31}-generated *in silico* modeling displaying predicted protein-protein interactions of SGRP, hSIRP α -V1 and mSIRP α -V1 with hCD47. Amino acid sequence information regarding hCD47, IL2sig, hSIRP α -V1, hSIRP γ -V1, IL2sig-SGRP, mSIRP α -V1 and SGRP are listed in Table 1.

5 **Fig. 4:** Maps of polycistronic lentiviral constructs encoding mCherry (mC)-labeled aCD19 CAR or aEGFRvIII CAR under the control of *EF1A* promoter +/- SGRP secretion.

Fig. 5: Overview of lentiviral vector maps used in the study. Nucleotide sequence information for a CD19 (FMC63.BBz), aEGFRvIII (3C10.BBz), aEGFRvIII-SGRP and aEGFRvIII-SGRP-His CAR constructs are listed in Table 2.

10 **Fig. 6:** Representative T cell lentiviral transduction efficiency for the different CAR lentiviral vectors used, based on the percentage of mCherry⁺ T cells assessed by flow cytometry 4 days after transduction (gated on live single cells). Mock-transduced T cells served as controls.

Fig. 7: Post-sort enrichment of CAR T cells after sorting for mCherry⁺ cells. These were subsequently expanded and used for downstream experiments.

15 **Fig. 8:** Representative plots of CAR surface expression on aCD19 CAR and aEGFRvIII CAR T cells and assessed by FC using streptavidin (SA)-FITC labeling of biotinylated (bt)-CD19 (top plots) or bt-EGFRvIII (bottom plots) bound to the respective CAR; n = 2 HDs.

Fig. 9: aCD19 or aEGFRvIII CAR T cells were exposed to wt-EGFR using biotinylated (bt) EGFR recombinant protein. No binding of CARs to bt-EGFR by flow-cytometric analysis of streptavidin (SA)-FITC could be detected.

20 **Fig. 10:** TATA-box binding protein (TBP)-normalized expression of mCherry and SGRP detected by real-time quantitative PCR (RT-qPCR) in aEGFRvIII CAR or aEGFRvIII-SGRP CAR T cells, showing mCherry expression in CARs transduced with either construct and SGRP expression specifically in aEGFRvIII-SGRP CARs; n = 4 HDs. Statistical differences were assessed by unpaired t tests with Welch's correction.

Fig. 11: Differentially secreted proteins in CAR T cell-conditioned media, detected by LC-MS, of resting aEGFRvIII-SGRP CAR vs aEGFRvIII CAR, highlighting the presence of SGRP exclusively in aEGFRvIII-SGRP CAR T cell-conditioned media; n = 2 HDs. Mean SGRP expression: $-\log_{10} \text{qValue} / \log_2 \text{fold-change} = 8.60/6.65$.

30 **Fig. 12:** Volcano plots of healthy-donor (HD) specific differential supernatant secretome analysis by LC-MS of aEGFRvIII-SGRP CAR vs aEGFRvIII CAR T cells. Log₂ (Fold change) indicates the mean expression level for each protein. Each dot represents one protein. The $-\log_{10} (\text{qValue})$ represents the adjusted level of significance for each protein. SGRP is

highly enriched in aEGFRvIII-SGRP CAR T cell-conditioned media from both healthy donors assessed. The raw expression data is available in Table S3.

Fig. 13: Representation of SGRP sequence coverage in LC–MS. SGRP was identified by 7 exclusive unique peptides and 8 exclusive unique spectra covering 47% of the 119 AA sequence.

Fig. 14: Flow cytometric representation of surface target expression of tumor cell lines U251vIII, U251, U87, BS153, Raji and control neural stem cell line NSC197. Note: all subsequent *in vitro* and *in vivo* experiments encompassing EGFRvIII⁺ U251 and U87 were performed using sorted cells on the EGFRvIII⁺ population.

Fig. 15: Cell culture microphotographs overlaying brightfield and fluorescent detection of nuclear (n) EGFP-reporter expression after lentiviral, puromycin-selective transduction of target cells BS153, U251 and U251vIII for later time-lapse co-culture experiments; Scale bar: 250 μ m.

Fig. 16: Assessment of CAR T cell on-target killing capacity by co-culture time-lapse of nEGFP⁺ U251vIII with mCherry⁺ target-specific (aEGFRvIII CAR +/- SGRP) or nonspecific (aCD19 CAR +/- SGRP) at a 1:1 E:T ratio for 72 h.

Fig. 17: 72 h timelapse co-culture experiment of endogenously EGFRvIII⁺ BS153 cells with aCD19 or aEGFRvIII CARs, respectively. Tumor confluence (cells/well) is plotted over time. Curves represent the mean of duplicate measurements. Differences between each co-culture compared to the ‘tumor alone’ control condition were analyzed using one-way ANOVA and Dunnett’s multiple comparisons tests.

Fig. 18: Tumor confluence (Green nuclei per well) in co-cultures of EGFRvIII⁺ U251 with either aCD19, aCD19-SGRP, aEGFRvIII or aEGFRvIII-SGRP CAR T cells in 3 different effector-target ratios at 24, 48 and 72 h timepoints. Differences between each co-culture compared to the ‘tumor alone’ control condition were analyzed using one-way ANOVA and Dunnett’s multiple comparisons tests.

Fig. 19: Dose-dependent CAR T cell killing capacity in a co-culture with U251vIII at defined time points. Straight horizontal lines represent the mean confluence in control wells with U251vIII only.

Fig. 20: Tumor confluence (Green nuclei per well) in co-cultures of EGFRvIII⁺ U251 with either aCD19, aCD19-SGRP, aEGFRvIII or aEGFRvIII-SGRP CAR T cells in 3 different effector-target ratios at 24, 48 and 72 h timepoints.

Fig. 21: FC assessment of CD4/CD8 T cell phenotypes of aCD19, aCD19-SGRP, aEGFRvIII and aEGFRvIII-SGRP CAR T cells used in this study (gated on live singlets) at the time of experimental use. One representative healthy donor CAR T cell batch is shown.

Fig. 22: Representative histograms displaying the percentage of CAR T cell degranulation (CD107a expression) in co-cultures with U251vIII, BS153, U251 or U87 GBM cell lines for 24 h (gated on live mCherry⁺ singlets). Conditions were performed in duplicates with n = 2 HDs and the experiment was repeated once

Fig. 23: IFN γ release detected by ELISA in supernatants of CAR T cells co-cultured with GBM cell lines expressing endogenous EGFRvIII (BS153), overexpressing EGFRvIII (U251vIII) or EGFRvIII⁻ (U251 and U87). Differences in IFN γ release between EGFRvIII⁺ and EGFRvIII⁻ co-cultures with each CAR were determined using Two-way ANOVA and Šidák's multiple comparisons tests. Conditions were performed in triplicates with n = 2 HDs and the experiment was repeated once.

Fig. 24: Schematic (top panel) of the experimental setup of an SGRP/aCD47 blocking assay on CD47⁺ target tumor cells where (1) BS153 were treated with aEGFRvIII-SGRP CAR conditioned-medium or aCD47, (2) exposed to bt-SIRP α which competitively bound to available CD47, and (3) CD47-bound bt-SIRP α was assessed by SA-FITC and MFIs calculated; Representative dot-plots (bottom panel) depicting CD47-blocking capacity determined by FC-based detection of SA-FITC coupled to GBM-bound bt-SIRP α . Conditions were performed in triplicates and the experiment was repeated once.

Fig. 25: FC representation of surface CD47 expression of aEGFRvIII CAR and aEGFRvIII-SGRP CAR T cells from 3 HDs. Unstained T cells served as a negative control.

Fig. 26: FC assessment of phagocytosis and macrophage polarization/effector function in co-cultures of EGFRvIII-mosaic tumor cells with donor-matched macrophages and CAR T cells from 4 HDs. Heatmap showing the MFI of markers and the fractions of phagocytosed U87 and U251vIII cells. Gated on CD11b⁺ cells. All differences between conventional CARs and SGRP-secreting CARs were not statistically significant using one-way ANOVA and Tukey's multiple comparisons tests.

Fig. 27: Experimental setup of the EGFRvIII⁺ xenograft GBM tumor model and subsequent monotherapeutic CAR and antibody treatment schemes. 7 and 14 d after orthotopic tumor implantation with U251vIII-NLuc tumor cells, animals were treated with either intratumoral (i.t.) CAR T cells or antibodies followed by BLi and scoring until the humane endpoint was reached.

Fig. 28: Overview of experimental groups/therapeutic conditions and treatment dosages

Fig. 29: Kaplan-Meier plot of overall survival (in days). Log-rank tests were used to compare selected treatment/control groups.

Fig. 30: Tumor progression in EGFRvIII⁺ xenografts was monitored for each individual mouse using BLi time course.

Fig. 31: Experimental treatment and monitoring schedule for EGFRvIII mosaic model. Animals were treated twice – at 7 and 14 days – after intracranial tumor implantation using the same stereotactic coordinates, and routinely monitored for clinical signs, weekly dual bioluminescence imaging (BLi) and morbidity/survival assessment. Plasma for cytokine analysis was collected on day 15 – 24 h after the second intratumoral (i.t.) treatment. Anti-CD47 therapy was prolonged for 4 additional intraperitoneal (i.p.) injections on days 19, 22, 26 and 29. Animals were euthanized upon reaching the humane endpoint. Upon reaching 90 d tumor-free survival, 5 aEGFRvIII-SGRP CAR-treated animals were tumor-rechallenged in the contralateral hemisphere using the same stereotactic coordinates.

Fig. 32: Experimental setup of orthotopic xenograft experiments in NSG mice encompassing co-implanted EGFRvIII⁺ U251vIII and EGFRvIII⁻ U87 GBM cell lines mimicking tumor heterogeneity, and therapeutic/control cohorts including local CAR T cell or antibody monotherapies or combinations with local SGRP or local and systemic CD47 blockade.

Fig. 33: Kaplan-Meier plot of overall survival (in days). Log-rank tests were used to compare selected treatment/control groups. Survival and bioluminescence data were pooled from 3 independent experiments.

Fig. 34: Kaplan-Meier plot of tumor-free survival (in weeks), combining survival assessment with BLi monitoring scores. Log-rank tests were used to compare selected treatment/control groups. Survival and bioluminescence data were pooled from 3 independent experiments.

Fig. 35: Tumor progression in EGFRvIII-mosaic xenografts was monitored for each individual mouse using differential BLi time course imaging with either FFz or D-luciferin substrates (in weeks); U251vIII NLuc-reporter BLi curves are depicted here.

Fig. 36: Tumor progression in EGFRvIII-mosaic xenografts was monitored for each individual mouse using differential BLi time course imaging with either FFz or D-luciferin substrates (in weeks); U87 Luc2-reporter BLi curves are depicted here.

Fig. 37: Cumulative differential monitoring in weeks by BLI for both grafted mosaic EGFRvIII⁺ and EGFRvIII⁻ tumors per experimental condition as outlined in Fig. 32. Growth of U251vIII⁺ tumors was measured by luminescence elicited by FFz, whereas growth

of U87vIII⁻ tumors was detected by D-luciferin luminescence. Curves end whenever the humane endpoint was reached.

Fig. 38: Representative overlay images of dual BLi studies at week 7 of aEGFRvIII CAR, aEGFRvIII CAR + aCD47 and aEGFRvIII-SGRP CAR treated animals highlighting the suppression of EGFRvIII⁻ tumors in aEGFRvIII-SGRP CAR treated animals.

Fig. 39: Quantification of BLi signal intensities (mean photon counts) as a surrogate for tumor burden for both EGFRvIII⁺ and EGFRvIII⁻ tumors from Fig.38 at 7 weeks after tumor implantation. Each dot represents an individual mouse. Statistical comparisons were performed with one-way ANOVA with multiple comparisons corrections.

Fig. 40: Outline of *in vivo* experiment comparing the effect of aCD19-SGRP CAR and aEGFRvIII-SGRP CAR on the GBM iTME in the context of EGFRvIII⁻ or EGFRvIII-mosaic intracerebral tumors.

Fig. 41: Heatmap of scaled median cluster-defining cell lineage marker expression on 10 immune cell populations indicated on the left y-axis. Cluster frequency is indicated on the right y-axis.

Fig. 42: Frequency of immune cell clusters in the tumor-injected brain hemispheres of U87 or U87+U251vIII tumor-engrafted animals treated with aCD19-SGRP CAR or aEGFRvIII-SGRP CAR (n = 5 per group).

Fig. 43: Kaplan-Meier plot of overall survival of aEGFRvIII-SGRP CAR-cured, tumor-rechallenged animals (in days). A Log-rank test was used to compare the rechallenge group to the historic Vehicle control group.

Fig. 44: Dual BLi monitoring of initially cured, mosaic tumor rechallenged mice in weeks after tumor rechallenge.

Fig. 45: IHC micrograph of a brain section from an aEGFRvIII-SGRP CAR-treated mouse that remained tumor-free until day 90 post tumor implantation, showing DAPI-stained cell nuclei (blue) and human CD3-stained grafted CAR T cells (brown); *Left:* Overview of brain section; Scale bar: 1 mm; *Right:* Close-up of the region defined by the white insert on the overview; White arrowheads indicate the location of CAR T cells; Scale bar: 50 μ m.

Fig. 46: Frequency of mCherry-labeled aEGFRvIII-SGRP CAR T cells gated on single live cells (Zombie Aqua[®]) in the brains of animals that remained tumor-free until day 90 post tumor implantation; Three brain regions were dissected and processed separately into single-cell suspensions: tumor-implanted (t.i.) hemisphere, contralateral (c.l.) hemisphere, and

meninges; Each dot represents one animal; Statistical comparisons were performed using a one-way ANOVA with Tukey's multiple comparison tests.

Fig. 47: Clustered heatmap of targeted post-therapy plasma proteomics showing the relative expression of proteins between all treatment groups; the analysis included samples from complementary datasets which were bridged and normalized (from lighter to darker grey: dataset 1, dataset 2, bridge samples). Individual protein expressions across the datasets are depicted on the y-axis, each cell represents the Z-score of all the measurements in this row. Data are clustered according to Euclidean distance. The barplot on the right shows the proportion of measurements under the limit of detection for each protein in each dataset.

Fig. 48: Non-metric multidimensional scaling (NMDS) plot of 92 examined human soluble proteins in mouse plasma determined by proximity extension assay. Each individual mouse is represented by one data point, with symbols and colors differentiating healthy controls (Healthy, white; $n = 6$), mock-injected (Vehicle, black; $n = 6$), mouse IgG1-treated (Isotype, yellow; $n = 6$), anti-human CD47-treated (aCD47, orange; $n = 6$), aCD19 CAR-treated (red; $n = 6$), aCD19-SGRP CAR-treated (dark red; $n = 6$), aEGFRvIII CAR-treated (blue; $n = 6$), aEGFRvIII CAR + aCD47-treated (green; $n = 6$) or aEGFRvIII-SGRP CAR-treated; (dark blue; $n = 6$). All samples were collected approximately 24 h after the last local treatment. The ellipses represent the 95% confidence interval of each treatment group. Source data are provided in Table S7.

Fig. 49: Volcano plot comparing aEGFRvIII-SGRP CAR and aEGFRvIII CAR treatment groups, showing significant enrichment of immune markers CCL3, IL13, and reduction of CD27. Significant differences in protein expression are represented by symbols: adj. $P < 0.001$, star; adj. $P < 0.05$, pentagon; $P < 0.05$, triangle; $P > 0.05$ or fold change (FC) < 0.5 , dot.

Fig. 50: Box plots showing the normalized protein expression of significant innate immune surrogate markers in plasma (CD27, CCL3, IL13, ARG1, IL1A and IFN γ). Each data point represents one mouse. Statistics were calculated using two-sided Mann-Whitney-U tests for the comparisons of interest (aEGFRvIII CAR vs aEGFRvIII CAR + aCD47, aEGFRvIII CAR vs aEGFRvIII-SGRP CAR, aEGFRvIII CAR vs aCD19 CAR, aCD19-SGRP CAR vs aEGFRvIII-SGRP CAR, aCD19 CAR vs aCD19-SGRP CAR, aEGFRvIII CAR + aCD47 vs aEGFRvIII-SGRP CAR, aCD47 vs aEGFRvIII CAR + aCD47) with Benjamini-Hochberg correction. Only significant statistics are shown.

Fig. 51: Experimental treatment and monitoring schedule of the CCL3 blockade *in vivo* cohort. Animals were treated i.t. twice – at 7 and 14 days – after i.c. implantation of EGFRvIII⁺ U251vIII and EGFRvIII⁻ U87 GBM cell lines. Tumor and CAR T cells were injected using the same stereotactic coordinates. Systemic aCCL3 therapy was administered 3x per week for 5 weeks, starting on day 8. Animals were euthanized upon reaching the humane endpoint or at day 90. CAR T cell dose: 5×10^5 cells delivered i.t.; Antibody dose: 50 ng delivered i.p.

Fig. 52: Kaplan–Meier plot of overall survival (in days). Log-rank tests were used to compare the indicated treatment/control groups.

Fig. 53: Overview of experimental groups/therapeutic conditions and treatment dosages for brain multiplex IF.

Fig. 54: H&E-stained sections of representative tumor-burdened brains at day 21 post tumor implantation and 7 days after the second treatment dose. Frontal sections of cerebrum from different experimental groups: (1) mock-injected, Vehicle, (2) mouse IgG1 (MOPC-21), Isotype, (3) anti-human CD47 (B6.H12), aCD47, (4) aCD19 CAR, (5) aEGFRvIII CAR, (6) aEGFRvIII CAR + aCD47, and (7) aEGFRvIII-SGRP CAR; Scale bars: 1000 μ m.

Fig. 55: DAPI nuclear-stained stitched assemblies of brain sections of experimental groups used for subsequent immunofluorescence multiplexing; Scale bars: 1000 μ m.

Fig. 56: Histomorphological brain tumor size assessment at day 21; n = 6 brains per condition, n = 5 brains in aEGFRvIII CAR + aCD47 group. All comparisons were non-significant using one-way ANOVA with Dunnett's multiple comparison tests.

Fig. 57: Brain collection intermediate post-therapeutic time points for conventional immunohistochemistry per experimental condition. Representative IHC images at d 13 for aEGFRvIII-SGRP CAR treatment and d 21 for all other groups showing CD3⁺ human T cells within the tumor core and adjacent brain (human CD3 IHC). *Left column:* overview of tumor-burdened brain sections, scale bar: 1 mm; *right column:* close-up according to the inserts at the tumor-brain interface. Scale bar: 100 μ m. Bar graphs: Quantification of CD3⁺ T cells in the tumor rim and tumor core.

Fig. 58: Pie charts displaying relative comparisons of the percentage of marker-positive cells/all cells within the whole tumor or tumor cores in experimental conditions. Two slides per condition were assessed creating a ratio between overall positive/negative cells. No tumors were detected in any of the aEGFRvIII-SGRP CAR-treated brains analyzed.

Fig. 59: Brain collection intermediate post-therapeutic time points for conventional immunohistochemistry per experimental condition. Representative IHC images at d 13 for aEGFRvIII-SGRP CAR treatment and d 21 for all other groups showing CD68 stained brain sections per therapeutic condition. *Left column:* overview of tumor-burdened brain sections, scale bar: 1 mm; *right column:* close-up according to the inserts at the tumor-brain interface. Scale bar: 100 μ m. Percentage of CD68⁺ cells within the tumor rim (defined as 100 μ m expansion around the tumor). Percentage of CD68⁺ cells within the tumor core (delineated based on nuclear stain (or DAPI) density).

Fig. 60: Assessment of spleen to bodyweight ratio on day 21 post tumor implantation, after two treatments on days 7 and 14. Comparisons of all treatment groups to vehicle were non-significant using a one-way ANOVA with Dunnett's multiple comparisons tests.

Fig. 61: Weekly weight monitoring of animals in the EGFRvIII-mosaic GBM survival experiment, showing aEGFRvIII CAR, aEGFRvIII CAR + aCD47, and aEGFRvIII-SGRP CAR groups. Dashed vertical lines mark the treatments in weeks 1 and 2 post tumor implantation.

Fig. 62: Box plots with individual data points showing the normalized protein expression (NPX) of IL6 in plasma the day after the second treatment (day 15 post tumor implantation) determined by proximity extension assay. Each data point represents one mouse (n = 6 per group). Statistics were calculated for the comparisons of conventional CAR to its SGRP-secreting CAR counterpart using two-sided Mann-Whitney-U tests with Benjamini-Hochberg correction; All comparisons were non-significant.

Fig. 63: Mouse CRP in plasma collected the day after the first treatment (day 8 post tumor implantation), detected by ELISA. Only the aEGFRvIII CAR + aCD47 comparison to vehicle was significant ($P = 0.0138$) using one-way ANOVA with Dunnett's multiple comparisons tests.

Fig. 64: Longitudinal monitoring of hematological parameters: erythrocyte count (RBC), platelet count (PLT) and neutrophil count (NEUT) for up to 20 days after a single treatment. The scale was normalized to Z-score to allow direct comparisons between time points. A mixed effects model was applied to each variable using the lme4 package in R. Post hoc tests for significant effects were conducted using estimated marginal means (emmeans package) to compare conditions within each time point. Significant differences between conditions were found only in neutrophil counts at day 3, 13, and 20.

Fig. 65: Luxol fast blue-stained micrographs of mouse brain sections per therapeutic condition at day 7 post treatment (single dose) showing myelin density; *Left column:* Overview of representative brain sections per condition; White arrowheads indicate the magnified regions of the corpus callosum on the overview of each condition; Scale bars: 1 mm; *Right column:*

5 Close-up of the regions defined by the inserts per condition; Scale bar: 100 μ m.

Fig. 66: Quantification of myelin-covered brain area on day 7 post therapy; n = 5 brains per condition, n = 6 brains in the aEGFRvIII CAR group. All comparisons were non-significant using one-way ANOVA with Dunnett's multiple comparison tests. **a-g,** Mice in all experiments were injected i.c. with a tumor mix of 2.5×10^4 U87 and 2.5×10^4 U251vIII

10 cells; Each treatment dose consisted of 5×10^5 CAR T cells and/or 5 μ g antibody injected i.t.

Fig. 67: Overview of titrated/optimized antibody panel and conjugated fluorophores employed in spectral flow cytometry.

Fig. 68: *Left panel:* pseudocolor plots of a representative sample gating strategy to yield live (Zombie NIR⁻), CD45⁺ or CD45⁻ singlets. *Right panel:* Scatter bar plot depicting influx of

15 overall increased CD45⁺ cells after CAR treatments (n = 6 tumor dissociations per experimental group; each dot represents measurements from an individual animal). Statistics: One-way ANOVA with Tukey's multiple comparison test * $P < 0.05$. The gating strategy is detailed in Fig. 71

Fig. 69: *Left panel:* Representative pseudocolor plots visualizing mCherry⁺ CAR T cells and mTagBFP2⁺ tumor cells per experimental conditions. *Right panel:* quantification of CAR T cells and tumor cells per condition. Statistics: One-way ANOVA with Tukey's multiple comparison test. * $P < 0.05$.

Fig. 70: Pseudocolor plots displaying the 4 most prominent CD45⁺ myeloid subsets in the TME of Vehicle or CAR-treated animals, differentiated by expression of P2RY12 and F4/80.

25 *Bottom plot:* Backgating of the 4 populations termed 'activated microglia' (dark green, P2RY12^{int}F4/80^{hi}), 'microglia' (light green, P2RY12^{hi}F4/80^{int}), 'moMacs' (orange, P2RY12^{lo}F4/80^{hi}), and 'granulocytes' (red, P2RY12⁻F4/80⁻).

Fig. 71: Gating strategy to yield CD45⁺ or CD45⁻ live singlets. Further myeloid subdivision on CD45⁺ cells was performed by identifying 4 populations with differential expression of P2RY12 and F4/80, respectively, named monocyte-derived cells (MdCs), activated microglia, resting microglia, and neutrophils. Human CAR T cells (moCD45⁻) and BFP2⁺ tumor cells were assessed in the CD45⁻ gate.

30

Fig. 72: Representative heatmap overlay of expression values of CD11c (top), CD86 (center) and MHCII (bottom) per experimental condition on the 4 different subsets from Fig. 70. The color bar insert represents MFI values.

Fig. 73: Quantification of mTagBFP2 signal intensities within different phagocytic subsets.

- 5 *Top panel:* Staggered histograms of the median fluorescent intensities of mTagBFP2 for all Vehicle (light blue), aEGFRvIII CAR (dark blue) and aEGFRvIII-SGRP CAR (red) -treated animals. *Bottom panel:* Scatter plots of mTagBFP2 MFI values per condition in activated microglia, microglia and macrophages. Each dot represents measurements from an individual brain (n = 6 per condition). Statistics: One-way ANOVA with Tukey's multiple comparison
- 10 test. Adj. *P* values * $P < 0.05$, ** $P = 0.0013$, *** $P < 0.005$, **** $P < 0.0001$.

Fig. 74: MFI of TNF and MHCII on the activated microglia subpopulation. Statistics: One-way ANOVA with Tukey's multiple comparison test. Adj. *P* values listed.

Fig. 75: TSNE (*top*) and UMAP (*bottom*) plots depicting the initial 15 clusters within the CD45⁺ pre-gated population.

- 15 **Fig. 76:** Clustered heatmap of scaled median marker expression per cluster. The number of cells per cluster is indicated on the right y-axis.

Fig. 77: Histograms of marker expression per cluster.

Fig. 78: Frequency of clusters in aEGFRvIII CAR-treated animals (E, n = 5 animals), and aEGFRvIII-SGRP CAR-treated animals (ES, n = 5 animals).

- 20 **Fig. 79:** TSNE plot after merging and annotation of initial clustering. 8 clusters (microglia, activated microglia, MoMac, MoDC, monocytes, pDC, neutrophils, and unknowns) were identified.

- Fig. 80:** UMAP (plots after merging and annotation of initial clustering. 8 clusters (microglia, activated microglia, MoMac, MoDC, monocytes, pDC, neutrophils, and unknowns) were
- 25 identified.

Fig. 81: Individual TSNEs per experimental condition and animal (E = aEGFRvIII CAR, ES = aEGFRvIII-SGRP CAR treatments).

Fig. 82: Stacked bar plots representing cluster frequencies per condition.

- Fig. 83:** Representative heatmap overlay of expression values of CD11c (top), CD86 (center) and MHCII (bottom) per experimental condition on the 4 different subsets identified by
- 30 conventional gating and flow cytometric analysis. The color bar insert represents MFI values.

Fig. 84: Overlay of P2RY12/F4/80 (upper panel) and P2RY12/CD11c on the TSNE plot from Fig. 79 displaying differential contribution of these markers to the microglia subclusters.

- Fig. 85:** TSNE plot of microglia-specific sub clustering, resulting in 3 microglia populations: resting microglia, MHCII-high microglia and activated microglia. Right upper panel: merged TSNE plot for aEGFRvIII CAR-treated animals; right lower panel: TSNE plot for aEGFRvIII-SGRP CAR treated animals.
- 5 **Fig. 86:** Histograms displaying individual marker expression per microglia subcluster.
- Fig. 87:** Stacked bar plots displaying the frequency distribution of microglia subpopulations per individual experimental animal.
- Fig. 88:** Median expression heatmap displaying XCR1 expression levels in microglia subpopulation per experimental condition/individual animal.
- 10 **Fig. 89:** Overview of median expression heatmaps per marker assessed per microglia subpopulations.
- Fig. 90:** EGFRvIII-expression analysis by quantitative real-time PCR (qPCR) in U87, U251 and U251vIII cell lines. RFU: relative fluorescent units.
- Fig. 91:** *Top panel:* Experimental setup of pharmacoscopy experiment. Batched, frozen
- 15 single-cell suspensions from GBM patients (tumor center) were thawed and plated at equal numbers into 384 well plates. Beforehand, EGFRvIII status was determined on RNA extracts of matching samples. Single-cell suspensions were co-cultured with aEGFRvIII, aEGFRvIII-SGRP, aCD19 or aCD19-SGRP CAR T cells for 48 h, fixed, stained and imaged via confocal microscopy. *Center panel:* Exemplary immunofluorescence readouts of co-cultures of CAR T
- 20 cells with single-cell suspensions derived from 5 GBM patients; Scale: 100 μ m. *Bottom panel:* **Left:** Mean EGFRvIII⁺ tumor cell count displayed as fold change relative to the aCD19 CAR control. **Middle:** Mean Nestin⁺ tumor cell count displayed as fold change relative to the aCD19 CAR control. **Right:** Mean CD14⁺ tumor cell count displayed as fold change relative to the aCD19 CAR control. Statistics: One-way ANOVA with multiple comparisons
- 25 correction.
- Fig. 92:** Results of EGFRvIII screening by qPCR of patient-derived GBM EGFRvIII positive samples.
- Fig. 93:** Results of EGFRvIII screening by qPCR of patient-derived GBM EGFRvIII negative samples.
- 30 **Fig. 94:** Boxplots summarizing expression of EGFRvIII (upper panel) and EGFR wt (lower panel) in the 2 cohorts.
- Fig. 95:** Experimental setup and timeline of interventions of peripheral CD19⁺ lymphoma model treated with systemic CAR T cell infusions. Three days after tumor implantation in the

right flank, mice were treated i.v. with CAR T cells, followed by 3 times weekly tumor volume assessment and clinical scoring. Mice were sacrificed upon reaching the humane endpoint.

Fig. 96: Overview of experimental groups/therapeutic conditions and treatment dosages.

5 **Fig. 97:** Kaplan-Meier plot of overall survival (in days). Log-rank tests were used to compare indicated treatment/control groups.

Fig. 98: Tumor volume measurements in mm³ of individual animals over time (in days post tumor implantation).

10 Detailed description of the invention

As outlined above, the present invention relates to a polynucleotide molecule comprising a nucleotide sequence encoding a chimeric antigen receptor (CAR) or a fragment thereof i) comprising an extracellular domain comprising an antigen-binding region which binds to epidermal growth factor receptor variant III (EGFRvIII); or ii) comprising an extracellular
15 domain comprising an antigen-binding region which binds to CD19; wherein the nucleotide sequence encoding the CAR is operably linked to a promoter and wherein the polynucleotide molecule further comprises a nucleotide sequence encoding a heterologous signal peptide fused to a signal regulatory protein gamma (SIRP γ)-related protein (SGRP). The present invention relates further to a chimeric antigen receptor (CAR)-T cell expressing the
20 polynucleotide molecule and methods of treating cancer using the polynucleotide molecule and/or the chimeric antigen receptor (CAR)-T cell expressing the polynucleotide molecule.

For the purposes of interpreting this specification, the following definitions will apply and whenever appropriate, terms used in the singular will also include the plural and vice versa. It
25 is to be understood that the terminology used herein is for the purpose of describing particular embodiments only and is not intended to be limiting.

Features, integers, characteristics, compounds described in conjunction with a particular aspect, embodiment or example of the invention are to be understood to be applicable to any
30 other aspect, embodiment or example described herein unless incompatible therewith. All of the features disclosed in this specification (including any accompanying claims, abstract and drawings), and/or all of the steps of any method or process so disclosed, may be combined in any combination, except combinations where at least some of such features and/or steps are

mutually exclusive. The invention is not restricted to the details of any foregoing embodiments.

The term “comprise” and variations thereof, such as, “comprises” and “comprising” is
5 generally used in the sense of include, that is, as “including, but not limited to”, that is to say permitting the presence of one or more features or components.

The singular forms “a,” “an,” and “the” include plural referents unless the content clearly dictates otherwise.

10

The term "about" refers to a range of values $\pm 10\%$ of a specified value. For example, the phrase "about 200" includes $\pm 10\%$ of 200, or from 180 to 220.

The term “fragment thereof” or “fragment” in relation to a chimeric antigen receptor (CAR)
15 refer to functionally active fragments of the CAR, preferably to functionally active fragments of the CAR which are capable of exercising the same physiological function as the CAR.

Thus, in a first aspect the present invention provides a polynucleotide molecule comprising a nucleotide sequence encoding a chimeric antigen receptor (CAR) or a fragment thereof i)
20 comprising an extracellular domain comprising an antigen-binding region which binds to epidermal growth factor receptor variant III (EGFRvIII); or ii) comprising an extracellular domain comprising an antigen-binding region which binds to CD19; wherein the nucleotide sequence encoding the CAR is operably linked to a promoter and wherein the polynucleotide molecule further comprises a nucleotide sequence encoding a heterologous signal peptide
25 fused to a signal regulatory protein gamma (SIRP γ)-related protein (SGRP).

In one embodiment the polynucleotide molecule further comprises a nucleotide sequence encoding a self-cleaving peptide.

30 In a preferred embodiment the polynucleotide molecule comprises in the following order from the 5' to the 3' end:
a) a promoter;
b) a nucleotide sequence encoding a chimeric antigen receptor (CAR) or a fragment thereof i)

- comprising an extracellular domain comprising an antigen binding region which binds to epidermal growth factor receptor variant III (EGFRvIII); or ii) comprising an extracellular domain comprising an antigen binding region which binds to CD19; wherein the nucleotide sequence encoding the chimeric antigen receptor (CAR) or a fragment thereof is operably
- 5 linked to the promoter of a);
- c) a nucleotide sequence encoding a self-cleaving peptide; and
- d) a nucleotide sequence encoding a heterologous signal peptide fused to a signal regulatory protein gamma (SIRP γ)-related protein (SGRP).
- 10 In one embodiment the heterologous signal peptide fused to a signal regulatory protein gamma (SIRP γ)-related protein (SGRP) is a signal peptide selected from the group consisting of interleukin 2 (IL-2) signal peptide, interleukin 4 (IL-4) signal peptide, interleukin 9 (IL-9) signal peptide and interferon gamma (IFN γ) signal peptide, preferably selected from the group consisting of human IL-2 signal peptide, human IL-4 signal peptide, human IL-9 signal
- 15 peptide and human IFN γ signal peptide.

In a preferred embodiment the heterologous signal peptide fused to a signal regulatory protein gamma (SIRP γ)-related protein (SGRP) is a human interleukin 2 (IL-2) signal peptide.

- 20 In a further preferred embodiment the heterologous signal peptide is fused to the N-terminal region of the signal regulatory protein gamma (SIRP γ)-related protein (SGRP).

- In one embodiment the promoter which is operably linked to the nucleotide sequence encoding the chimeric antigen receptor (CAR) or a fragment thereof is the elongation factor 1
- 25 alpha (EF1A) promoter or the elongation factor 1 alpha short (EFS) promoter, preferably the EF1A promoter.

- In a more preferred embodiment the chimeric antigen receptor (CAR) or a fragment thereof comprises i) an extracellular domain comprising an antigen binding region which binds to
- 30 epidermal growth factor receptor variant III (EGFRvIII); or ii) an extracellular domain comprising an antigen binding region which binds to CD19; wherein the CAR or a fragment thereof further comprises a CD8 α leader, CD8 α hinge and transmembrane domains, a TNF receptor superfamily member 9 costimulatory domain and a CD3 ζ signaling domain.

In an even more preferred embodiment the chimeric antigen receptor (CAR) or a fragment thereof comprises i) an extracellular domain comprising an antigen binding region which binds to epidermal growth factor receptor variant III (EGFRvIII); or ii) an extracellular domain comprising an antigen binding region which binds to CD19; wherein the the CAR or a
5 fragment thereof further comprises a CD8 α leader, CD8 α hinge and transmembrane domains, a 4-1BB costimulatory domain and a CD3 ζ signaling domain.

In one embodiment i) the extracellular domain comprising an antigen binding region which
10 binds to epidermal growth factor receptor variant III (EGFRvIII) is a single-chain variable fragment (scFv); or ii) the extracellular domain comprising an antigen binding region which binds to CD19 is a single-chain variable fragment (scFv).

In one embodiment the self-cleaving peptide is a T2A peptide or a P2A peptide, preferably a
15 T2A peptide.

In a preferred embodiment the polynucleotide molecule comprises the sequence as shown in SEQ ID NO: 1:

```
GGCTCCGGTGCCCGTCAGTGGGCAGAGCGCACATCGCCACAGTCCCGAGAAGT
20 TGGGGGGAGGGGTTCGGCAATTGAACCGGTGCCTAGAGAAGGTGGCGCGGGGTAA
ACTGGGAAAGTGATGTCGTGTACTGGCTCCGCCTTTTTCCCGAGGGTGGGGGAGA
ACCGTATATAAGTGCAGTAGTCGCCGTGAACGTTCTTTTTCGCAACGGGTTTGCCG
CCAGAACACAGGTAAGTGCCGTGTGTGGTTCCCGCGGGCCTGGCCTCTTTACGGG
TTATGGCCCTTGCGTGCCTTGAATTACTTCCACCTGGCTGCAGTACGTGATTCTTG
25 ATCCCGAGCTTCGGGTTGGAAGTGGGTGGGAGAGTTCGAGGCCTTGCGCTTAAGG
AGCCCCTTCGCCTCGTGCTTGAGTTGAGGCCTGGCCTGGGCGCTGGGGCCGCCGC
GTGCGAATCTGGTGGCACCTTCGCGCCTGTCTCGCTGCTTTCGATAAGTCTCTAGC
CATTTAAAATTTTTGATGACCTGCTGCGACGCTTTTTTTCTGGCAAGATAGTCTTG
TAAATGCGGGCCAAGATCTGCACACTGGTATTTTCGGTTTTTTGGGGCCGCGGGCGG
30 CGACGGGGCCCGTGCGTCCCAGCGCACATGTTTCGGCGAGGCGGGGCCTGCGAGC
GCGGCCACCGAGAATCGGACGGGGGTAGTCTCAAGCTGGCCGGCCTGCTCTGGTG
CCTGGTCTCGCGCCGCGGTATCGCCCCGCCCTGGGCGGCAAGGCTGGCCCGGT
CGGCACCAGTTGCGTGAGCGGAAAGATGGCCGCTTCCCGGCCCTGCTGCAGGGA
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GCTCAAAATGGAGGACGCGGCGCTCGGGAGAGCGGGCGGGTGAGTCACCCACAC
AAAGGAAAAGGGCCTTTCCGTCTCAGCCGTCGCTTCATGTGACTCCACGGAGTA
CCGGGCGCCGTCCAGGCACCTCGATTAGTTCTCGAGCTTTTGGAGTACGTCGTCTT
TAGGTTGGGGGGAGGGGTTTTATGCGATGGAGTTTCCCCACACTGAGTGGGTGGA
5 GACTGAAGTTAGGCCAGCTTGGCACTTGATGTAATTCTCCTTGGAATTTGCCCTTT
TTGAGTTTGGATCTTGGTTCATTCTCAAGCCTCAGACAGTGGTTCAAAGTTTTTTT
CTTCCATTTAGGTGTCGTGACAAGTTTGTACAAAAAGCAGGCTGCCACCATGG
CCTTACCAGTGACCGCCTTGCTCCTGCCGCTGGCCTTGCTGCTCCACGCCGCCAGG
CCGGGATCCGAGATTCAGCTGCAGCAATCTGGGGCAGAACTTGTGAAGCCAGGG
10 GCCTCAGTCAAGCTGTCCTGCACAGGTTCTGGCTTCAACATTGAAGACTACTATAT
TCACTGGGTGAAGCAGAGGACTGAACAGGGCCTGGAATGGATTGGAAGGATTGA
TCCTGAGAATGATGAACTAAATATGGCCCAATATTCCAGGGCAGGGCCACTATA
ACAGCAGACACATCCTCCAACACAGTCTACCTGCAACTCAGCAGCCTGACATCTG
AGGACACTGCCGTCTATTACTGTGCCTTTTCGCGGTGGAGTCTACTGGGGGCCAGG
15 AACCCTCTCACAGTCTCCTCAGGAGGTGGTGGTTCCGGTGGTGGTGGTTCCGGA
GGTGGTGGTTCACATATGGATGTTGTGATGACCCAGTCTCCACTCACTCTATCGGT
TGCCATTGGACAATCAGCCTCCATCTCTTGCAAGTCAAGTCAGAGCCTCTTAGAT
AGTGATGGAAAGACATATTTGAATTGGTTGTTACAGAGGCCAGGCCAGTCTCCAA
AGCGCCTAATCTCTCTGGTGTCTAACTGGACTCTGGAGTCCCTGACAGGTTCACT
20 GGCAGTGGATCAGGGACAGATTTCACTGAGAATCAGCAGAGTGGAGGCTGAG
GATTTGGGAATTTATTATTGCTGGCAAGGTACACATTTTCTGGGACGTTCCGGTGG
AGGGACCAAGCTGGAGATAAAAGCTAGCACACGACGCCAGCGCCGCGACCACC
AACACCGGCGCCACCATCGCGTCGCAGCCCTGTCCCTGCGCCCAGAGGCGTGC
CGGCCAGCGGCGGGGGGCGCAGTGCACACGAGGGGGCTGGACTTCGCCTGTGAT
25 ATCTACATCTGGGCGCCCTTGCCGGGACTTGTGGGGTCTTCTCCTGTCACTGGT
TATCACCTTTACTGCAAACGGGGCAGAAAGAACTCCTGTATATATTCAAACAA
CCATTTATGAGACCAGTACAACTACTCAAGAGGAAGATGGCTGTAGCTGCCGAT
TTCCAGAAGAAGAAGAAGGAGGATGTGAACTGAGAGTGAAGTTCAGCAGGAGCG
CAGACGCCCCCGCTACAAGCAGGGCCAGAACCAGCTCTATAACGAGCTCAATCT
30 AGGACGAAGAGAGGAGTACGATGTTTTGGACAAGAGACGTGGCCGGGACCCTGA
GATGGGGGGAAAGCCGAGAAGGAAGAACCTCAGGAAGGCCTGTACAATGAACT
GCAGAAAGATAAGATGGCGGAGGCCTACAGTGAGATTGGGATGAAAGGCGAGCG
CCGGAGGGGCAAGGGGCACGATGGCCTTTACCAGGGTCTCAGTACAGCCACCAA

GGACACCTACGACGCCCTTCACATGCAGGCCCTGCCCCCTCGCGGAAGCGGAGAG
GGCAGGGGAAGTCTTCTAACATGCGGGGACGTGGAGGAAAATCCCGGCCCATG
TACAGGATGCAACTCCTGTCTTGCAATTGCACTAAGTCTTGCACTTGTCACGAATTC
GGAGGAGGAGCTGCAGATCATCCAGCCAGAGAAGCTGCTGCTGGTGACCGTGGG
5 CAAGACGGCCACTCTTCATTGTACCATCACCTCTTTGTTCCCCGTGGGTCCCATCC
AGTGGTTCCGCGGGGTCGGACCCGGGCGGGTGCTCATCTACAACCAGAAGGACG
GCCACTTTCCTAGGGTCACCACAGTAAGCGACGGCACCAAGCGCAACAATATGG
ATTTACGATTCGCATCTCTTCCATTACTCCGGCGGACGTGGGCACCTATTACTGC
GTTAAATTTTCGTAAGGGATCCCCGGAAGACGTGGAGTTCAAATCCGGGCCCTGGTA
10 CGGAGATGGCTCTGGGCGCCAAGCCCTCG.

In a further preferred embodiment the polynucleotide molecule comprises the sequence as shown in SEQ ID NO: 2:

GGCTCCGGTGCCCGTCAGTGGGCAGAGCGCACATCGCCACAGTCCCCGAGAAGT
15 TGGGGGGAGGGGTCGGCAATTGAACCGGTGCCTAGAGAAGGTGGCGCGGGGTAA
ACTGGGAAAGTGATGTCGTGTACTGGCTCCGCCTTTTTCCCGAGGGTGGGGGAGA
ACCGTATATAAGTGCAGTAGTCGCCGTGAACGTTCTTTTTTCGCAACGGGTTTGCCG
CCAGAACACAGGTAAGTGCCGTGTGTGGTTCCCGCGGGCCTGGCCTCTTTACGGG
TTATGGCCCTTGCCTGCTTGAATTACTTCCACCTGGCTGCAGTACGTGATTCTTG
20 ATCCCGAGCTTCGGGTTGGAAGTGGGTGGGAGAGTTCGAGGCCTTGCGCTTAAGG
AGCCCCCTTCGCCTCGTGCTTGAGTTGAGGCCTGGCCTGGGCGCTGGGGCCGCCG
GTGCGAATCTGGTGGCACCTTCGCGCCTGTCTCGCTGCTTTTCGATAAGTCTCTAGC
CATTTAAAATTTTGTATGACCTGCTGCGACGCTTTTTTTCTGGCAAGATAGTCTTG
TAAATGCGGGCCAAGATCTGCACACTGGTATTTTCGGTTTTTGGGGCCGCGGGCGG
25 CGACGGGGCCCGTGCGTCCCAGCGCACATGTTTCGGCGAGGCGGGGCCTGCGAGC
GCGGCCACCGAGAATCGGACGGGGGTAGTCTCAAGCTGGCCGGCCTGCTCTGGTG
CCTGGTCTCGCGCCGCCGTGTATCGCCCCGCCCTGGGCGGCAAGGCTGGCCCGGT
CGGCACCAAGTTGCGTGAGCGGAAAGATGGCCGCTTCCCGGCCCTGCTGCAGGGA
GCTCAAAATGGAGGACGCGGCGCTCGGGAGAGCGGGCGGGTGAGTCACCCACAC
30 AAAGGAAAAGGGCCTTTCCGTCCTCAGCCGTCGCTTCATGTGACTCCACGGAGTA
CCGGGCGCCGTCCAGGCACCTCGATTAGTTCTCGAGCTTTTGGAGTACGTCGTCTT
TAGGTTGGGGGGAGGGTTTTATGCGATGGAGTTTCCCCACACTGAGTGGGTGGA
GACTGAAGTTAGGCCAGCTTGGCACTTGATGTAATTCTCCTTGAATTTGCCCTTT

TTGAGTTTGGATCTTGGTTCATTCTCAAGCCTCAGACAGTGGTTCAAAGTTTTTTT
CTTCCATTTCAAGGTGTCGTGACAAGTTTGTACAAAAAAGCAGGCTGCCACCATGG
CCTTACCAGTGACCGCCTTGCTCCTGCCGCTGGCCTTGCTGCTCCACGCCGCCAGG
CCGGGATCCGACATCCAGATGACACAGACTACATCCTCCCTGTCTGCCTCTCTGG
5 GAGACAGAGTCACCATCAGTTGCAGGGCAAGTCAGGACATTAGTAAATATTTAA
ATTGGTATCAGCAGAAACCAGATGGAACCTGTTAAACTCCTGATCTACCATACATC
AAGATTACACTCAGGAGTCCCATCAAGGTTCAAGTGGCAGTGGGTCTGGAACAGAT
TATTCTCTCACCATTAGCAACCTGGAGCAAGAAGATATTGCCACTTACTTTTGCCA
ACAGGGTAATACGCTTCCGTACACGTTCCGAGGGGGGACTAAGTTGGAATAAC
10 AGGCTCCACCTCTGGATCCGGCAAGCCCGGATCTGGCGAGGGATCCACCAAGGG
CGAGGTGAAACTGCAGGAGTCAGGACCTGGCCTGGTGGCGCCCTCACAGAGCCT
GTCCGTACATGCACTGTCTCAGGGGTCTCATTACCCGACTATGGTGTAAGCTGG
ATTCGCCAGCCTCCACGAAAGGGTCTGGAGTGGCTGGGAGTAATATGGGGTAGTG
AAACCACATACTATAATTCAGCTCTCAAATCCAGACTGACCATCATCAAGGACAA
15 CTCCAAGAGCCAAGTTTTCTTAAAAATGAACAGTCTGCAAACCTGATGACACAGCC
ATTTACTACTGTGCCAAACATTATTACTACGGTGGTAGCTATGCTATGGACTACTG
GGGTCAAGGAACCTCAGTCACCGTCTCCTCAGCGGCCGCAGCTAGCACCCACGACG
CCAGCGCCGCGACCAACACCGGCGCCACCATCGCGTCGCAGCCCCTGTCCC
TGCGCCCAGAGGCGTGCCGGCCAGCGGCGGGGGGCGCAGTGACACGAGGGGGG
20 TGGACTTCGCCTGTGATATCTACATCTGGGCGCCCTTGGCCGGGACTTGTGGGGTC
CTTCTCCTGTCACTGGTTATCACCTTTTACTGCAAACGGGGCAGAAAGAACTCCT
GTATATATTCAAACAACCATTTATGAGACCAGTACAACTACTCAAGAGGAAGAT
GGCTGTAGCTGCCGATTTCCAGAAGAAGAAGAGGAGGATGTGAACTGAGAGTG
AAGTTCAGCAGGAGCGCAGACGCCCCCGCTACAAGCAGGGCCAGAACCAGCTC
25 TATAACGAGCTCAATCTAGGACGAAGAGAGGAGTACGATGTTTTGGACAAGAGA
CGTGGCCGGGACCCTGAGATGGGGGGAAAGCCGAGAAGGAAGAACCCTCAGGA
AGGCCTGTACAATGAACTGCAGAAAGATAAGATGGCGGAGGCCTACAGTGAGAT
TGGGATGAAAGGCGAGCGCCGGAGGGGCAAGGGGCACGATGGCCTTTACCAGGG
TCTCAGTACAGCCACCAAGGACACCTACGACGCCCTTCACATGCAGGCCCTGCCC
30 CCTCGCGGAAGCGGAGAGGGCAGGGGAAGTCTTCTAACATGCGGGGACGTGGAG
GAAAATCCCGGCCCATGTACAGGATGCAACTCCTGTCTTGCAATTGCACTAAGTC
TTGCACTTGTCACGAATTCGGAGGAGGAGCTGCAGATCATCCAGCCAGAGAAGCT
GCTGCTGGTGACCGTGGGCAAGACGGCCACTCTTCATTGTACCATCACCTCTTTGT

TCCCCGTGGGTCCCATCCAGTGGTTCCGCGGGGTCGGACCCGGGCGGGTGCTCAT
 CTACAACCAGAAGGACGGCCACTTTCCTAGGGTCACCACAGTAAGCGACGGCAC
 CAAGCGCAACAATATGGATTTAGCATTCGCATCTCTTCCATTACTCCGGCGGAC
 GTGGGCACCTATTACTGCGTTAAATTCGTAAGGGATCCCCGGAAGACGTGGAGT
 5 TCAATCCGGCCCTGGTACGGAGATGGCTCTGGGCGCCAAGCCCTCG.

In one embodiment the polynucleotide molecule further comprises a peptide tag, preferably a tag selected from the group consisting of CD34, FLAG and MYC.

- 10 In a further aspect, the present invention provides an amino sequence comprising a chimeric antigen receptor (CAR) or a fragment thereof comprising an extracellular domain comprising an antigen-binding region which binds to epidermal growth factor receptor variant III (EGFRvIII); wherein the nucleotide sequence encoding the chimeric antigen receptor (CAR) is operably linked to a promoter and wherein the polynucleotide molecule further comprises a
- 15 nucleotide sequence encoding a heterologous signal peptide fused to a signal regulatory protein gamma (SIRP γ)-related protein (SGRP), wherein the amino acid comprises preferably the sequence as shown in SEQ ID NO: 3:

MALPVTALLLPLALLLHAARPGSEIQLQQSGAELVKPGASVKLSCTGSGFNIEDYYIH
 WVVKQRTEQGLEWIGRIDPENDETKYGPIFQGRATITADTSSNTVYLQLSSLTSEDYAV
 20 YYCAFRGGVYWGPQTTLTVSSGGGGSGGGGSGGGGSHMDVVMQTQSPLTSLVAIGQS
 ASISCKSSQSLDSDGKTYLNWLLQRPQGSPKRLISLVSKLDSGVPDRFTGSGSGTDFT
 LRISRVEAEDLGIYYCWQGFTHFGTFGGGTKLEIKASTTTPAPRPPTPAPTIASQPLSLR
 PEACRPAAGGAVHTRGLDFACDIYWAPLAGTCGVLLSLVITLYCKRGRKLLYIFK
 QPFMRPVQTTQEEDGCSCRFPEEEEGGCELRVKFSRSADAPAYKQGQNQLYNELNLG
 25 RREEYDVLDKRRGRDPEMGGKPRRKNPQEGLYNELQKDKMAEAYSEIGMKGERRR
 GKGHDLGLYQGLSTATKDTYDALHMQALPPRGSGEGRSLLTCGDVEENPGPMYRM
 QLLSCIALSLALVTNSEEELQIIQPEKLLLVTVGKTATLHCTITSLFPVGPIQWFRGVGP
 GRVLIYNQKDGHFPRVTTVSDGTKRNNMDFSIRISSITPADVGTYCYVKFRKGSPEDV
 EFKSGPGTEMALGAKPS.

30

In a further aspect, the present invention provides an amino sequence comprising a chimeric antigen receptor (CAR) or a fragment thereof comprising an extracellular domain comprising an antigen-binding region which binds to CD19; wherein the nucleotide sequence encoding

the CAR is operably linked to a promoter and wherein the polynucleotide molecule further comprises a nucleotide sequence encoding a heterologous signal peptide fused to a signal regulatory protein gamma (SIRP γ)-related protein (SGRP), wherein the amino acid comprises preferably the sequence as shown in SEQ ID NO: 4:

5 MALPVTALLLPLALLLHAARPGSDIQMTQTTSSLSASLGDRVTISCRASQDISKYLNW
YQQKPDGTVKLLIYHTSRLHSGVPSRFSGSGSGTDYSLTISNLEQEDIATYFCQQGNTL
PYTFGGGKLEITGSTSGSGKPGSGEGSTKGEVKLQESGPGLVAPSQSLSVTCTVSGVS
LPDYGVSWIRQPPRKGLEWLGVWGETTYNSALKSRLTIKDNSKSQVFLKMNSLQ
TDDTAIYYCAKHYYYGGSYAMDYWGQTSVTVSSAAAASSTTPAPRPPTPAPTIASQ
10 PLSLRPEACRPAAGGAVHTRGLDFACDIYWAPLAGTCGVLLLSLVITLYCKRGRKKL
LYIFKQPFMRPVQTTQEEDGCSCRFPEEEEEGGCELRVKFSRSADAPAYKQGQNQLYN
ELNLGRREEYDVLDKRRGRDPEMGGKPRRKNPQEGLYNELQKDKMAEAYSEIGMK
GERRRGKGHDGLYQGLSTATKDTYDALHMQALPPRGSGEGRGSLLTCGDVEENPGP
MYRMQLLSICIALSLALVTNSEEELQIIQPEKLLLVTVGKTATLHCTITSLFPVGPIQWF
15 RGVGPGRVLIYNQKDGHFPRVTTVSDGTKRNNMDFSIRISSITPADVGTYYCVKFRKG
SPEDVEFKSGPGTEMALGAKPS.

In a further aspect, the present invention provides a construct comprising the polynucleotide molecule as described herein. In one embodiment the construct is comprised by a viral
20 expression vector, preferably by a lentiviral, retroviral or adenoviral expression vector or by a non-viral mammalian expression system, preferably by a PiggyBac, Sleeping Beauty or Tol2 expression system.

In a further aspect, the present invention provides a chimeric antigen receptor (CAR)-T cell
25 comprising T cells expressing the polynucleotide molecule as described herein and/or the construct as described herein.

In one embodiment the T cells are present in a therapeutically effective amount for the prevention and/or treatment of a cancer entity expressing epidermal growth factor receptor
30 variant III (EGFRvIII) and/or CD19.

In one embodiment at least a portion of the T cells are activated and produce one or more cytokines.

In one embodiment the one or more cytokines are selected from the group consisting of interferon gamma (IFN γ), TNF, CCL3, IL-13 and IL-1A.

- 5 In one embodiment at least a portion of the T cells express one or more surface markers selected from the group consisting of CD25, CD69 and CD107a.

In one embodiment the chimeric antigen receptor (CAR)-T cell further comprises a pharmaceutically acceptable excipient, carrier, and/or diluent which supports maintenance of
10 the T cells.

In a further aspect, the present invention provides a method of treating cancer in a subject suffering from an epidermal growth factor receptor (EGFR)-associated cancer or suffering from a CD19-associated cancer, the method comprising administering to the subject a
15 therapeutically effective amount of a chimeric antigen receptor (CAR)-T cell as described herein.

Also provided is a chimeric antigen receptor (CAR)-T cell as described herein, for use in a method for the treatment of cancer in a subject suffering from an epidermal growth factor receptor (EGFR)-associated cancer or suffering from a CD19-associated cancer, the method
20 comprising administering to the subject a therapeutically effective amount of a chimeric antigen receptor (CAR)-T cell as described herein.

Also provided is the use of a chimeric antigen receptor (CAR)-T cell as described herein for
25 the manufacture of a medicament for the treatment of cancer in a subject suffering from an epidermal growth factor receptor (EGFR)-associated cancer or suffering from a CD19-associated cancer.

Also provided is the use of a therapeutically effective amount of a chimeric antigen receptor
30 (CAR)-T cell as described herein for the treatment of cancer in a subject suffering from an epidermal growth factor receptor (EGFR)-associated cancer or suffering from a CD19-associated cancer.

In a further aspect, the present invention provides a method of treating a solid cancer in a subject, the method comprising administering to the subject a therapeutically effective amount of a chimeric antigen receptor (CAR)-T cell as described herein.

5 Also provided is a chimeric antigen receptor (CAR)-T cell as described herein, for use in a method for the treatment of a solid cancer in a subject, the method comprising administering to the subject a therapeutically effective amount of a chimeric antigen receptor (CAR)-T cell as described herein.

10 Also provided is the use of a chimeric antigen receptor (CAR)-T cell as described herein for the manufacture of a medicament for the treatment of a solid cancer in a subject.

Also provided is the use of a therapeutically effective amount of a chimeric antigen receptor (CAR)-T cell as described herein for the treatment of a solid cancer in a subject comprising
15 administering to the subject a therapeutically effective amount of a chimeric antigen receptor (CAR)-T cell as described herein.

In a further aspect, the present invention provides a method of treating cancer in a subject, wherein the cancer is located in the central nervous system and wherein the method comprises
20 administering to the subject a therapeutically effective amount of a chimeric antigen receptor (CAR)-T cell as described herein, wherein the CAR or a fragment thereof of the CAR-T cell comprises a CAR or a fragment thereof comprising an extracellular domain comprising an antigen binding region which binds to epidermal growth factor receptor variant III (EGFRvIII).

25 Also provided is a chimeric antigen receptor (CAR)-T cell as described herein, wherein the CAR or a fragment thereof of the CAR-T cell comprises a CAR or a fragment thereof comprising an extracellular domain comprising an antigen binding region which binds to epidermal growth factor receptor variant III (EGFRvIII), for use in a method for the treatment
30 of cancer in a subject, wherein the cancer is located in the central nervous system, the method comprising administering to the subject a therapeutically effective amount of a chimeric antigen receptor (CAR)-T cell as described herein.

Also provided is the use of a chimeric antigen receptor (CAR)-T cell as described herein, wherein the CAR or a fragment thereof of the CAR-T cell comprises a CAR or a fragment thereof comprising an extracellular domain comprising an antigen binding region which
5 binds to epidermal growth factor receptor variant III (EGFRvIII), for the manufacture of a medicament for the treatment of cancer in a subject, wherein the cancer is located in the central nervous system,.

Also provided is the use of a therapeutically effective amount of a chimeric antigen receptor
10 (CAR)-T cell as described herein, wherein the CAR or a fragment thereof of the CAR-T cell comprises a CAR or a fragment thereof comprising an extracellular domain comprising an antigen binding region which binds to epidermal growth factor receptor variant III (EGFRvIII), for the treatment cancer in a subject, wherein the cancer is located in the central nervous system, comprising administering to the subject a therapeutically effective amount of
15 a chimeric antigen receptor (CAR)-T cell as described herein.

In one embodiment the cancer or solid cancer is selected from the group consisting of glioma, glioblastoma (GBM), medulloblastoma, ependymoma or diffuse intrinsic pontine glioma (DIPG), and is preferably GBM.
20

In a further aspect, the present invention provides a method of treating cancer in a subject, wherein the cancer is selected from the group consisting of a breast cancer, lung cancer, melanoma, lymphoma, acute lymphocytic leukemia (ALL), and non-Hodgkin's lymphoma (NHL), preferably lymphoma, and wherein the method comprises administering to the subject
25 a therapeutically effective amount of a chimeric antigen receptor (CAR)-T cell as described herein, wherein the CAR or a fragment thereof of the CAR-T cell comprises a CAR or a fragment thereof comprising an extracellular domain comprising an antigen binding region which binds to CD19.

Also provided is a chimeric antigen receptor (CAR)-T cell as described herein, wherein the
30 CAR or a fragment thereof of the CAR-T cell comprises a CAR or a fragment thereof comprising an extracellular domain comprising an antigen binding region which binds to CD19, for use in a method for the treatment of cancer in a subject, wherein the cancer is

selected from the group consisting of a breast cancer, lung cancer, melanoma, lymphoma, acute lymphocytic leukemia (ALL), and non-Hodgkin's lymphoma (NHL), preferably lymphoma.

- 5 Also provided is the use of a therapeutically effective amount of a chimeric antigen receptor (CAR)-T cell as described herein, wherein the CAR or a fragment thereof of the CAR-T cell comprises a CAR or a fragment thereof, comprising an extracellular domain comprising an antigen binding region which binds to CD19, for the manufacture of a medicament for the treatment of cancer in a subject, wherein the cancer is selected from the group consisting of a
- 10 breast cancer, lung cancer, melanoma, lymphoma, acute lymphocytic leukemia (ALL), and non-Hodgkin's lymphoma (NHL), preferably lymphoma.

- Also provided is the use of a therapeutically effective amount of a chimeric antigen receptor (CAR)-T cell as described herein, wherein the CAR or a fragment thereof of the CAR-T cell
- 15 comprises a CAR or a fragment thereof, comprising an extracellular domain comprising an antigen binding region which binds to CD19, for the treatment of cancer in a subject, wherein the cancer is selected from the group consisting of a breast cancer, lung cancer, melanoma, lymphoma, acute lymphocytic leukemia (ALL), and non-Hodgkin's lymphoma (NHL), preferably lymphoma.

20

In one embodiment the cancer is a target antigen-mosaic tumor or a target antigen-homogeneous tumor, preferably a target antigen-mosaic tumor.

- In a further aspect, the present invention provides a method of preparing a population of
- 25 activated T cells comprising a polynucleotide molecule as described herein or a construct as described herein, the method comprising: (i) contacting in vitro one or more T cells that have been modified to comprise a polynucleotide molecule as described herein or a construct as described herein with a stimulus that induces expansion of the T cells to provide an expanded T cell population; and (ii) activating in vitro the T cells to produce an activated T cell
- 30 population. In one embodiment, the T cells are the chimeric antigen receptor (CAR)-T cell as described herein.

In a further aspect, the present invention provides a nucleotide sequence encoding a signal regulatory protein gamma (SIRP γ)-related protein (SGRP) comprising the sequence as shown in SEQ ID NO: 5:

TACAGGATGCAACTCCTGTCTTGCATTGCACTAAGTCTTGCACTTGTCACGAATTC
5 GGAGGAGGAGCTGCAGATCATCCAGCCAGAGAAGCTGCTGCTGGTGACCGTGGG
CAAGACGGCCACTCTTCATTGTACCATCACCTCTTTGTTCCCCGTGGGTCCCATCC
AGTGGTTCCGCGGGGTCGGACCCGGGCGGGTGCTCATCTACAACCAGAAGGACG
GCCACTTTCCTAGGGTCACCACAGTAAGCGACGGCACCAAGCGCAACAATATGG
ATTTTCAGCATTCGCATCTCTTCCATTACTCCGGCGGACGTGGGCACCTATTACTGC
10 GTTAAATTTTCGTAAGGGATCCCCGGAAGACGTGGAGTTCAAATCCGGCCCTGGTA
CGGAGATGGCTCTGGGCGCCAAGCCCTCG.

In a further aspect, the present invention provides a amino sequence comprising a signal regulatory protein gamma (SIRP γ)-related protein (SGRP) comprising the sequence as shown in SEQ ID NO: 6:

MYRMQLLSICIALSLALVTNSEEELQIIQPEKLLLVTVGKTATLHCTITSLFPVGPIQWF
RGVGPGRVLIYNQKDGHFPRVTTVSDGTKRNNMDFSIRISSITPADVGTYCYVKFRKG
SPEDVEFKSGPGTEMALGAKPS.

20 In a further aspect, the present invention provides a nucleotide sequence encoding a signal regulatory protein gamma (SIRP γ)-related protein (SGRP) comprising the sequence as shown in SEQ ID NO: 18:

ATGGAGGAGGAGCTGCAGATCATCCAGCCAGAGAAGCTGCTGCTGGTGACCGTG
GGCAAGACGGCCACTCTTCATTGTACCATCACCTCTTTGTTCCCCGTGGGTCCCAT
25 CCAGTGGTTCCGCGGGGTCGGACCCGGGCGGGTGCTCATCTACAACCAGAAGGA
CGGCCACTTTCCTAGGGTCACCACAGTAAGCGACGGCACCAAGCGCAACAATATG
GATTTTCAGCATTCGCATCTCTTCCATTACTCCGGCGGACGTGGGCACCTATTACTG
CGTTAAATTTTCGTAAGGGATCCCCGGAAGACGTGGAGTTCAAATCCGGCCCTGGT
ACGGAGATGGCTCTGGGCGCCAAGCCCTCG.

Examples

Materials and Methods

SGRP engineering for production and release by CAR T cells

To generate SGRP, 9 amino acid (AA) substitutions were made to the endogenous human
5 SIRP γ binding domain (hSIRP γ -V1) AA sequence, as previously described by Ring and
colleagues²⁹. A human IL-2 signal peptide (IL2sig) sequence was added to the N-terminus of
SGRP to allow its secretion by T cells to the extracellular space. The SGRP AA sequence was
optimized for production by human T cells using GenSmart Codon Optimization (GenScript
Biotech, USA) and reverse-translated into a nucleotide (nt) sequence. All AA and nt sequences
10 are listed in **Table 1** below.

CD47-SIRP/SGRP protein interaction modeling

Protein structures of SGRP, human SIRP α binding domain (hSIRP α -V1), murine SIRP α
binding domain (mSIRP α -V1) and human CD47 (hCD47) were predicted by AlphaFold^{30,31},
using the source code, trained weights and inference script available under an open-source
15 license: <https://github.com/deepmind/alphafold>. AA sequences of SGRP, hSIRP α -V1,
mSIRP α -V1 and hCD47 were entered and folded using the multimer model. Sequences were
superimposed against a genetic database to generate multiple sequence alignment statistics.
Predictions ran on 'relax mode without GPU' and generated 3D interaction models for SGRP
and hCD47, hSIRP α -V1 and hCD47, or mSIRP α -V1 and hCD47. Predicted local distance
20 difference tests (pLDDT) were calculated to evaluate local distance differences of all atoms in
each model and validate stereochemical plausibility (data not shown). All AA and nt sequences
are listed in **Table 1** below:

Table 1

Protein/peptide	SEQ ID NO:	Uniprot Ref	AA sequence
Human CD47	7	Q08722	QLLFNKTKSVEFTFCNDTVVIPCFVTNMEAQ NTTEVYVKWKFKGRDIYTFDGALNKSTVPT DFSSAKIEVSQLLKGDASLKMDKSDAVSHT GNYTCEVTELTREGETIIEELKYRVVSWFSPN ENILIVIFPIFAILLFWGQFGIKTLKYRSGGMD EKTIALLVAGLVITVIVIVGAILFVPGEYSLKN

			ATGLGLIVTSTGILILLHYVVFSTAIGLTSFVIA ILVIQVIAYILAVVGLSLCIAACIPMHGPLLISG LSILALAQLLGLVYMKFVASNQKTIQPPRKA VEEPLNAFKESKGMNDE
Human IL2sig	8	P60568	MYRMQLLSICIALSLALVTNS
Human SIRP α -V1	9	P78324	EELQVIQPDKSVLVAAGETATLRCTATSLIPV GPIQWFRGAGPGRELIYNQKEGHFPRVTTV SDLTKRNNMDFSIRIGNITPADAGTYCYVKF RKGSPDDVEFKSG
Human SIRP γ -V1	10	Q9P1W8	EELQMIQPEKLLLVTVGKTATLHCTVTSLLP VGPVLWFRGVGPGRELIYNQKEGHFPRVT TVSDLTKRNNMDFSIRISSITPADVGTYCYCV KFRKGSPENVEFKSG
IL2sig-SGRP	6	NA	MYRMQLLSICIALSLALVTNSEELQIIQPEKL LLLVTVGKTATLHCTITSLFPVGPIQWFRGVG PGRVLIYNQKDGHFPRVTTVSDGTKRNNM DFSIRISSITPADVGTYCYVKFRKGSPEDVE FKSGPGTEMALGAKPS
Murine SIRP α -V1	11	P97797	KELKVTQPEKSVSVAAGDSTVLNCTLTSLLP VGPIRWYRGVGPSRLLIYSFAGEYVPRIRNV SDTTKRNNMDFSIRISNVTPADAGIYYCVKF QKGSSEPDTEIQS
SGRP	12	NA	EEELQIIQPEKLLLVTVGKTATLHCTITSLFPV GPIQWFRGVGPGRVLIYNQKDGHFPRVTTV SDGTKRNNMDFSIRISSITPADVGTYCYVKF RKGSPEDVEFKSGPGTEMALGAKPS

Table 1 - continued

Protein/peptide	SEQ ID NO:	NCBI Ref Seq	nt sequence
Human CD47	13	NM_001777.4	CAGCTACTATTTAATAAAACAAAATCTGTA GAATTCACGTTTTGTAATGACACTGTCGTC ATTCCATGCTTTGTTACTAATATGGAGGCA CAAAACACTACTGAAGTATACGTAAAGTG GAAATTTAAAGGAAGAGATATTTACACCTT TGATGGAGCTCTAAACAAGTCCACTGTCC CCACTGACTTTAGTAGTGCAAAAATTGAA GTCTCACAATTACTAAAAGGAGATGCCTC TTTGAAGATGGATAAGAGTGATGCTGTCT CACACACAGGAACTACACTTGTGAAGTA ACAGAATTAACCAGAGAAGGTGAAACGAT CATCGAGCTAAAATATCGTGTTGTTTCATG GTTTTCTCCAAATGAAAATATTCTTATTGTT ATTTTCCCAATTTTGTACTACTCCTGTTT TGGGGACAGTTTGGTATTAACAACTTAA ATATAGATCCGGTGGTATGGATGAGAAAA CAATTGCTTTACTTGTGCTGGACTAGTGA TCACTGTCATTGTCATTGTTGGAGCCATT TTTTCGTCCCAGGTGAATATTCATTAAAGA ATGCTACTGGCCTTGGTTTAATTGTGACTT CTACAGGGATATTAATATTACTTCACTACT ATGTGTTTAGTACAGCGATTGGATTAACCT CCTTCGTCAATGCCATATTGGTTATTCAGG TGATAGCCTATATCCTCGCTGTGGTTGGA CTGAGTCTCTGTATTGCGGCGTGATACC

			AATGCATGGCCCTCTTCTGATTTTCAGGTTT GAGTATCTTAGCTCTAGCACAATTACTTG GACTAGTTTATATGAAATTTGTGGCTTCCA ATCAGAAGACTATACAACCTCCTAGGAAA GCTGTAGAGGAACCCCTTAATGCATTCAA AGAATCAAAAGGAATGATGAATGATGAA
Human IL2sig	14	NM_000586 .4	ATGTACAGGATGCAACTCCTGTCTTGCAT TGCACTAAGTCTTGCACTTGTCACGAATT CG
Human SIRP α -V1	15	NM_001040 022.1	GAGGAGGAGCTGCAGGTGATTCAGCCTG ACAAGTCCGTGTTGGTTGCAGCTGGAGA GACAGCCACTCTGCGCTGCACTGCGACC TCTCTGATCCCTGTGGGGCCCATCCAGTG GTTTCAGAGGAGCTGGACCAGGCCGGGAA TTAATCTACAATCAAAAAGAAGGCCACTTC CCCCGGGTAACAACCTGTTTCAGACCTCAC AAAGAGAAACAACATGGACTTTTCCATCC GCATCGGTAACATCACCCAGCAGATGC CGGCACCTACTACTGTGTGAAGTTCCGGA AAGGGAGCCCCGATGACGTGGAGTTTAA GTCTGGA
Human SIRP γ -V1	16	NM_018556 .4	GAGGAGGAGCTACAGATGATTCAGCCTG AGAAGCTCCTGTTGGTCACAGTTGGAAAG ACAGCCACTCTGCACTGCACTGTGACCTC CCTGCTTCCCGTGGGACCCGTCCTGTGG TTCAGAGGAGTTGGACCAGGCCGGGAAT TAATCTACAATCAAAAAGAAGGCCACTTC CCCAGGGTAACAACAGTTTCAGACCTCAC AAAGAGAAACAACATGGACTTTTCCATCC GCATCAGTAGCATCACCCAGCAGATGTC GGCACATACTACTGTGTGAAGTTTCGAAA AGGGAGCCCTGAGAACGTGGAGTTTAAAG TCTGGA
IL2sig-SGRP	5	NA	TACAGGATGCAACTCCTGTCTTGCATTGC ACTAAGTCTTGCACTTGTCACGAATTCGG AGGAGGAGCTGCAGATCATCCAGCCAGA GAAGCTGCTGCTGGTGACCGTGGGCAAG ACGGCCACTCTTCATTGTACCATCACCTC TTTGTTCCCGTGGGTCCCATCCAGTGGT TCCGCGGGGTGCGACCCGGGCGGGTGC TCATCTACAACCAGAAGGACGGCCACTTT CCTAGGGTCACCACAGTAAGCGACGGCA CCAAGCGCAACAATATGGATTTTCAGCATT CGCATCTCTTCCATTACTCCGGCGGACGT GGGCACCTATTACTGCGTTAAATTTTCGTA AGGGATCCCCGGAAGACGTGGAGTTCAA ATCCGGCCCTGGTACGGAGATGGCTCTG GGCGCCAAGCCCTCG
Murine SIRP α -V1	17	NM_007547 .5	AAGGAAGTGAAGGTGACTCAGCCTGAGAA ATCAGTGTCTGTTGCTGCTGGGATTTCGA CCGTTCTGAACTGCACTTTGACCTCCTTG TTGCCGGTGGGACCCATTAGGTGGTACA GAGGAGTAGGGCCAAGCCGGCTGTTGAT

			CTACAGTTTCGCAGGAGAATACGTTCCCTC GAATTAGAAATGTTTCAGATACTACTAAGA GAAACAATATGGACTTTTCCATCCGTATCA GTAATGTCACCCCAGCAGATGCTGGCATC TACTACTGTGTGAAGTTCCAGAAAGGATC ATCAGAGCCTGACACAGAAATACAATCT
SGRP	18	NA	ATGGAGGAGGAGCTGCAGATCATCCAGC CAGAGAAGCTGCTGCTGGTGACCGTGGG CAAGACGGCCACTCTTCATTGTACCATCA CCTCTTTGTTCCCCGTGGGTCCCATCCAG TGGTTCCGCGGGGTCTGGACCCGGGCGG GTGCTCATCTACAACCAGAAGGACGGCC ACTTTCCTAGGGTCACACAGTAAGCGAC GGCACCAAGCGCAACAATATGGATTTCAG CATTTCGATCTCTTCCATTACTCCGGCGG ACGTGGGCACCTATTACTGCGTTAAATTT CGTAAGGGATCCCCGGAAGACGTGGAGT TCAAATCCGGCCCTGGTACGGAGATGGC TCTGGGCGCCAAGCCCTCG

CAR construct and lentiviral expression vector design

For human T cell transduction, replication-defective lentiviruses were produced using a second-generation lentiviral system with transfer plasmids encoding a 3C10.BBz^{32,33} (anti-EGFRvIII) or FMC63.BBz^{34,35} (anti-CD19) CAR, an mCherry fluorescence reporter protein and, in some iterations, SGRP^{29,36}. The CAR structure consisted of a CD8 α leader, a single-chain variable fragment (scFv), CD8 α hinge and transmembrane domains, a 4-1BB costimulatory domain and a CD3 ζ signaling domain. Transgene expression was driven by the *EF1A* promoter and polyprotein sequences were cleaved by T2A or P2A peptides. All sequences were assembled with Vector Design Studio (VectorBuilder, USA). The vectors included an ampicillin resistance gene for positive selection of transformed *E. coli*. The lentiviral plasmids were purchased as bacterial glycerol stocks from VectorBuilder (VectorBuilder, USA). CAR vector maps are illustrated in **Fig. 5** and CAR vector sequences are listed in **Table 2** below:

Table 2

CAR construct	CAR design	bp	SEQ ID NO:	nt sequence
aCD19 CAR	FMC63.BBz	2262	19	ATGGCCTTACCAAGTGACCGCCTTGCTCC TGCCGCTGGCCTTGCTGCTCCACGCCG CCAGGCCGGGATCCGACATCCAGATGA CACAGACTACATCCTCCCTGTCTGCCTC TCTGGGAGACAGAGTCACCATCAGTTGC AGGGCAAGTCAGGACATTAGTAAATATT TAAATTGGTATCAGCAGAAACCAGATGG AACTGTAAACTCCTGATCTACCATACAT

				CAAGATTACACTCAGGAGTCCCATCAAG GTTCAAGTGGCAGTGGGTCTGGAACAGA TTATTCTCTCACCATTAGCAACCTGGAG CAAGAAGATATTGCCACTTACTTTTGCCA ACAGGGTAATACGCTTCCGTACACGTTC GGAGGGGGGACTAAGTTGAAATAACA GGCTCCACCTCTGGATCCGGAAGCCC GGATCTGGCGAGGGATCCACCAAGGGC GAGGTGAACTGCAGGAGTCAGGACCT GGCCTGGTGGCGCCCTCACAGAGCCTG TCCGTACATGCACTGTCTCAGGGGTCT CATTACCCGACTATGGTGTAAAGCTGGAT TCGCCAGCCTCCACGAAAGGGTCTGGA GTGGCTGGGAGTAATATGGGGTAGTGA AACCACATACTATAATTCAGCTCTCAAAT CCAGACTGACCATCATCAAGGACAACTC CAAGAGCCAAGTTTTCTTAAAAATGAACA GTCTGCAAACTGATGACACAGCCATTTA CTACTGTGCCAAACATTATTACTACGGT GGTAGCTATGCTATGGACTACTGGGGTC AAGGAACCTCAGTCACCGTCTCCTCAGC GGCCGCAGCTAGCACCACGACGCCAGC GCCGCGACCACCAACACCGGCGCCAC CATCGCGTCGCAGCCCTGTCCCTGCG CCCAGAGGCGTGCCGGCCAGCGGCGG GGGGCGCAGTGCACACGAGGGGGCTG GACTTCGCCTGTGATATCTACATCTGGG CGCCCTTGGCCGGGACTTGTGGGGTCC TTCTCCTGTCACTGGTTATCACCCTTAC T GCAAACGGGGCAGAAAGAACTCCTGT ATATATTCAAACAACCATTTATGAGACCA GTACAACTACTCAAGAGGAAGATGGCT GTAGCTGCCGATTTCCAGAAGAAGA AGGAGGATGTGAACTGAGAGTGAAGTTC AGCAGGAGCGCAGACGCCCCCGCGTAC AAGCAGGGCCAGAACCAGCTCTATAAC GAGCTCAATCTAGGACGAAGAGAGGAG TACGATGTTTTGGACAAGAGACGTGGCC GGGACCCTGAGATGGGGGGAAAGCCGA GAAGGAAGAACCCTCAGGAAGGCCTGT ACAATGAACTGCAGAAAGATAAGATGGC GGAGGCCTACAGTGAGATTGGGATGAA AGGCGAGCGCCGAGGGGCAAGGGGC ACGATGGCCTTTACCAGGGTCTCAGTAC AGCCACCAAGGACACCTACGACGCCCT TCACATGCAGGCCCTGCCCCCTCGCGG AAGCGGAGAGGGCAGGGGAAGTCTTCT AACATGCGGGGACGTG GAG GAAAATCCCGGCCCCATGGTGAGCAAG GGCGAGGAGGATAACATGGCCATCATC AAGGAGTTCATGCGCTTCAAGGTGCACA TGGAGGGCTCCGTGAACGGCCACGAGT TCGAGATCGAGGGCGAGGGCGAGGGC CGCCCCCTACGAGGGCACCCAGACCGCC AAGCTGAAGGTGACCAAGGGTGGCCCC CTGCCCTTCGCCTGGGACATCCTGTCCC CTCAGTTCATGTACGGCTCCAAGGCCTA
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				CGTGAAGCACCCCGCCGACATCCCCGA CTACTTGAAGCTGTCCTTCCCCGAGGGC TTCAAGTGGGAGCGCGTGATGAACTTCG AGGACGGCGGCGTGGTGACCGTGACCC AGGACTCCTCCCTGCAGGACGGCGAGT TCATCTACAAGGTGAAGCTGCGCGGCA CCAACTTCCCCTCCGACGGCCCCGTAAT GCAGAAGAAGACCATGGGCTGGGAGGC CTCCTCCGAGCGGATGTACCCCGAGGA CGGCGCCCTGAAGGGCGA GAT CAAGCAGAGGCTGAAGCTGAAGGACGG CGGCCACTACGACGCTGAGGTCAAGAC CACCTACAAGGCCAAGAAGCCCGTGCA GCTGCCCCGGCGCTACAACGTCAACAT CAAGTTGGACATCACCTCCCACAACGAG GACTACACCATCGTGGAACAGTACGAAC GCGCCGAGGGCGGCCACTCCACCGGC GGCATGGACGAGCTGTACAAGTAA
aCD19-SGRP CAR	FMC63. BBz	2745	20	ATGGCCTTACCAGTGACCGCCTTGCTCC TGCCGCTGGCCTTGCTGCTCCACGCCG CCAGGCCGGGATCCGACATCCAGATGA CACAGACTACATCCTCCCTGTCTGCCTC TCTGGGAGACAGAGTCACCATCAGTTGC AGGGCAAGTCAGGACATTAGTAAATATT TAAATTGGTATCAGCAGAAACCAGATGG AACTGTTAACTCCTGATCTACCATACAT CAAGATTACACTCAGGAGTCCCATCAAG GTTCAGTGGCAGTGGGTCTGGAACAGA TTATTCTCTCACCATTAGCAACCTGGAG CAAGAAGATATTGCCACTTACTTTTGCCA ACAGGGTAATACGCTTCCGTACACGTTT GGAGGGGGGACTAAGTTGGAATAACA GGCTCCACCTCTGGATCCGGCAAGCCC GGATCTGGCGAGGGATCCACCAAGGGC GAGGTGAAACTGCAGGAGTCAGGACCT GGCCTGGTGGCGCCCTCACAGAGCCTG TCCGTACATGCACTGTCTCAGGGTCT CATTACCCGACTATGGTGTAAGCTGGAT TCGCCAGCCTCCACGAAAGGTCTGGA GTGGCTGGGAGTAATATGGGGTAGTGA AACCACATACTATAATTCAGCTCTCAAAT CCAGACTGACCATCATCAAGGACAATC CAAGAGCCAAGTTTTCTTAAAAATGAACA GTCTGCAAACTGATGACACAGCCATTTA CTACTGTGCCAAACATTATTACTACGGT GGTAGCTATGCTATGGACTACTGGGGTC AAGGAACCTCAGTCACCGTCTCCTCAGC GGCCGCAGCTAGCACACGACGCCAGC GCCGCGACCACCAACCCGGCGCCAC CATCGCGTCGCAGCCCCTGTCCCTGCG CCCAGAGGCGTGCCGGCCAGCGGCGG GGGGCGCAGTGACACGAGGGGGCTG GACTTCGCCTGTGATATCTACATCTGGG CGCCCTTGGCCGGGACTTGTGGGGTCC TTCTCCTGTCACTGGTTATCACCCTTTAC TGCAAACGGGGCAGAAAGAACTCCTGT ATATATTCAAACAACCATTTATGAGACCA GTACAACTACTCAAGAGGAAGATGGCT

				GTAGCTGCCGATTTCCAGAAGAAGAAGA AGGAGGATGTGAACTGAGAGTGAAGTTC AGCAGGAGCGCAGACGCCCCCGCGTAC AAGCAGGGCCAGAACCAGCTCTATAAC GAGCTCAATCTAGGACGAAGAGAGGAG TACGATGTTTTGGACAAGAGACGTGGCC GGGACCCTGAGATGGGGGGAAAGCCGA GAAGGAAGAACCCTCAGGAAGGCCTGT ACAATGAACTGCAGAAAGATAAGATGGC GGAGGCCTACAGTGAGATTGGGATGAA AGGCGAGCGCCGGAGGGGCAAGGGGC ACGATGGCCTTTACCAGGGTCTCAGTAC AGCCACCAAGGACACCTACGACGCCCT TCACATGCAGGCCCTGCCCCCTCGCGG AAGCGGAGAGGGCAGGGGAAGTCTTCT AACATGCGGGGACGTGGAG GAAAATCCCGGCCCCATGGTGAGCAAG GGCGAGGAGGATAACATGGCCATCATC AAGGAGTTCATGCGCTTCAAGGTGCACA TGGAGGGCTCCGTGAACGGCCACGAGT TCGAGATCGAGGGCGAGGGCGAGGGC CGCCCCCTACGAGGGCACCCAGACCGCC AAGCTGAAGGTGACCAAGGGTGGCCCC CTGCCCTTCGCCTGGGACATCCTGTCCC CTCAGTTCATGTACGGCTCCAAGGCCTA CGTGAAGCACCCCGCCGACATCCCCGA CTACTTGAAGCTGTCTTCCCCGAGGGC TTCAAGTGGGAGCGCGTGATGAACTTCG AGGACGGCGGCGTGGTGACCGTGACCC AGGACTCCTCCCTGCAGGACGGCGAGT TCATCTACAAGGTGAAGCTGCGCGGCA CCAACTTCCCCTCCGACGGCCCCGTAAT GCAGAAGAAGACCATGGGCTGGGAGGC CTCCTCCGAGCGGATGTACCCCGAGGA CGGCGCCCTGAAGGGCGA GAT CAAGCAGAGGCTGAAGCTGAAGGACGG CGGCCACTACGACGCTGAGGTCAAGAC CACCTACAAGGCCAAGAAGCCCGTGCA GCTGCCCCGGCGCTACAACGTCAACAT CAAGTTGGACATCACCTCCCACAACGAG GACTACACCATCGTGGAACAGTACGAAC GCGCCGAGGGCCGCCACTCCACCGGC GGCATGGACGAGCTGTACAAGGGAAGC GGAGCCACGAACTTCTCTCTGTTAAAGC AAGCAGGAGATGTTGAAGAAAACCCCG GGCCTATGTACAGGATGCAACTCCTGTC TTGCATTGCACTAAGTCTTGCACTTGTC CGAATTCGGAGGAGGAGCTGCAGATCA TCCAGCCAGAGAAGCTGCTGCTGGTGA CCGTGGGCAAGACGGCCACTCTTCATT GTACCATCACCTCTTTGTTCCCCGTGGG TCCCATCCAGTGGTTCCGCGGGGTCGG ACCCGGGCGGGTGCTCATCTACAACCA GAAGGACGGCCACTTTC CTA GGGTCACCACAGTAAGCGACGGCACCA AGCGCAACAATATGGATTTGAGCATTCG CATCTCTCCATTACTCCGGCGGACGTG
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				GGCACCTATTACTGCGTTAAATTCGTAA GGGATCCCCGGAAGACGTGGAGTTCAA ATCCGGCCCTGGTACGGAGATGGCTCT GGGCGCCAAGCCCTCGTAA
aEGFRvIII CAR	3C10.B Bz	2247	21	ATGGCCTTACCA GTGACCGCCTTGCTCC TGCCGCTGGCCTTGCTGCTCCACGCCG CCAGGCCGGGATCCGAGATTCAGCTGC AGCAATCTGGGGCAGA ACTTGTAAGC CAGGGGCCTCAGTCAAGCTGTCCTGCA CAGGTTCTGGCTTCAACATTGAAGACTA CTATATTCAGTGGGTGAAGCAGAGGACT GAACAGGGCCTGGAATGGATTGGAAGG ATTGATCCTGAGAATGATGAACTAAATA TGGCCCAATATTCCAGGGCAGGGCCAC TATAACAGCAGACACATCCTCCAACACA GTCTACCTGCAACTCAGCAGCCTGACAT CTGAGGACACTGCCGTCTATTACTGTGC CTTTCGCGGTGGAGTCTACTGGGGGCC AGGAACCACTCTCACAGTCTCCTCAGGA GGTGGTGGTTCCGGTGGTGGTGGTTCC GGAGGTGGTGGTTCACATATGGATGTTG TGATGACCCAGTCTCCACTCACTCTATC GGTTGCCATTGGACAATCAGCCTCCATC TCTTGCAAGTCAAGTCAGAGCCTCTTAG ATAGTGATGGAAGACATATTTGAATTG GTTGTTACAGAGGCCAGGCCAGTCTCCA AAGCGCCTAATCTCTCTGGTGTCTAAAC TGGACTCTGGAGTCCCTGACAGGTTCAC TGGCAGTGGATCAGGGACAGATTTACAC CTGAGAATCAGCAGAGTGGAGGCTGAG GATTTGGGAATTTATTATTGCTGGCAAG GTACACATTTTCCTGGGACGTTGCGGTGG AGGGACCAAGCTGGAGATAAAAGCTAG CACCACGACGCCAGCGCCGCGACCACC AACACCGGCGCCCACCATCGCGTCGCA GCCCCTGTCCCTGCGCCCAGAGGCGTG CCGGCCAGCGGCGGGGGGCGCAGTGC ACACGAGGGGGCTGGACTTCGCCTGTG ATATCTACATCTGGGCGCCCTTGGCCGG GACTTGTGGGGTCCTTCTCCTGTCACTG GTTATCACCCTTTACTGCAAACGGGGCA GAA AGAAACTCCTGTATATATTCAAACAACCA TTTATGAGACCAAGTACAACTACTCAAG AGGAAGATGGCTGTAGCTGCCGATTTC AGAAGAAGAAGAAGGAGGATGTGA GAGAGTGAAGTTCAGCAGGAGCGCAGA CGCCCCCGCGTACAAGCAGGGCCAGAA CCAGCTCTATAACGAGCTCAATCTAGGA CGAAGAGAGGAGTACGATGTTTTGGACA AGAGACGTGGCCGGGACCCTGAGATGG GGGGAAGCCGAGAAGGAAGAACCCTC AGGAAGGCCTGTACAATGAACTGCAGAA AGATAAGATGGCGGAGGCCTACAGTGA GATTGGGATGAAAGGCGAGCGCCGGAG GGGCAAGGGGCACGATGGCCTTTACCA GGGTCTCAGTACAGCCACCAAGGACAC CTACGACGCCCTTCACATGCAGGCCCT

				GCCCCCTCGCGGAAGCGGAGAGGGCA GGGGAAGTCTTCTAACATGCGGGGACG TGGAGGAAAATCCCCGC CCC ATGGTGAGCAAGGGCGAGGAGGATAAC ATGGCCATCATCAAGGAGTTCATGCGCT TCAAGGTGCACATGGAGGGCTCCGTGA ACGGCCACGAGTTCGAGATCGAGGGCG AGGGCGAGGGCCGCCCTACGAGGGC ACCCAGACCGCCAAGCTGAAGGTGACC AAGGGTGGCCCCCTGCCCTTCGCCTGG GACATCCTGTCCCCTCAGTTCATGTACG GCTCCAAGGCCTACGTGAAGCACCCCG CCGACATCCCCGACTACTTGAAGCTGTC CTTCCCCGAGGGCTTCAAGTGGGAGCG CGTGATGAACTTCGAGGACGGCGGCGT GGTGACCGTGACCCAGGACTCCTCCCT GCAGGACGGCGAGTTCATCTACAAGGT GAAGCTGCGCGGCACCAACTTCCCCTC CGACGGCCCCGTAATGCAGAAGAAGAC CATGGGCTGGGAGGCCTCCTCCGAGCG GATGTACCCCGAGGACGGCGCCCTGAA GGGCGAGATCAAGCAGAGGCT GAA GCTGAAGGACGGCGGCCACTACGACGC TGAGGTCAAGACCACCTACAAGGCCAA GAAGCCCGTGACAGTGCCCGGCGCCTA CAACGTCAACATCAAGTTGGACATCACC TCCCACAACGAGGACTACACCATCGTGG AACAGTACGAACGCGCCGAGGGCCGCC ACTCCACCGCGGCATGGACGAGCTGT ACAAGTAA
aEGFRvIII- SGRP CAR	3C10.B Bz	2730	22	ATGGCCTTACCAAGTACCGCCTTGCTCC TGCCGCTGGCCTTGCTGCTCCACGCCG CCAGGCCGGGATCCGAGATTGAGCTGC AGCAATCTGGGGCAGAACTTGTGAAGC CAGGGGCCCTCAGTCAAGCTGTCCTGCA CAGGTTCTGGCTTCAACATTGAAGACTA CTATATTTCACTGGGTGAAGCAGAGGACT GAACAGGGCCTGGAATGGATTGGAAGG ATTGATCCTGAGAATGATGAACTAAATA TGGCCCAATATTCCAGGGCAGGGCCAC TATAACAGCAGACACATCCTCCAACACA GTCTACCTGCAACTCAGCAGCCTGACAT CTGAGGACACTGCCGTCTATTACTGTGC CTTTCGCGGTGGAGTCTACTGGGGGCC AGGAACCACTCTCACAGTCTCCTCAGGA GGTGGTGGTTCCGGTGGTGGTGGTTCC GGAGGTGGTGGTTCACATATGGATGTTG TGATGACCCAGTCTCCACTCACTCTATC GGTTGCCATTGGACAATCAGCCTCCATC TCTTGCAAGTCAAGTCAGAGCCTCTTAG ATAGTGATGGAAAGACATATTTGAATTG GTTGTTACAGAGGCCAGGCCAGTCTCCA AAGCGCCTAATCTCTCTGGTGTCTAAAC TGGACTCTGGAGTCCCTGACAGGTTTCA TGGCAGTGGATCAGGGACAGATTTTACA CTGAGAATCAGCAGAGTGGAGGCTGAG GATTTGGGAATTTATTATTGCTGGCAAG

				GTACACATTTTCCTGGGACGTTCCGGTGG AGGGACCAAGCTGGAGATAAAAGCTAG CACCACGACGCCAGCGCCGCGACCAACC AACACCGGGCGCCCAACATCGCGTCGCA GCCCCTGTCCCTGCGCCCAAGAGGCGTG CCGGCCAGCGGCGGGGGGCGCAGTGC ACACGAGGGGGCTGGACTTCGCCTGTG ATATCTACATCTGGGCGCCCTTGGCCGG GACTTGTGGGGTCCTTCTCCTGTCACTG GTTATCACCCCTTTACTGCAAACGGGGCA GAAAGAACTCCTGTATATATTCAAACAA CCATTTATGAGACCAAGTACAACTACTC AAGAGGAAGATGGCTGTAGCTGCCGATT TCCAGAAGAAGAAGAAGGAGGATGTGA ACTGAGAGTGAAGTTTACGACAGGAGCGC AGACGCCCCCGCGTACAAGCAGGGCCA GAACCAGCTCTATAACGAGCTCAATCTA GGACGAAGAGAGGAGTACGATGTTTTG GACAAGAGACGTGGCCGGGACCTGAG ATGGGGGGAAAGCCGAGAAGGAAGAAC CCTCAGGAAGGCCTGTACAATGAACTGC AGAAAGATAAGATGGCGGAGGCCTACA GTGAGATTGGGATGAAAGGCGAGCGCC GGAGGGGCAAGGGGCGACGATGGCCTTT ACCAGGGTCTCAGTACAGCCACCAAGG ACACCTACGACGCCCTTCACATGCAGGC CCTGCCCCCTCGCGGAAGCGGAGAGGG CAGGGGAAGTCTTCTAACATGCGGGGA CGTGGAGGAAAATCCCGGCCCC ATGGTGAGCAAGGGCGAGGAGGATAAC ATGGCCATCATCAAGGAGTTCATGCGCT TCAAGGTGCACATGGAGGGCTCCGTGA ACGGCCACGAGTTCGAGATCGAGGGCG AGGGCGAGGGCCGCCCTACGAGGGC ACCCAGACCGCCAAGCTGAAGGTGACC AAGGGTGGCCCCCTGCCCTTCGCCTGG GACATCCTGTCCCCTCAGTTCATGTACG GCTCCAAGGCCCTACGTGAAGCACCCCG CCGACATCCCCGACTACTTGAAGCTGTC CTTCCCCGAGGGCTTCAAGTGGGAGCG CGTGATGAACTTCGAGGACGGCGGCGT GGTGACCGTGACCCAGGACTCCTCCCT GCAGGACGGCGAGTTCATCTACAAGGT GAAGCTGCGCGGCACCAACTTCCCCTC CGACGGCCCCGTAATGCAGAAGAAGAC CATGGGCTGGGAGGCCTCCTCCGAGCG GATGTACCCCGAGGACGGCGCCCTGAA GGGCGAGATCAAGCAGAGGCT GAA GCTGAAGGACGGCGGCCACTACGACGC TGAGGTCAAGACCACCTACAAGGCCAA GAAGCCCGTGACGCTGCCCGGCGCCTA CAACGTCAACATCAAGTTGGACATCACC TCCCACAACGAGGACTACACCATCGTGG AACAGTACGAACGCGCCGAGGGCCGCC ACTCCACCGGCGGCATGGACGAGCTGT ACAAGGGAAGCGGAGCCACGAAGTTCT CTCTGTTAAAGCAAGCAGGAGATGTTGA AGAAAACCCCGGCCTATGTACAGGAT
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				GCAACTCCTGTCTTGCATTGCACTAAGT CTTGCACTTGTACGAATTCGGAGGAGG AGCTGCAGATCATCCAGCCAGAGAAGCT GCTGCTGGTGACCGTGGGCAAGACGGC CACTCTTCATTGTACCATCACCTCTTTGT TCCCCGTGGGTCCCATCCAGTGGTTCC GCGGGGTCCGACCCGGGCGGGTGCTC ATCTACAACCAGAAGGACGGCCACTTTC CTAGGGTCACCACAG TAA GCGACGGCACCAAGCGCAACAATATGG ATTTCAGCATTTCGCATCTCTTCCATTACT CCGGCGGACGTGGGCACCTATTACTGC GTAAATTTCTGTAAGGGATCCCCGGAAG ACGTGGAGTTCAAATCCGGCCCTGGTAC GGAGATGGCTCTGGGCGCCAAGCCCTC GTAA
aEGFRvIII- SGRP-His CAR	3C10.B Bz	2748	23	ATGGCCTTACCAGTGACCGCCTTGCTCC TGCCGCTGGCCTTGCTGCTCCACGCCG CCAGGCCGGGATCCGAGATTCACTGC AGCAATCTGGGGCAGAACTTGTGAAGC CAGGGGCCCTCAGTCAAGCTGTCCTGCA CAGGTTCTGGCTTCAACATTGAAGACTA CTATATTCAGTGGGTGAAGCAGAGGACT GAACAGGGCCTGGAATGGATTGGAAGG ATTGATCCTGAGAATGATGAACTAAATA TGGCCCAATATTCCAGGGCAGGGCCAC TATAACAGCAGACACATCCTCCAACACA GTCTACCTGCAACTCAGCAGCCTGACAT CTGAGGACACTGCCGTCTATTACTGTGC CTTTCGCGGTGGAGTCTACTGGGGGCC AGGAACCACTCTCACAGTCTCCTCAGGA GGTGGTGGTTCGGTGGTGGTGGTTC GGAGGTGGTGGTTCACATATGGATGTTG TGATGACCCAGTCTCCACTCACTCTATC GGTTGCCATTGGACAATCAGCCTCCATC TCTTGCAAGTCAAGTCAGAGCCTCTTAG ATAGTGATGGAAAGACATATTTGAATTG GTTGTTACAGAGGCCAGGCCAGTCTCCA AAGCGCCTAATCTCTCTGGTGTCTAAAC TGGACTCTGGAGTCCCTGACAGGTTTCA TGGCAGTGGATCAGGGACAGATTTTACA CTGAGAATCAGCAGAGTGGAGGCTGAG GATTTGGGAATTTATTATTGCTGGCAAG GTACACATTTTCTGGGACGTTTCGGTGG AGGGACCAAGCTGGAGATAAAAGCTAG CACCACGACGCCAGCGCCGCGACCACC AACACCGGCGCCACCATCGCGTCGCA GCCCCTGTCCCTGCGCCAGAGGCGTG CCGGCCAGCGGCGGGGGGCGCAGTGC ACACGAGGGGGCTGGACTTCGCCTGTG ATATCTACATCTGGGCGCCCTTGGCCGG GACTTGTGGGGTCCTTCTCCTGTCACTG GTTATCACCCCTTTACTGCAAACGGGGCA GAAAGAACTCCTGTATATATTCAAACAA CCATTTATGAGACCAGTACAACTACTC AAGAGGAAGATGGCTGTAGCTGCCGATT TCCAGAAGAAGAAGAAGGAGGATGTGA ACTGAGAGTGAAGTTCAGCAGGAGCGC

				AGACGCCCCCGCGTACAAGCAGGGCCA GAACCAGCTCTATAACGAGCTCAATCTA GGACGAAGAGAGGAGTACGATGTTTTG GACAAGAGACGTGGCCGGGACCCTGAG ATGGGGGGAAAGCCGAGAAGGAAGAAC CCTCAGGAAGGCCTGTACAATGAACTGC AGAAAGATAAGATGGCGGAGGCCTACA GTGAGATTGGGATGAAAGGCGAGCGCC GGAGGGGCAAGGGGCACGATGGCCTTT ACCAGGGTCTCAGTACAGCCACCAAGG ACACCTACGACGCCCTTCACATGCAGGC CCTGCCCCCTCGCGGAAGCGGAGAGGG CAGGGGAAGTCTTCTAACATGCGGGGA CGTGGAGGAAAATCCCGGCCCC ATGGTGAGCAAGGGCGAGGAGGATAAC ATGGCCATCATCAAGGAGTTCATGCGCT TCAAGGTGCACATGGAGGGCTCCGTGA ACGGCCACGAGTTCGAGATCGAGGGCG AGGGCGAGGGCCGCCCTACGAGGGC ACCCAGACCGCCAAGCTGAAGGTGACC AAGGGTGGCCCCCTGCCCTTCGCCTGG GACATCCTGTCCCCTCAGTTCATGTACG GCTCCAAGGCCTACGTGAAGCACCCCG CCGACATCCCCGACTACTTGAAGCTGTC CTTCCCCGAGGGCTTCAAGTGGGAGCG CGTGATGAACTTCGAGGACGGCGGCGT GGTGACCGTGACCCAGGACTCCTCCCT GCAGGACGGCGAGTTCATCTACAAGGT GAAGCTGCGCGGCACCAACTTCCCCTC CGACGGCCCCGTAATGCAGAAGAAGAC CATGGGCTGGGAGGCCTCCTCCGAGCG GATGTACCCCGAGGACGGCGCCCTGAA GGGCGAGATCAAGCAGAGGCT GAA GCTGAAGGACGGCGGCCACTACGACGC TGAGGTCAAGACCACCTACAAGGCCAA GAAGCCCGTGACGCTGCCCGGCGCCTA CAACGTCAACATCAAGTTGGACATCACC TCCCACAACGAGGACTACACCATCGTGG AACAGTACGAACGCGCCGAGGGCCGCC ACTCCACCGGCGGCATGGACGAGCTGT ACAAGGGAAGCGGAGCCACGAATTCT CTCTGTTAAAGCAAGCAGGAGATGTTGA AGAAAACCCCGGGCCTATGTACAGGAT GCAACTCCTGTCTTGCAATTGCACTAAGT CTTGCACTTGTCACGAATTCGCATCACC ATCACCATCACGAGGAGGAGCTGCAGA TCATCCAGCCAGAGAAGCTGCTGCTGGT GACCGTGGGCAAGACGGCCACTCTTCA TTGTACCATCACCTCTTTGTTCCCGTG GGTCCCATCCAGTGTTCCGCGGGGTC GGACCCGGGCGGGTGCTCATCTACAAC CAGAAGGACGGCCACT TTC CTAGGGTCACCACAGTAAGCGACGGCA CCAAGCGCAACAATATGGATTTAGCAT TCGCATCTCTTCCATTACTCCGGCGGAC GTGGGCACCTATTACTGCGTTAAATTC GTAAGGGATCCCCGGAAGACGTGGAGT
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				TCAAATCCGGCCCTGGTACGGAGATGG CTCTGGGCGCCAAGCCCTCGTAA
EF1A-driven untagged aEGFRvIII CAR	3C10.B Bz	3162	1	GGCTCCGGTGCCCGTCAGTGGGCAGAG CGCACATCGCCACAGTCCCCGAGAAG TTGGGGGGAGGGGTGGCAATTGAACC GGTGCTAGAGAAGGTGGCGCGGGGTA AACTGGGAAAGTGATGTCGTGTACTGGC TCCGCCTTTTTCCCGAGGGTGGGGGAG AACCGTATATAAGTGCAGTAGTCGCCGT GAACGTTCTTTTTCGCAACGGGTTTGCC GCCAGAACACAGGTAAGTGCCGTGTGT GGTTCCCGCGGGCCTGGCCTCTTTACG GGTTATGGCCCTTGCGTGCCTTGAATTA CTTCCACCTGGCTGCAGTACGTGATTCT TGATCCCGAGCTTCGGGTTGGAAGTGG GTGGGAGAGTTCGAGGCCTTGCGCTTA AGGAGCCCCCTTCGCCTCGTGCTTGAGTT GAGGCCTGGCCTGGGCGCTGGGGCCG CCGCGTGCGAATCTGGTGGCACCTTCG CGCCTGTCTCGCTGCTTTGATAAGTCT CTAGCCATTTAAAATTTTTGATGACCTGC TGCGACGCTTTTTTTCTGGCAAGATAGT CTTGTAATGCGGGCCAAGATCTGCACA CTGGTATTTTCGGTTTTTGGGGCCGCGG GCGGCGACGGGGCCCGTGCGTCCCAG CGCACATGTTTCGGCGAGGCGGGGCCTG CGAGCGCGGCCACCGAGAATCGGACGG GGGTAGTCTCAAGCTGGCCGGCCTGCT CTGGTGCTGGTCTCGCGCCGCCGTGT ATCGCCCCGCCCTGGGCGGCAAGGCTG GCCCCGTGCGCACCAAGTTGCGTGAGCG GAAAGATGGCCGCTTCCCGGCCCTGCT GCAGGGAGCTCAAAATGGAGGACGCGG CGCTCGGGAGAGCGGGCGGGTGAGTC ACCCACACAAAGGAAAAGGGCCTTCCG TCCTCAGCCGTCGCTTCATGTGACTCCA CGGAGTACCGGGCGCCGTCCAGGCACC TCGATTAGTTCTCGAGCTTTTGAGTAC GTCGTCTTTAGGTTGGGGGAGGGGTT TTATGCGATGGAGTTTCCCCACACTGAG TGGGTGGAGACTGAAGTTAGGCCAGCT TGGCACTTGATGTAATTCTCCTTGGAATT TGCCCTTTTTGAGTTTGATCTTGGTTCA TTCTCAAGCCTCAGACAGTGGTTCAAAG TTTTTTTCTTCCATTTAGGTGTCGTGAC AAGTTTGTACAAAAAGCAGGCTGCCAC CATGGCCTTACCAAGTGACCGCCTTGCTC CTGCCGCTGGCCTTGCTGCTCCACGCC GCCAGGCCGGGATCCGAGATTGAGCTG CAGCAATCTGGGGCAGAACTTGTGAAG CCAGGGGCCTCAGTCAAGCTGTCCTGC ACAGGTTCTGGCTTCAACATTGAAGACT ACTATATTCACTGGGTGAAGCAGAGGAC TGAACAGGGCCTGGAATGGATTGGAAG

				GATTGATCCTGAGAATGATGAAACTAAA TATGGCCCAATATTCCAGGGCAGGGCC ACTATAACAGCAGACACATCCTCCAACA CAGTCTACCTGCAACTCAGCAGCCTGAC ATCTGAGGACACTGCCGTCTATTACTGT GCCTTTCGCGGTGGAGTCTACTGGGGG CCAGGAACCACTCTCACAGTCTCCTCAG GAGGTGGTGGTTCCGGTGGTGGTGGTT CCGGAGGTGGTGGTTCACATATGGATGT TGTGATGACCCAGTCTCCACTCACTCTA TCGGTTGCCATTGGACAATCAGCCTCCA TCTCTTGCAAGTCAAGTCAGAGCCTCTT AGATAGTGATGGAAAGACATATTTGAATT GGTTGTTACAGAGGCCAGGCCAGTCTC CAAAGCGCCTAATCTCTCTGGTGTCTAA ACTGGACTCTGGAGTCCCTGACAGGTTCT ACTGGCAGTGGATCAGGGACAGATTTCA CACTGAGAATCAGCAGAGTGGAGGCTG AGGATTTGGGAATTTATTATTGCTGGCA AGGTACACATTTTCCTGGGACGTTCCGT GGAGGGACCAAGCTGGAGATAAAAGCT AGCACACGACGCCAGCGCCGCGACCA CCAACACCGGCGCCACCATCGCGTCG CAGCCCCTGTCCCTGCGCCAGAGGCG TGCCGGCCAGCGGCGGGGGGCGCAGT GCACACGAGGGGGCTGGACTTCGCCTG TGATATCTACATCTGGGCGCCCTTGGCC GGGACTTGTGGGGTCCCTTCTCCTGTCAC TGTTATCACCCCTTACTGCAAACGGGG CAGAAAGAACTCCTGTATATATTTCAAAC AACCATTTATGAGACCAGTACAACTACT CAAGAGGAAGATGGCTGTAGCTGCCGA TTTCCAGAAGAAGAAGAAGGAGGATGTG AACTGAGAGTGAAGTTCAGCAGGAGCG CAGACGCCCCCGCGTACAAGCAGGGCC AGAACCAGCTCTATAACGAGCTCAATCT AGGACGAAGAGAGGAGTACGATGTTTTG GACAAGAGACGTGGCCGGGACCCTGAG ATGGGGGGAAAGCCGAGAAGGAAGAAC CCTCAGGAAGGCCTGTACAATGAACTGC AGAAAGATAAGATGGCGGAGGCCTA CA GTGAGATTGGGATGAAAGGCGAGCGCC GGAGGGGCAAGGGGCACGATGGCCTTT ACCAGGGTCTCAGTACAGCCACCAAGG ACACCTACGACGCCCTTACATGCAGGC CCTGCCCCCTCGCGGAAGCGGAGAGGG CAGGGGAAGTCTTCTAACATGCGGGGA CGTGGAGGAAAATCCCGGCCCATGTA CAGGATGCAACTCCTGTCTTGCAATTGCA CTAAGTCTTGCACTTGTCACGAATTCGG AGGAGGAGCTGCAGATCATCCAGCCAG AGAAGCTGCTGCTGGTGACCGTGGGCA AGACGGCCACTCTTCATTGTACCATCAC CTCTTTGTTCCCGTGGGTCCCATCCAG TGTTCCGCGGGGTCCGACCCGGGCG GGTGCTCATCTACAACCAGAAGGACGG CCTTTTCTAGGGTCACCACAGTAAGC GACGGCACCAAGCGCAACAATATGGATT
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				TCAGCATTGCGATCTCTTCCATTACTCC GGCGGACGTGGGACCT AT TACTGCGTTAAATTTCTGAAGGGATCCC CGGAAGACGTGGAGTTCAAATCCGGCC CTGGTACGGAGATGGCTCTGGGCGCCA AGCCCTCG
EF1A-driven untagged aCD19 CAR	FMC63. BBz	3177	2	GGCTCCGGTGCCCGTCAGTGGGCAGAG CGCACATCGCCACAGTCCCCGAGAAG TTGGGGGAGGGGTGCGCAATTGAACC GGTGCTAGAGAAGGTGGCGCGGGGTA AACTGGGAAAGTGATGTCGTGTAAGG TCCGCCTTTTTCCCGAGGGTGGGGGAG AACCGTATATAAGTGCAGTAGTCGCCGT GAACGTTCTTTTTCGCAACGGGTTTGCC GCCAGAACACAGGTAAGTGCCGTGTGT GGTTCCCGCGGGCCTGGCCTCTTTACG GGTTATGGCCCTTGCGTGCCTTGAATTA CTTCCACCTGGCTGCAGTACGTGATTCT TGATCCCGAGCTTCGGGTTGGAAGTGG GTGGGAGAGTTCGAGGCCTTGCGCTTA AGGAGCCCCCTTCGCCTCGTGCTTGAGTT GAGGCCTGGCCTGGGCGCTGGGGCCG CCGCGTGCGAATCTGGTGGCACCTTCG CGCCTGTCTCGCTGCTTTGATAAGTCT CTAGCCATTTAAAATTTTGATGACCTGC TGCGACGCTTTTTTTCTGGCAAGATAGT CTTGTAATGCGGGCCAAGATCTGCACA CTGGTATTTTCGGTTTTTGGGGCCGCGG GCGGCGACGGGGCCCCGTGCGTCCCAG CGCACATGTTTCGGCGAGGCGGGGCTG CGAGCGCGGCCACCGAGAATCGGACGG GGGTAGTCTCAAGCTGGCCGGCCTGCT CTGGTGCTGGTCTCGCGCCGCCGTGT ATCGCCCCGCCCTGGGCGGCAAGGCTG GCCCCGTGCGCACCAAGTTGCGTGAGCG GAAAGATGGCCGCTTCCCGGCCCTGCT GCAGGGAGCTCAAAATGGAGGACGCGG CGCTCGGGAGAGCGGGCGGGTGAGTC ACCCACACAAAGGAAAAGGGCCTTTCCG TCCTCAGCCGTGCTTCATGTGACTCCA CGGAGTACCGGGCGCCGTCCAGGCACC TCGATTAGTTCTCGAGCTTTTGAGTAC GTCGTCTTTAGGTTGGGGGAGGGGTT TTATGCGATGGAGTTTCCCACTGAG TGGGTGGAGACTGAAGTTAGGCCAGCT TGGCACTTGATGTAATTCTCCTTGGAATT TGCCCTTTTTGAGTTTGGATCTTGTTCA TTCTCAAGCCTCAGACAGTGGTTCAAAG TTTTTTCTTCCATTTAGGTGTCGTGAC AAGTTTGTACAAAAAGCAGGCTGCCAC CATGGCCTTACCAGTGACCGCCTTGCTC CTGCCGCTGGCCTTGCTGCTCCACGCC GCCAGGCCGGGATCCGACATCCAGATG ACACAGACTACATCCTCCCTGTCTGCCT CTCTGGGAGACAGAGTCACCATCAGTTG CAGGGCAAGTCAGGACATTAGTAAATAT TTAAATTGGTATCAGCAGAAACCAGATG GAACTGTAAACTCCTGATCTACCATACA

				TCAAGATTACACTCAGGAGTCCCATCAA GGTTCAGTGGCAGTGGGTCTGGAACAG ATTATTCTCTCACCATTAGCAACCTGGA GCAAGAAGATATTGCCACTTACTTTTGC CAACAGGGTAATACGCTTCCGTACACGT TCGGAGGGGGGACTAAGTTGGAATAA CAGGCTCCACCTCTGGATCCGGCAAGC CCGGATCTGGCGAGGGATCCACCAAGG GCGAGGTGAAACTGCAGGAGTCAGGAC CTGGCCTGGTGGCGCCCTCACAGAGCC TGTCCGTACATGCACTGTCTCAGGGGT CTCATTACCCGACTATGGTGTAAAGCTGG ATTCGCCAGCCTCCACGAAAGGGTCTG GAGTGGCTGGGAGTAATATGGGGTAGT GAAACCACATACTATAATTCAGCTCTCAA ATCCAGACTGACCATCATCAAGGACAAC TCCAAGAGCCAAGTTTTCTTAAAAATGAA CAGTCTGCAAACCTGATGACACAGCCATT TACTACTGTGCCAAACATTATTACTACGG TGGTAGCTATGCTATGGACTACTGGGGT CAAGGAACCTCAGTCACCGTCTCCTCAG CGGCCGCGAGCTAGCACCACGACGCCAG CGCCGCGACCACCAACACCGGCGCCCA CCATCGCGTGCAGCCCCTGTCCCTGC GCCCAGAGGCGTGCCGGCCAGCGGCG GGGGGCGCAGTGCACACGAGGGGGCT GGACTTCGCCTGTGATATCTACATCTGG GCGCCCTTGCCCGGACTTGTGGGGTC CTTCTCCTGTCACTGGTTATCACCCTTTA CTGCAAACGGGGCAGAAAGAACTCCT GTATATATTCAAACAACCATTATGAGAC CAGTACAACTACTCAAGAGGAAGATGG CTGTAGCTGCCGATTTCCAGAAGAAGAA GAAGGAGGATGTGAACTGAGAGTGAAG TTCAGCAGGAGCGCAGACGCCCCCGCG TACAAGCAGGGCCAGAACCAGCTCTATA ACGAGCTCAATCTAGGACGAAGAGAGG AGTACGATGTTTTGGACAAGAGACGTGG CCGGGACCCTGAGATGGGGGAAAGCC GAGAAGGAAGAACCCTCAGGAAGGCCT GTACAATGAACTGCAGAAAGATAA GA TGGCGGAGGCCTACAGTGAGATTGGGA TGAAAGGCGAGCGCCGGAGGGGCAAG GGGCACGATGGCCTTTACCAGGGTCTC AGTACAGCCACCAAGGACACCTACGAC GCCCTTCACATGCAGGCCCTGCCCCCT CGCGGAAGCGGAGAGGGCAGGGGAAG TCTTCTAACATGCGGGGACGTGGAGGA AAATCCCGGCCCCATGTACAGGATGCAA CTCCTGTCTTGCAATTGCACTAAGTCTTG CACTTGTCACGAATTCGGAGGAGGAGCT GCAGATCATCCAGCCAGAGAAGCTGCT GCTGGTGACCGTGGGCAAGACGGCCAC TCTTCATTGTACCATCACCTCTTTGTTCC CCGTGGGTCCCATCCAGTGGTTCCGCG GGGTCGGACCCGGGCGGGTGCTCATCT ACAACCAGAAGGACGGCCACTTTCCTAG GGTCACCACAGTAAGCGACGGCACCAA
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				GCGCAACAATATGGATTTTCAGCATTCGC ATCTCTTCCATTACTCCGG CG GACGTGGGCACCTATTACTGCGTTAAAT TTCGTAAGGGATCCCCGGAAGACGTGG AGTTCAAATCCGGCCCTGGTACGGAGAT GGCTCTGGGCGCCAAGCCCTCG
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Table 2 - continued

3C10.BBz CAR construct domain	bp	SEQ ID NO:	nt sequence
3C10.BBz	1473	24	ATGGCCTTACCAGTGACCGCCTTGCTCCTGCCGCT GGCCTTGCTGCTCCACGCCGCCAGGCCGGGATCC GAGATTCAGCTGCAGCAATCTGGGGCAGAATTGT GAAGCCAGGGGCCTCAGTCAAGCTGTCCTGCACA GGTTCTGGCTTCAACATTGAAGACTACTATATTCAC TGGGTGAAGCAGAGGACTGAACAGGGCCTGGAAT GGATTGGAAGGATTGATCCTGAGAATGATGAACT AAATATGGCCCAATATTCCAGGGCAGGGCCACTAT AACAGCAGACACATCCTCCAACACAGTCTACCTGC AACTCAGCAGCCTGACATCTGAGGACACTGCCGTC TATTACTGTGCCTTTCGCGGTGGAGTCTACTGGGG GCCAGGAACCACTCTCACAGTCTCCTCAGGAGGT GGTGGTTCCGGTGGTGGTGGTTCGGAGGTGGTG GTTACATATGGATGTTGTGATGACCCAGTCTCCA CTCACTCTATCGGTTGCCATTGG ACA ATCAGCCTCCATCTCTTGCAAGTCAAGTCAGAGCC TCTTAGATAGTGATGGAAAGACATATTTGAATTGGT TGTTACAGAGGCCAGGCCAGTCTCCAAAGCGCCTA ATCTCTCTGGTGCTAAACTGGACTCTGGAGTCCC TGACAGGTTCACTGGCAGTGGATCAGGGACAGATT TCACACTGAGAATCAGCAGAGTGGAGGCTGAGGA TTTGGGAATTTATTATTGCTGGCAAGGTACACATTT TCCTGGGACGTTCCGTGGAGGGACCAAGCTGGAG ATAAAAGCTAGCACCACGACGCCAGCGCCGCGAC CACCAACACCGGCGCCCAACATCGCGTCGCAGCC CCTGTCCCTGCGCCCAGAGGCGTGCCGGCCAGCG GCGGGGGGCGCAGTGCACACGAGGGGGCTGGAC TTCGCCTGTGATATCTACATCTGGGCGCCCTTGGC CGGGACTTGTGGGGTCTTCTCCTGTCACTGGTTA TCACCTTTACTGCAAACGGGGCA GAA AGAAACTCCTGTATATATTCAAACAACCATTTATGA GACCAGTACAACTACTCAAGAGGAAGATGGCTGT AGCTGCCGATTTCCAGAAGAAGAAGAAGGAGGAT GTGAACTGAGAGTGAAGTTCAGCAGGAGCGCAGA CGCCCCCGCGTACAAGCAGGGCCAGAACCAGCTC TATAACGAGCTCAATCTAGGACGAAGAGAGGAGTA CGATGTTTTGGACAAGAGACGTGGCCGGGACCCT GAGATGGGGGGAAAGCCGAGAAGGAAGAACCCTC AGGAAGGCCTGTACAATGAACTGCAGAAAAGATAAG ATGGCGGAGGCCTACAGTGAGATTGGGATGAAAG GCGAGCGCCGAGGGGGCAAGGGGCACGATGGCC

			TTTACCAGGGTCTCAGTACAGCCACCAAGGACACC TACGACGCCCTTCACATGCAGGCCCTGCCCCCTC GC
CD8a leader	60	25	GCCTTACCAGTGACCGCCTTGCTCCTGCCGCTGG CCTTGCTGCTCCACGCCGCCAGGCCG
3C10	729	26	GAGATTCACTGCAGCAATCTGGGGCAGAACTTGT GAAGCCAGGGGCCTCAGTCAAGCTGTCCTGCACA GGTTCTGGCTTCAACATTGAAGACTACTATATTCAC TGGGTGAAGCAGAGGACTGAACAGGGCCTGGAAT GGATTGGAAGGATTGATCCTGAGAATGATGAAACT AAATATGGCCCAATATTCCAGGGCAGGGCCACTAT AACAGCAGACACATCCTCCAACACAGTCTACCTGC AACTCAGCAGCCTGACATCTGAGGACACTGCCGTC TATTACTGTGCCTTTCGCGGTGGAGTCTACTGGGG GCCAGGAACCACTCTCACAGTCTCCTCAGGAGGT GGTGGTTCCGGTGGTGGTGGTTCGGGAGGTGGTG GTTACATATGGATGTTGTGATGACCCAGTCTCCA CTCACTCTATCGGTTGCCATTGGACAATCAGCCTC CATCTCTTGCAAGTCAAGTCAGAGCCTCTTAGATA GTGATGGAAAGACATATTTGAA TTG GTTGTTACAGAGGCCAGGCCAGTCTCCAAAGCGC CTAATCTCTCTGGTGTCTAAACTGGACTCTGGAGT CCCTGACAGGTTCACTGGCAGTGGATCAGGGACA GATTTCACTGAGAATCAGCAGAGTGGAGGCTGA GGATTTGGGAATTTATTATTGCTGGCAAGGTACAC ATTTTCCTGGGACGTTCCGTGGAGGGACCAAGCT GGAGATAAAA
CD8a hinge	135	27	ACCACGACGCCAGCGCCGCGACCACCAACACCGG CGCCCACCATCGCGTCGCGAGCCCCTGTCCCTGCG CCCAGAGGCGTGCCGGCCAGCGGCGGGGGGCGC AGTGCACACGAGGGGGCTGGACTTCGCCTGTGAT
CD8a transmembrane	72	28	ATCTACATCTGGGCGCCCTTGCCGGGAGTGTG GGGTCTTCTCCTGTCACTGGTTATCACCTTTACT GC
4-1BB	126	29	AAACGGGGCAGAAAGAACTCCTGTATATATTCAA ACAACCATTTATGAGACCAGTACAACTACTCAAGA GGAAGATGGCTGTAGCTGCCGATTTCCAGAAGAAG AAGAAGGAGGATGTGAACTG
CD3z	336	30	AGAGTGAAGTTCAGCAGGAGCGCAGACGCCCCCG CGTACCAGCAGGGCCAGAACCAGCTCTATAACGA GCTCAATCTAGGACGAAGAGAGGAGTACGATGTTT TGACAAGAGACGTGGCCGGGACCCCTGAGATGGG GGGAAAGCCGAGAAGGAAGAACCCTCAGGAAGGC CTGTACAATGAACTGCAGAAAGATAAGATGGCGGA GGCCTACAGTGAGATTGGGATGAAAGGCGAGCGC CGGAGGGGCAAGGGGACGATGGCCTTTACCAGG GTCTCAGTACAGCCACCAAGGACACCTACGACGC CCTTCACATGCAGGCCCTGCCCCCTCGC
T2A	63	31	GGAAGCGGAGAGGGCAGGGGAAGTCTTCTAACAT CGGGGACGTGGAGGAAAATCCCGGCCCC

mCherry	708	32	ATGGTGAGCAAGGGCGAGGAGGATAACATGGCCA TCATCAAGGAGTTCATGCGCTTCAAGGTGCACATG GAGGGCTCCGTGAACGGCCACGAGTTCGAGATCG AGGGCGAGGGCGAGGGCCGCCCTACGAGGGCA CCCAGACCGCCAAGCTGAAGGTGACCAAGGGTGG CCCCCTGCCCTTCGCCTGGGACATCCTGTCCCCTC AGTTCATGTACGGCTCCAAGGCCTACGTGAAGCAC CCCGCCGACATCCCCGACTACTTGAAGCTGTCTT CCCCGAGGGCTTCAAGTGGGAGCGCGTGATGAAC TTCGAGGACGGCGGCGTGGTGACCGTGACCCAGG ACTCCTCCCTGCAGGACGGCGAGTTCATCTACAAG GTGAAGCTGCGCGGCACCAACTTCCCCTCCGACG GCCCCGTAATGCAGAAGAAGACCATGGGCTGGGA GGCCTCCTCCGAGCGGATGTACCCCGAGGACGGC GCCCTGAAGGGCGAGATCAAGCAGAGGCT GAA GCTGAAGGACGGCGGCCACTACGACGCTGAGGTC AAGACCACCTACAAGGCCAAGAAGCCCGTGACGC TGCCCGGCGCCTACAACGTCAACATCAAGTTGGAC ATCACCTCCCACAACGAGGACTACACCATCGTGGA ACAGTACGAACGCGCCGAGGGCCGCCACTCCACC GGCGGCATGGACGAGCTGTACAAG
P2A	66	33	GGAAGCGGAGCCACGAACTTCTCTGTAAAGCA AGCAGGAGATGTTGAAGAAAACCCCGGGCCT
IL2sig-SGRP	417	34	ATGTACAGGATGCAACTCCTGTCTTGCACTTGCACT AAGTCTTGCACTTGTACGAATTTCGAGGAGGAGC TGCAGATCATCCAGCCAGAGAAGCTGCTGCTGGT GACCGTGGGCAAGACGGCCACTTTCATTGTACCA TCACCTCTTTGTTCCCGTGGGTCCCATCCAGTGG TTCCGCGGGGTGCGACCCGGGCGGGTGCTCATCT ACAACCAGAAGGACGGCCACTTTCCTAGGGTCAAC ACAGTAAGCGACGGCACCAAGCGCAACAATATAG ATTTACGATTTCGCATCTCTTCCATTACTCCGGCG GACGTGGGCACCTATTACTGCGTTAAATTTTCGTAA GGGATCCCCGGAAGACGTGGAGTTCAAATCCGGC CCTGGTACGGAGATGGCTCTGGGCGCCAAGCCCT CG
IL2sig-His-SGRP	435	35	ATGTACAGGATGCAACTCCTGTCTTGCACTTGCACT AAGTCTTGCACTTGTACGAATTTCGCATCACCATCA CCATCACGAGGAGGAGCTGCAGATCATCCAGCCA GAGAAGCTGCTGCTGGTGACCGTGGGCAAGACGG CCACTTTCATTGTACCATCACCTCTTTGTTCCCG TGGGTCCCATCCAGTGGTTCCGCGGGGTGCGACC CGGGCGGGTGCTCATCTACAACCAGAAGGACGGC CACTTTCCTAGGGTCACCACAGTAAGCGACGGCAC CAAGCGCAACAATATGGATTTTCAGCATTTCGCATCT CTTCCATTACTCCGGCGGACGTGGGCACCTATTAC TGCGTTAAATTTTCGTAAAGGGATCCCCGGAAGACGT GGAGTTCAAATCCGGCCCTGGTACGGAGATGGCT CTGGGCGCCAAGCCCTCG
IL2sig	57	36	TACAGGATGCAACTCCTGTCTTGCACTTGCACTAAG TCTTGCACTTGTACGAATTCG
His	18	37	CATCACCATCACCATCAC
SGRP	357	38	GAGGAGGAGCTGCAGATCATCCAGCCAGAGAAGC TGCTGCTGGTGACCGTGGGCAAGACGGCCACTCT TCATTGTACCATCACCTCTTTGTTCCCGTGGGTC CCATCCAGTGGTTCCGCGGGGTGCGACCCGGGCG GGTGCTCATCTACAACCAGAAGGACGGCCACTTTC

			CTAGGGTCACCACAGTAAGCGACGGCACCAAGCG CAACAATATGGATTTCAGCATTCGCATCTCTTCCAT TACTCCGGCGGACGTGGGCACCTATTACTGCGTTA AATTTTCGTAAGGGATCCCCGGAAGACGTGGAGTTC AAATCCGGCCCTGGTACGGAGATGGCTCTGGGCG CCAAGCCCTCG
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Table 2 – continued

FMC63.BBz CAR construct domain	bp	SEQ ID NO:	nt sequence
FMC63.BBz	1488	39	ATGGCCTTACCAGTGACCGCCTTGCTCCTGCCGCT GGCCTTGCTGCTCCACGCCGCCAGGCCGGGATCC GACATCCAGATGACACAGACTACATCCTCCCTGTC TGCCTCTCTGGGAGACAGAGTCACCATCAGTTGCA GGGCAAGTCAGGACATTAGTAAATATTTAAATTGGT ATCAGCAGAAACCAGATGGAAGTGTAAACTCCTG ATCTACCATAACATCAAGATTACACTCAGGAGTCCCA TCAAGGTTTCAGTGGCAGTGGGTCTGGAACAGATTA TTCTCTCACCATTAGCAACCTGGAGCAAGAAGATA TTGCCACTTACTTTTGCCAACAGGGTAATACGCTTC CGTACACGTTCCGAGGGGGGACTAAGTTGGAAT AACAGGCTCCACCTCTGGATCCGGCAAGCCCGGA TCTGGCGAGGGATCCACCAAGGGCGAGGTGAAAC TGCAGGAGTCAGGACCTGGCCTGGTGGCGCCCTC ACAGAGCCTGTCCGTACATG CAC TGTCCTCAGGGGTCTCATTACCCGACTATGGTGTA GCTGGATTCCGACGCTCCACGAAAGGGTCTGGA GTGGCTGGGAGTAATATGGGGTAGTGAAACCACAT ACTATAATTCAGCTCTCAAATCCAGACTGACCATCA TCAAGGACAACCTCCAAGAGCCAAGTTTTCTTAAAA TGAACAGTCTGCAAACCTGATGACACAGCCATTTAC TACTGTGCCAAACATTATTACTACGGTGGTAGCTAT GCTATGGACTACTGGGGTCAAGGAACCTCAGTCAC CGTCTCCTCAGCGGCCGCGAGCTAGCACCACGACG CCAGCGCCGCGACCAACACCGGCGGCCACCA TCGCGTCGCAGCCCCCTGTCCCTGCGCCCAGAGGC GTGCCGGCCAGCGGCGGGGGGCGCAGTGACACAC GAGGGGGCTGGACTTCGCCTGTGATATCTACATCT GGGCGCCCTTGGCCGGGACTTGTGGGGTCCTTCT CCTGTCACTGGTTATCACCCCTT ACT GCAAACGGGGCAGAAAGAACTCCTGTATATATTC AAACAACCATTTATGAGACCAGTACAACTACTCAA GAGGAAGATGGCTGTAGCTGCCGATTTCCAGAAGA AGAAGAAGGAGGATGTGAACTGAGAGTGAAGTTCA GCAGGAGCGCAGACGCCCCCGCGTACAAGCAGG GCCAGAACCAGCTCTATAACGAGCTCAATCTAGGA CGAAGAGAGGAGTACGATGTTTTGGACAAGAGAC GTGGCCGGGACCCTGAGATGGGGGGAAAGCCGA GAAGGAAGAACCCTCAGGAAGGCCTGTACAATGAA CTGCAGAAAGATAAGATGGCGGAGGCCTACAGTG AGATTGGGATGAAAGGCGAGCGCCGGAGGGGCAA

			GGGGCACGATGGCCTTTACCAGGGTCTCAGTACA GCCACCAAGGACACCTACGACGCCCTTCACATGCA GGCCCTGCCCCCTCGC
CD8a leader	60	25	GCCTTACCAGTGACCGCCTTGCTCCTGCCGCTGG CCTTGCTGCTCCACGCCGCCAGGCCG
FMC63	744	40	GACATCCAGATGACACAGACTACATCCTCCTGTC TGCCTCTCTGGGAGACAGAGTCACCATCAGTTGCA GGGCAAGTCAGGACATTAGTAAATATTTAAATTGGT ATCAGCAGAAACCAGATGGAAGTGTAAACTCCTG ATCTACCATACATCAAGATTACACTCAGGAGTCCCA TCAAGGTTCAGTGGCAGTGGGTCTGGAACAGATTA TTCTCTCACCATTAGCAACCTGGAGCAAGAAGATA TTGCCACTTACTTTTGCCAACAGGGTAATACGCTTC CGTACACGTTCCGAGGGGGGACTAAGTTGAAAT AACAGGCTCCACCTCTGGATCCGGCAAGCCCGGA TCTGGCGAGGGATCCACCAAGGGCGAGGTGAAAC TGCAGGAGTCAGGACCTGGCCTGGTGGCGCCCTC ACAGAGCCTGTCCGTACATGCACTGTCTCAGGG GTCTCATTACCCGACTATGGTGTAAAGCTGGATTCC CCAGCCTCCACGAAAGGGTCT GGA GTGGCTGGGAGTAATATGGGGTAGTGAAACCACAT ACTATAATTCAGCTCTCAAATCCAGACTGACCATCA TCAAGGACAACTCCAAGAGCCAAGTTTTCTTAAAAA TGAACAGTCTGCAAACTGATGACACAGCCATTTAC TACTGTGCCAAACATTATTACTACGGTGGTAGCTAT GCTATGGACTACTGGGGTCAAGGAACCTCAGTCAC CGTCTCCTCAGCGGCCGCA
CD8a hinge	135	27	ACCACGACGCCAGCGCCGCGACCACCAACACCGG CGCCCACCATCGCGTCGCAGCCCCTGTCCCTGCG CCCAGAGGCGTGCCGGCCAGCGGCGGGGGGCGC AGTGCACACGAGGGGGCTGGACTTCGCCTGTGAT
CD8a transmembrane	72	28	ATCTACATCTGGGCGCCCTTGCCGGGACTTGTG GGGTCTTCTCCTGTCACTGGTTATCACCTTTACT GC
4-1BB	126	29	AAACGGGGCAGAAAGAACTCCTGTATATATTCAA ACAACCATTTATGAGACCAGTACAACTACTCAAGA GGAAGATGGCTGTAGCTGCCGATTTCCAGAAAGA AAGAAGGAGGATGTGAACTG
CD3z	336	30	AGAGTGAAGTTCAGCAGGAGCGCAGACGCCCCCG CGTACCAGCAGGGCCAGAACCAGCTCTATAACGA GCTCAATCTAGGACGAAGAGAGGAGTACGATGTTT TGGACAAGAGACGTGGCCGGGACCCTGAGATGGG GGGAAAGCCGAGAAGGAAGAACCCTCAGGAAGGC CTGTACAATGAACTGCAGAAAGATAAGATGGCGGA GGCCTACAGTGAGATTGGGATGAAAGGCGAGCGC CGGAGGGGCAAGGGGACGATGGCCTTTACCAGG GTCTCAGTACAGCCACCAAGGACACCTACGACGC CCTTCACATGCAGGCCCTGCCCCCTCGC
T2A	63	31	GGAAGCGGAGAGGGCAGGGGAAGTCTTCTAACAT GCGGGGACGTGGAGGAAAATCCCGGCCCC
mCherry	708	32	ATGGTGAGCAAGGGCGAGGAGGATAACATGGCCA TCATCAAGGAGTTCATGCGCTTCAAGGTGCACATG GAGGGCTCCGTGAACGGCCACGAGTTCGAGATCG AGGGCGAGGGCGAGGGCCGCCCTACGAGGGCA CCCAGACCGCCAAGCTGAAGGTGACCAAGGGTGG

			CCCCCTGCCCTTCGCCTGGGACATCCTGTCCCCTC AGTTCATGTACGGCTCCAAGGCCTACGTGAAGCAC CCCGCCGACATCCCCGACTACTTGAAGCTGTCCTT CCCCGAGGGCTTCAAGTGGGAGCGCGTGATGAAC TTCGAGGACGGCGGCGTGGTGACCGTGACCCAGG ACTCCTCCCTGCAGGACGGCGAGTTCATCTACAAG GTGAAGCTGCGCGGCACCAACTTCCCCTCCGACG GCCCCGTAATGCAGAAGAAGACCATGGGCTGGGA GGCCTCCTCCGAGCGGATGTACCCCGAGGACGGC GCCCTGAAGGGCGAGATCAAGCAGAGGCTGA A GCTGAAGGACGGCGGCCACTACGACGCTGAGGTC AAGACCACCTACAAGGCCAAGAAGCCCGTGCAGC TGCCCGGCGCCTACAACGTCAACATCAAGTTGGAC ATCACCTCCCACAACGAGGACTACACCATCGTGGA ACAGTACGAACGCGCCGAGGGCCGCCACTCCACC GGCGGCATGGACGAGCTGTACAAG
P2A	66	33	GGAAGCGGAGCCACGAACTTCTCTGTAAAGCA AGCAGGAGATGTTGAAGAAAACCCCGGGCCT
IL2sig-SGRP	417	34	ATGTACAGGATGCAACTCCTGTCTTGCACTTGC AAGTCTTGCACTTGTCACGAATTCGGAGGAGGAGC TGCAGATCATCCAGCCAGAGAAGCTGCTGCTGGT GACCGTGGGCAAGACGGCCACTCTTCATTGTACCA TCACCTCTTTGTTCCCCGTGGGTCCCATCCAGTGG TTCCGCGGGGTGCGACCCGGGCGGGTGCTCATCT ACAACCAGAAGGACGGCCACTTTCTAGGGTCAAC ACAGTAAGCGACGGCACCAAGCGCAACAATATGG ATTTGAGCATTGCGATCTCTTCCATTACTCCGGCG GACGTGGGCACCTATTACTGCGTTAAATTTGTA GGGATCCCCGGAAGACGTGGAGTTCAAATCCGGC CCTGGTACGGAGATGGCTCTGGGCGCCAAGCCCT CG
IL2sig-His-SGRP	435	35	ATGTACAGGATGCAACTCCTGTCTTGCACTTGC AAGTCTTGCACTTGTCACGAATTCGCATCACCATCA CCATCACGAGGAGGAGCTGCAGATCATCCAGCCA GAGAAGCTGCTGCTGGTGACCGTGGGCAAGACGG CCACTCTTCATTGTACCATCACCTCTTTGTTCCCCG TGGGTCCCATCCAGTGGTTCGCGGGGTGCGACC CGGGCGGGTGCTCATCTACAACCAGAAGGACGGC CACTTTCTAGGGTCAACACAGTAAGCGACGGCAC CAAGCGCAACAATATGGATTTGAGCATTGCGATCT CTTCCATTACTCCGGCGGACGTGGGCACCTATTAC TGCGTTAAATTTGTAAGGGATCCCCGGAAGACGT GGAGTTCAAATCCGGCCCTGGTACGGAGATGGCT CTGGGCGCCAAGCCCTCG
IL2sig	57	36	TACAGGATGCAACTCCTGTCTTGCACTTGCCTAAG TCTTGCACTTGTCACGAATTCTG
SGRP	357	38	GAGGAGGAGCTGCAGATCATCCAGCCAGAGAAGC TGCTGCTGGTGACCGTGGGCAAGACGGCCACTCT TCATTGTACCATCACCTCTTTGTTCCCCGTGGGTC CCATCCAGTGGTTCGCGGGGTGCGACCCGGGCG GGTGCTCATCTACAACCAGAAGGACGGCCACTTTC CTAGGGTCACCACAGTAAGCGACGGCACCAAGCG CAACAATATGGATTTGAGCATTGCGATCTTCCAT TACTCCGGCGGACGTGGGCACCTATTACTGCGTTA AATTTGTAAGGGATCCCCGGAAGACGTGGAGTTC AAATCCGGCCCTGGTACGGAGATGGCTCTGGGCG CCAAGCCCTCG

Lentivirus production

Plasmid DNA was extracted with a QIAprep Spin Miniprep Kit (#27104, QIAGEN, Netherlands) from bacterial cultures grown overnight in the presence of 100 µg/mL ampicillin (#A5354, Sigma-Aldrich, USA). Lentiviral particles were generated by co-delivery of a transfer plasmid, a plasmid encoding a VSV-G envelope (pMD2.G; #12259, Addgene, USA) and an empty backbone plasmid (psPAX2; #12260, Addgene, USA) into HEK293T cells. Cells were maintained in DMEM (#11995065, Gibco, USA) supplemented with 10% inactivated fetal bovine serum (FBS; #P30-3302, PAN-Biotech, Germany), 1% pen strep (#15140-122, Gibco, USA) and 2 mM GlutaMAX-I (#35050-038, Gibco, USA). They were cultured as adherent monolayers, at 37°C, in a 5% CO₂ atmosphere and regularly sub-cultured when reaching approximately 70-80% confluence. For the transfections, 5×10^5 HEK293T cells were seeded per well in a 6-well plate and rested for 24 h. Growth media were replaced with antibiotic-free growth media, DNA plasmids were complexed with polyethyleneimine (PEI; #408727, Sigma-Aldrich, USA) for 15 min and added dropwise to the cells, followed by a 48 h incubation. The viral supernatants were collected and cleared from cells and debris by centrifugation at $500 \times g$ for 10 min, followed by filtration through a 0.45 µm polyethersulfone filter (#SLHPM33RS, MilliporeSigma, USA). Virus particles were precipitated with Lenti-X Concentrator (#631232, Takara Bio, Japan), suspended in phosphate buffer saline (PBS; #D8537, Sigma-Aldrich, USA), quantified with Lenti-X GoStix Plus (#631280, Takara Bio, Japan), aliquoted and stored at -80°C.

Healthy-donor T cell and monocyte isolation

Peripheral blood leukocytes from healthy donors (HDs) were obtained from the Blood Donation Center of the University Hospital Basel, Switzerland, after informed consent was obtained from all participants before blood collection. Peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll Paque-PLUS (#GE17-1440-02, Cytiva, Germany) and density centrifugation. After up to 2 rounds of ACK-lysis (#A10492-01, Gibco, USA) for removal of erythrocytes, PBMC were washed with PBS. CD3⁺ T cells were magnetically separated by negative selection with a Human Pan T cell isolation Kit (#130-096-535, Miltenyi Biotec, Germany) and stored long-term in Bmbanker serum-free cell freezing medium (#BB01, GC Lymphotec, Japan) in liquid nitrogen (LN₂). CD14⁺ cells used in phagocytosis assays were

magnetically separated by positive selection using Human CD14 MicroBeads (#130-050-201, Miltenyi Biotec, Germany) and stored in Bambanker serum-free cell freezing medium in LN₂.

CAR T cell production

HD T cells were thawed, washed with PBS and rested in X-VIVO 15 (#BE02-060F, Lonza, Switzerland) at a density of 1×10^6 cells per mL at 37°C, in a 5% CO₂ atmosphere. After 24 h, the T cells were activated in X-VIVO 15 containing 150 U/mL of human IL-2 (#Ro 23-6019, Roche, Switzerland), 10 ng/mL of recombinant IL-7 (#200-07, PeproTech, USA), 10 ng/mL of recombinant IL-15 (#200-15, PeproTech, USA), 20 ng/mL of recombinant IL-21 (#200-21, PeproTech, USA) and Dynabeads human T-activator CD3/CD28 (aCD3/CD28; #11131D, Gibco, USA) in a 1:1 cell-bead ratio. After 48 h, aCD3/CD28 beads were magnetically removed and T cells were resuspended in X-VIVO 15 with 5 µg/mL polybrene (#TR-1003-G, Sigma-Aldrich, USA) at a density of 3×10^6 cells per mL. Lentiviral suspensions were added to the T cells at different multiplicity of infection (MOI) ratios and spininfected at 2500 rpm, at 30°C for 90 min. After spininfection, the T cells were washed and maintained at a density of 1×10^6 cells per mL in X-VIVO 15 containing 500 U/mL IL-2 for 5-7 days. After this post-transduction cell expansion, T cells were sorted for mCherry expression using a BD FACSMelody Cell Sorter (BD Biosciences, USA). After sorting, the CAR T cell cultures were expanded in X-VIVO 15 containing 500 U/mL IL-2 and kept at a density of 1×10^6 cells per mL by adjusting the cell density every 2-3 days based on automated cell counting with a LUNA-FL Dual Fluorescence Cell Counter (Logos Biosystems, South Korea) for 5-10 days until used in downstream assays.

CAR-biotinylated target protein binding assay

CAR T cell viability and count were assessed by Trypan blue exclusion. Cells were washed with PBS and seeded into a 96-well plate at a density of 2×10^5 live cells per well. Cells were immediately stained with a Zombie NIR Viability kit (#423106, BioLegend, USA) diluted 1:5000 in PBS for 20 min in the dark at RT. After viability staining, the cells were washed with autoMACS Running Buffer (#130-091-221, Miltenyi Biotec, Germany) and then resuspended in 100 µL per well of 10 µg/mL dilutions of biotinylated CD19, EGFR or EGFRvIII proteins (#CD9-H82E9, #EGR-H82E3 and #EGR-H82E0, ACROBiosystems, USA) for 1 h in the dark at 4°C. After CAR-target exposure, the cells were washed with autoMACS Running Buffer and stained with 100 µL per well of FITC Streptavidin (#405202, BioLegend, USA) diluted 1:50 in autoMACS Running Buffer, for 1 h in the dark at 4°C. Afterward, the cells were washed 3 times

with autoMACS Running Buffer and resuspended in 100 µL of autoMACS Running Buffer. Samples were acquired with a CytoFLEX Flow Cytometer (Beckman Coulter, USA) and data were analyzed using FlowJo v10 Software (BD, USA).

5 CAR T cell secretome analysis

Supernatant isolation

Expanded aEGFRvIII CAR and aEGFRvIII-SGRP CAR cultures were rested in X VIVO medium without additional supplements for 24 h. The following day, cell viability and count were assessed by Trypan blue exclusion, after which cells were washed in PBS, resuspended in
10 RPMI at a density of 1×10^6 live cells per mL, and incubated for another 24 h at 37°C. Afterward, cultures were centrifuged at $300 \times g$ for 5 min, and supernatants were collected, passed through 0.22 µm filters, and stored at -20°C.

Protein digestion

15 Supernatant samples were TCA-precipitated following the procedure: one volume of TCA was added to every 4 volumes of sample, mixed by vortexing, and incubated for 10 min at 4°C followed by precipitate collection by centrifugation for 5 min at $23000 \times g$. The supernatant was discarded, and the pellet was washed two times with acetone precooled to -20°C. The washed pellets were incubated in the open tube for 2 min at RT to allow residual acetone to
20 evaporate. The pellets were resuspended in 2M guanidine hydrochloride (GUA), 10 mM TCEP, and 100 mM Ammonium Bicarbonate (AmBIC; pH = 8.5) by sonication. The samples were incubated for 10 min at 95°C, let to cool down to RT, followed by the addition of chloroacetamide in a final concentration of 15 mM. After an incubation of 30 min at 37°C, the samples were diluted with 100 mM AmBIC to achieve a final concentration of 0.5 M GUA.
25 Sequencing-grade modified trypsin (1/50, w/w; #V5280, Promega, USA) was added, and the proteins were digested for 12 h at 37°C shaking at 300 rpm. Digests were acidified (pH < 3) using TFA and desalted using C18 spin columns (#74-4101, Harvard Apparatus, USA) according to the manufacturer's instructions. Peptides were dried under vacuum and stored at -20°C.

Peptide mixture loading

A total of 1 µg of peptides were subjected to LC–MS/MS analysis using a Q Exactive Plus Mass Spectrometer (ThermoFisher Scientific, USA) fitted with an EASY-nLC 1000 (ThermoFisher Scientific, USA) and a custom-made column heater set to 60°C. Peptides were resolved using
5 RP-HPLC columns (75 µm × 30 cm) packed in-house with C18 resin (ReproSil-Pur C18–AQ, 1.9 µm resin; Dr. Maisch, Germany) at a flow rate of 0.2 µL per min. The following gradient was used for peptide separation: from 5% B to 10% B over 5 min to 35% B over 40 min to 50% B over 15 min to 95% B over 2 min followed by 18 min at 95% B. Buffer A was 0.1% formic acid in water and buffer B was 80% acetonitrile, 0.1% formic acid in water.

10 Acquisition

The mass spectrometer was operated in DDA mode with a total cycle time of approximately 1 sec. For MS1, 3e6 ions were accumulated in the Orbitrap over a maximum time of 100 ms and scanned at a resolution of 70000 FWHM at 200 m/z. Each MS1 scan was followed by high-collision-dissociation (HCD) of the 10 most abundant precursor ions with dynamic exclusion
15 set to 45 sec. MS2 scans were acquired at a target setting of 1e5 ions, a maximum accumulation time of 100 ms, and a resolution of 35000 FWHM at 200 m/z. Singly charged ions, ions with charge state ≥ 6 and ions with unassigned charge state were excluded from triggering MS2 events. The normalized collision energy was set to 27%; the mass isolation window was set to 1.4 m/z, and one microscope was acquired for each spectrum.

20 Data preprocessing and analysis

Following the recommendation of Jin et al.¹⁷, we performed importation of the LCMS data using the random forest approach implemented in the missForest R library¹⁰. After imputation, the data was normalized using the MSnbase^{18,19} R library (version 2.23.0). These analyses were conducted using R4.2.1. Raw data were imported into Progenesis QI v2.0 software (Nonlinear
25 Dynamics, UK), which extracted peptide precursor ion intensities across all samples with the default parameters. The generated mgf file was searched using MASCOT against a human database (consisting of 41094 forward and reverse protein sequences downloaded from Uniprot in April 2020), a manually entered recombinant SGRP AA sequence as well as 392 commonly observed contaminants using the following search criteria: full tryptic specificity was required
30 (cleavage after lysine or arginine residues, unless followed by proline); 3 missed cleavages were

allowed; carbamidomethylation (C) was set as fixed modification; oxidation (M) and acetyl (Protein N-term) were applied as variable modifications; mass tolerance of 10 ppm (precursor) and 0.6 Da (fragments). The database search results were filtered using the ion score to set the false discovery rate (FDR) to 1% on the peptide and protein level, respectively, based on the number of reverse protein sequence hits in the dataset. Quantitative analysis results from label-free quantification were processed using the SafeQuant R package v.2.3.2 (<https://github.com/eahrne/SafeQuant/>) (Ahrne, E., Molzahn, L., Glatter, T., & Schmidt, A. (2013). Critical assessment of proteome-wide label-free absolute abundance estimation strategies. *Proteomics. Journal of Proteome Research* <https://www.ncbi.nlm.nih.gov/pubmed/23794183>) to obtain peptide relative abundances. This analysis included global data normalization by equalizing the total peak/reporter areas across all LC-MS runs, data imputation using the knn algorithm, summation of peak areas per protein and LC-MS/MS run, followed by calculation of peptide abundance ratios. Only isoform-specific peptide ion signals were considered for quantification. To meet additional assumptions (normality and homoscedasticity) underlying the use of linear regression models and t-tests, MS-intensity signals were transformed from the linear to the log scale. The summarized peptide expression values were used to test for differentially abundant peptides between conditions. Here, empirical Bayes-moderated t-tests were applied, as implemented in the R/Bioconductor limma package (<http://bioconductor.org/packages/release/bioc/html/limma.html>)²⁰. The resulting per protein and condition comparison p-values were adjusted for multiple testing using the Benjamini-Hochberg method. Differential expression analysis compared aEGFRvIII-SGRP CAR and aEGFRvIII CAR samples from 2 HDs.

Cell lines and cell culture

BS153, U87, U251 and U251vIII are human glioma cell lines. BS153 cells were maintained in DMEM (#10938025, Gibco, USA) supplemented with 10% inactivated FBS, 1% pen strep, 2 mM GlutaMAX-I and 1 mM sodium pyruvate (#S8636, Sigma-Aldrich, USA). U87, U251 and U251vIII cells were maintained in MEM (#M4655, Sigma-Aldrich, USA) supplemented with 10% inactivated FBS, 1% pen strep, 1X MEM NEAA (#11140-035, Gibco, USA), 2 mM GlutaMAX-I and 1 mM sodium pyruvate. Raji is a lymphoma cell line cultured in DMEM supplemented with 10% inactivated FBS, 1X MEM NEAA and 1% pen strep. GBM cells were cultured as adherent monolayers whereas Raji cells were cultured in suspension. Cells were maintained at 37°C, in a 5% CO₂ atmosphere and regularly sub-cultured when reaching

approximately 70-80% confluence. All cell lines were routinely tested for mycoplasma contamination using a MycoAlert PLUS Mycoplasma Detection Kit (#LT07-710, Lonza, Switzerland).

GBM cell line lentiviral transduction

- 5 Parental EGFRvIII-negative U87 and U251 cell lines were transduced with a pmp71 lentiviral vector encoding a full-length EGFRvIII, to generate stable EGFRvIII-expressing U87vIII and U251vIII cell lines, respectively. For *in vitro* cytotoxicity assays, BS153, U251 and U251vIII cells were transduced with an Incucyte NucLight Green lentivirus (#4624, Sartorius, Germany) to express a nuclear-restricted EGFP (nEGFP) fluorescence viability reporter protein. For *in vivo* tumor monitoring by bioluminescence imaging (BLi) and fluorescence labeling of tumor cells in downstream assays, U87 cells were transduced with a Luc2-mTagBFP2 lentivirus and U251vIII cells were transduced with an iRFP713-NLuc lentivirus. GBM cell line vector sequences are listed in **Table 3** below:
- 10

Table 3

GBM constructs	Transgene	Selection cassette	SEQ ID NO:	nt sequence
EGFRvIII	EGFRvIII	Puromycin	47	ATGCGACCCCTCCGGGACGGCCGGG GCAGCGCTCCTGGCGCTGCTGGCT GCGCTCTGCCCCGGCGAGTCGGGCT CTGGAGGAAAAGAAAGGTAATTATG TGGTGACAGATCACGGCTCGTGCG TCCGAGCCTGTGGGGCCGACAGCT ATGAGATGGAGGAAGACGGCGTCC GCAAGTGTAAGAAGTGCGAAGGGC CTTGCCGCAAAGTGTGTAAACGGAAT AGGTATTGGTGAATTTAAAGACTCA CTCTCCATAAATGCTACGAATATTAA ACACTTCAAAAAGTGCACCTCCATC AGTGGCGATCTCCACATCCTGCCG GTGGCATTAGGGGTGACTCCTTCA CACATACTCCTCCTCTGGATCCACA GGAAGTGGATATTCTGAAAACCGTA AAGGAAATCACAGGGTTTTTGCTGA TTCAGGCTTGGCCTGAAAACAGGAC GGACCTCCATGCCTTTGAGAACCTA GAAATCATACGCGGCAGGACCAAG CAACATGGTCAGTTTTCTC T TGCAGTCGTCAGCCTGAACATAACA TCCTTGGGATTACGCTCCCTCAAGG AGATAAGTGATGGAGATGTGATAAT TTCAGGAAACAAAAATTTGTGCTAT GCAAATACAATAAACTGGAAAAAAC

				TGTTTGGGACCTCCGGTCAGAAAAAC CAAAATTATAAGCAACAGAGGTGAA AACAGCTGCAAGGCCACAGGCCAG GTCTGCCATGCCTTGTGCTCCCCCG AGGGCTGCTGGGGCCCGGAGCCC AGGGACTGCGTCTCTTGCCGGAAT GTCAGCCGAGGCAGGGAATGCGTG GACAAGTGCAACCTTCTGGAGGGT GAGCCAAGGGAGTTTGTGGAGAAC TCTGAGTGCATACAGTGCCACCCAG AGTGCCCTGCCCTCAGGCCATGAACAT CACCTGCACAGGACGGGGACCAGA CAACTGTATCCAGTGTGCCCACTAC ATTGACGGCCCCCACTGCGTCAAG ACCTGCCCCGGCAGGAGTCATGGGA GAAAACAACACCCTGGTCTGG A AGTACGCAGACGCCGGCCATGTGT GCCACCTGTGCCATCCAACTGCAC CTACGGATGCACTGGGCCAGGTCT TGAAGGCTGTCCAACGAATGGGCC TAAGATCCCGTCCATCGCCACTGG GATGGTGGGGGCCCTCCTCTTGCT GCTGGTGGTGGCCCTGGGGATCGG CCTCTTCATGCGAAGGCGCCACATC GTTCGGAAGCGC
iRFP713- P2A-Nluc	iRFP713, NanoLucifer ase	Puromycin	41	ATGGCTGAAGGATCCGTCGCCAGG CAGCCTGACCTCTTGACCTGCGAC GATGAGCCGATCCATATCCCCGGT GCCATCCAACCGCATGGACTGCTG CTCGCCCTCGCCGCCGACATGACG ATCGTTGCCGGCAGCGACAACCTT CCCGAACTCACCGGACTGGCGATC GGCGCCCTGATCGGCCGCTCTGCG GCCGATGTCTTCGACTCGGAGACG CACAACCGTCTGACGATCGCCTTG GCCGAGCCCCGGGGCGGCCGTCGG AGCACCGATCACTGTCTGGCTTCAC GATGCGAAAGGACGCAGGCTTCAT CGGCTCCTGGCATCGCCATGATCA GCTCATCTTCCTCGAGCTCGAGCCT CCCCAGCGGGACGTCGCCGAGCC GCAGGCGTTCTTCGCCCGCACCAA CAGCGCCATCCGCCGCCTGCAGGC CGCCGAAACCTTGAAAGCGCCTG CGCCGCCGCGGCGCAAGAGGTGC GGAAGATTACCGGCTTCGATCGGG TGATGAT CTA TCGCTTCGCCTCCGACTTCAGCGG CGAAGTGATCGCAGAGGATCGGTG CGCCGAGGTCGAGTCAAACTAGG CCTGCACTATCCTGCCTCAACCGTG CCGGCGCAGGCCCGTCGGCTCTAT ACCATCAACCCGGTACGGATCATTC CCGATATCAATTATCGGCCGGTGCC GGTCACCCCAGACCTCAATCCGGT CACCGGGCGGCCGATTGATCTTAG CTTCGCCATCCTGCGCAGCGTCTC

				GCCCGTCCATCTGGAATTCATGCGC AACATAGGCATGCACGGCACGATG TCGATCTCGATTTTGC GCGCGAGC GACTGTGGGGATTGATCGTTTGCCA TCACCGAACGCCGTACTACGTCGAT CTCGATGGCCGCCAAGCCTGCGAG CTAGTCGCCCAGGTTCTGGCCTGG CAGATCGGCGTGATGGAAGAGGGA AGCGGAGCCACGAAC TTCTCTGT TAAAGCAAGCAGGAGATGTTGAAGA AAACCCCGGGCCTATGGTCT TCA CACTCGAAGATTTTCGTTGGGGACTG GCGACAGACAGCCGGCTACAACCT GGACCAAGTCCTTGAACAGGGAGG TGTGTCCAGTTTGTTCAGAATCTC GGGGTGTCCGTA ACTCCGATCCAA AGGATTGTCCTGAGCGGTGAAAATG GGCTGAAGATCGACATCCATGTCAT CATCCCGTATGAAGGTCTGAGCGG CGACCAAATGGGCCAGATCGAAAA AATTTTAAAGGTGGTGTACCCTGTG GATGATCATCACTTTAAGGTGATCC TGCACTATGGCACACTGGTAATCGA CGGGGTTACGCCGAACATGATCGA CTATTTCCGACGGCCGTATGAAGG CATCGCCGTGTTGACGGCAAAAA GATCACTGTAACAGGGACCCTGTG GAACGGCAACAAAATTATCGACGAG CGCCTGATCAACCCCGACGGCTCC CTGCTGTTCCGAGTAACCATCAACG GAGTGACCGGCTGGCGGCTGTGCG AACGCATTCTGGCG
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Luc2-T2 A- mTagBFP2	mTagBFP2, Luciferase2	Puromycin	42	ATGGAAGATGCCAAAAACATTAAGA AGGGCCCAGCGCCATTCTACCCAC TCGAAGACGGGACCGCCGGCGAGC AGCTGCACAAAAGCCATGAAGCGCT ACGCCCTGGTGCCCGGCACCATCG CCTTTACCGACGCACATATCGAGGT GGACATTACCTACGCCGAGTACTTC GAGATGAGCGTTCGGCTGGCAGAA GCTATGAAGCGCTATGGGCTGAATA CAAACCATCGGATCGTGGTGTGCA GCGAGAATAGCTTGCAGTTCTTCAT GCCCGTGTTGGGTGCCCTGTTTCATC GGTGTGGCTGTGGCCCCAGCTAAC GACATCTACAACGAGCGCGAGCTG CTGAACAGCATGGGCATCAGCCAG CCCACCGTCGTATTTCGTGAGCAAGA AAGGGCTGCAAAAAGATCCTCAACGT GCAAAAAGAAGCTACCGATCATACAA AAGATCATCATCATGGATAGCAAGA CCGACTACCAGGGCTTCCAAAGCAT GTACACCTTCGTGACTTCCCATTG CCACCCGGCTTCAACGAGTACGAC TTCGTGCCCCGAGAGCTTCGACCGG GACAAAACCATCGCCCTGATCATGA ACAGTAGTGGCAGTACCGGATTGC CCAAGGGCGTAGCCCTACCGCACC GCACCGCTTGTGTCCGATTCAGTCA TGCCCGCGACCCCATCTTCGGCAA CCAGATCATCCCCGACACCGCTATC CTCAGCGTGGTGCCATTCACCACG GCTTCGGCATGTTACACGCTGG GCTACTTGATCTGCGGCTTTCGGGT CGTGCTCATGTACCGCTTCGAGGA GGAGCTATTCTTGCGCAGCTTGCAA GACTATAAGATTCAATCTGCCCTGC TGGTGCCCACTATTTAGCTTCTT CGCTAAGAGCACTCTCATCGACAAG TACGACCTAAGCAACTTGACAGAGA TCGCCAGCGGCGGGGCGCCGCTC AGCAAGGAGGTAGGTGAGGCCGTG GCCAAACGCTTCCACCTACCAGGC ATCCGCCAGGGCTACGGCC TGACAGAAACAACCAGCGCCATTCT GATCACCCCCGAAGGGGACGACAA GCCTGGCGCAGTAGGCAAGGTGGT GCCCTTCTTCGAGGCTAAGGTGGT GGACTTGGACACCGGTAAGACACT GGGTGTGAACCAGCGCGGCGAGCT GTGCGTCCGTGGCCCCATGATCAT GAGCGGCTACGTTAACAACCCCGA GGCTACAAACGCTCTCATCGACAAG GACGGCTGGCTGCACAGCGGCGAC ATCGCCTACTGGGACGAGGACGAG CACTTCTTCATCGTGACCGGCTGA AGAGCCTGATCAAATACAAGGGCTA CCAGGTAGCCCCAGCCGAAGTGA GAGCATCCTGCTGCAACACCCCAA CATCTTCGACGCCGGGTGCGCCG CCTGCCCCGACGACGATGCCGGCGA
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				GCTGCCCCGCCGAGTCGTCTGTGCT GGAACACGGTAAAACCATGACCGA GAAGGAGATCGTGGACTATGTGGC CAGCCAGGTTACAACCGCCAAGAA G CTG CGCGGTGGTGTGTGTTCTGTGGAC GAGGTGCCTAAAGGACTGACCGGC AAGTTGGACGCCCCGCAAGATCCGC GAGATTCTCATTAAAGGCCAAGAAGG GCGGCAAGATCGCCGTGGGAAGCG GAGAGGGCAGGGGAAGTCTTCTAA CATGCGGGGACGTGGAGGAAAATC CCGGCCCCATGAGCGAGCTGATTA AGGAGAACATGCACATGAAGCTGTA CATGGAGGGCACCCTGGACAACCA TCACTTCAAGTGCACATCCGAGGGC GAAGGCAAGCCCTACGAGGGCACC CAGACCATGAGAATCAAGGTGGTC GAGGGCGGCCCTCTCCCCTTCGCC TTCGACATCCTGGCTACTAGCTTCC TCTACGGCAGCAAGACCTTCATCAA CCACACCCAGGGCATCCCCGACTT CTTCAAGCAGTCCTTCCCTGAGGGC TTCACATGGGAGAGAGTCACCACAT ACGAAGACGGGGGCGTGCTGACCG CTACCCAGGACACCAGCCTCCA GGA CGGCTGCCTCATCTACAACGTCAAG ATCAGAGGGGTGAACTTCACATCCA ACGGCCCTGTGATGCAGAAGAAAA CACTCGGCTGGGAGGCCTTCACCG AGACGCTGTACCCCGCTGACGGCG GCCTGGAAGGCAGAAACGACATGG CCCTGAAGCTCGTGGGCGGGAGCC ATCTGATCGCAAACGCCAAGACCAC ATATAGATCCAAGAAACCCGCTAAG AACCTCAAGATGCCTGGCGTCTACT ATGTGGACTACAGACTGGAAGAAT CAAGGAGGCCAACAACGAGACCTA CGTCGAGCAGCACGAGGTGGCAGT GGCCAGATACTGCGACCTCCCTAG CAAACCTGGGGCACAAGCTTAAT
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For lentiviral transduction, tumor cells were seeded at 1×10^5 cells per well of a 24-well plate and rested for 24 h. Growth media were replaced with antibiotic-free growth media containing 8 $\mu\text{g/mL}$ polybrene. Lentiviral suspensions were added to the cells at different MOIs and

5 incubated for 6 h at 37°C. Afterward, transduction media were replaced with fresh growth media and the cells were expanded for 1-2 weeks. Cells expressing the relevant surface receptor or fluorescence protein were sorted using a BD FACSMelody or a BD FACS Aria SORP Cell Sorter (BD Biosciences, USA). Luc2 and NLuc expression was confirmed by exposing cells

seeded in the wells of a flat-bottom white 96-well plate to 1 volume of 15 mg/mL D-luciferin (#LUCNA-1G, Goldbio, USA) or 1 volume of 0.5 mg/mL Nano-Glo *In Vivo* Substrate, fluorofurimazine (FFz; #CS320501, Promega, USA), respectively. Cells were imaged after a 10 min incubation protected from the light and imaged with a Fusion FX System (Vilber, France).

Cell surface marker expression analysis

The expression of cell surface markers was determined by FC. Briefly, single-cell suspensions were counted by Trypan blue exclusion and seeded in 96-well plates at a density of 2×10^5 cells per well. Cells were washed with PBS and resuspended in 100 μ L of antibody staining solution. Unless stated otherwise, a viability staining step was performed before (Zombie NIR Viability kit diluted 1:5000) or after (DAPI Solution (#564907, BD Pharmigen, USA) diluted to 0.5X or DRAQ7 (#424001, BioLegend, USA) diluted 1:1000). All antibodies, viability stains and respective dilutions are listed in **Table 4** below.

Table 4

Marker	Fluorophore	Clone	Reactivity	Catalog reference	Company
CCL3	PE	REA257	Human	130-103-629	Miltenyi Biotec
CCR5	APC/Fire750	J418F1	Human	359134	BioLegend
CD4	BV711	OK14	Human	317440	BioLegend
CD8a	FITC	HIT8a	Human	300906	BioLegend
CD11b	Pacific Blue	M1/70	Mouse/Human	101224	BioLegend
CD19	BV510	HIB19	Human	302241	BioLegend
CD47	FITC	CC2C6	Human	323106	BioLegend
CD107a	BB700	H4A3	Human	566558	BD
EGFRvII	FITC	DH8.3	Human	NBP2-50599	Novus Biologicals
TNF	APC	MP6-XT22	Mouse	506308	BioLegend

CAR T cell cytotoxicity assay

Killing assays were performed using an Incucyte S3 Live-Cell Analysis System (Sartorius, Germany). nEGFP-labeled target cells were seeded in flat-bottom, clear, 96-well plates at a density of 1×10^4 cells per well and incubated for 24 h to allow the formation of a cell monolayer. mCherry-labeled CAR T cells were added at various effector-target (E:T) ratios and co-cultures were followed for 72 h. Brightfield and fluorescence images were recorded every 4 h with a 10X objective. Target cell viability kinetics were analyzed via time-lapse videos generated with Incucyte Software (Sartorius, Germany) and quantified using GraphPad Prism v10 Software (GraphPad, USA). Target cells incubated in medium alone were used to determine the baseline viability kinetics of each target cell line. Specific target cell lysis was calculated for each 4 h timepoint as green object area or count, depending on the experiment. All conditions were performed as duplicates or triplicates.

CAR T cell degranulation assay

T cell degranulation in co-cultures with GBM cells was assessed by FC. Briefly, tumor cells were washed with PBS, dissociated and counted by Trypan blue exclusion. GBM cells were seeded in a flat-bottom 96-well plate at a density of 1×10^4 cells per well in 100 μ L of growth medium and incubated for 24 h to allow the formation of a cell monolayer. Afterward, media in the wells was discarded, replaced by 100 μ L of CAR T cell or mock-transduced T cell suspensions in GBM growth medium in 1:1 E:T and incubated for 24 h. At 24 h of co-culture, suspension cells were gently mixed in the supernatant and collected into a round-bottom 96-well plate. Cells were washed with PBS and stained with a BB700 anti-CD107a (clone H4A3, #566558, BD Biosciences, USA) for 20 min in the dark at 4°C. After surface staining, the cells were washed 3 times with autoMACS Running Buffer and resuspended in 100 μ L per well of 0.5X DAPI diluted in autoMACS Running Buffer. Samples were acquired with a CytoFLEX Flow Cytometer and data were analyzed with FlowJo v10 Software (BD, USA). Positively stained cells were differentiated from the background using unstained controls. All conditions were performed as triplicates.

GBM-CAR T cell co-culture supernatant ELISA

Flat-bottom F96 MAXISORP NUNC-IMMUNO plates (#439454, Thermo Scientific, USA) were coated with 50 μ L per well of Purified anti-human IFN γ Antibody (clone MD-1, #507502,

BioLegend, USA) diluted 1:200 in 1X PBS (#5460-0023, BioConcept, Switzerland) and incubated overnight at 4°C. The following day, wells were washed 3 times with PBS-T buffer (PBS with 0.05% Tween 20) and blocked for unspecific binding with 100 µL per well of 1% SureBlock solution (#SB232010, LubioScience, Switzerland) for 1 h at RT. After the blocking, plates were washed 3 times with PBS-T, then 50 µL of 24 h co-culture supernatants or serial dilutions of Recombinant Human IFN γ standard (#300-02, Peprotech, USA) were added to the wells and incubated for 2 h at RT. Wells were washed 3 times before adding 50 µL per well of Biotin anti-human IFN- γ Antibody (clone 4S.B3, #502504, BioLegend, USA) diluted 1:400 in 1% SureBlock solution and incubated 1 h at RT. Wells were again washed 3 times before adding 50 µL per well of HRP Streptavidin (#405210, BioLegend, USA) diluted 1:2000 in 1% SureBlock solution and incubated 1 h at RT. Wells were washed 3 times and 100 µL per well of SIGMAFAST OPD tablet (#P9187, Sigma-Aldrich, USA) solution was added. The chromogenic reactions were stopped by adding 50 µL per well of 10% sulfuric acid (H₂SO₄) and absorbance values were measured by a Synergy H1 Hybrid microplate reader (BioTek, USA). Absolute IFN γ concentrations in test samples were interpolated from a standard curve and represented using GraphPad Prism v10 Software.

On-cell CD47 blocking assay

BS153 viability and count were assessed by Trypan blue exclusion, after which cells were seeded in flat-bottom 96-well plates at a density of 3×10^5 cells per well. Cells were then treated for 30 min at 4°C with 50 µL per well of 10 µg/mL of InVivoMAb anti-human CD47 (clone B6.H12, #BE0019-1, Bio X Cell, USA) or InVivoMAb mouse IgG1 isotype control (clone MOPC-21, #BE0083, Bio X Cell, USA) or conditioned-media from 24 h-rested, antigen-naïve aEGFRvIII or aEGFRvIII-SGRP CAR T cells seeded at a density of 1×10^6 cells per mL of unsupplemented RPMI. Pre-treated tumor cells were washed with PBS and incubated for 30 min at 4°C with biotinylated SIRP α (bt-SIRP α ; #CDA-H82F2, ACROBiosystems, USA), which bound to the unblocked CD47 on BS153 cells. Finally, APC Streptavidin (#405207, BioLegend, USA) staining was performed, followed by 3 washes with autoMACS Running Buffer and resuspension in 100 µL per well of 0.5X DAPI in autoMACS Running Buffer. Samples were acquired by a CytoFLEX Flow Cytometer and data were analysed with FlowJo v10 Software.

CD14⁺ monocyte differentiation and culture

Primary monocytes were thawed, washed in PBS and plated at 0.8×10^6 cells per mL in RPMI 1640 with 1X GlutaMAX (#61870-036, Gibco, USA), 10% FBS (#P30-3302, PAN-Biotech, Germany), 5% human serum (#H4522, Sigma-Aldrich, USA) and 25 ng/mL M-CSF (#300-25, PeptoTech, USA), and incubated at 37°C in a 5% CO₂ atmosphere. After 48 h, the medium was replaced with human serum-free growth media and refreshed every 2-3 days for a maximum of 14 days until phagocytosis assays.

Phagocytosis assay

Macrophages were detached from culture dishes using cell scrapers after a 15-minute incubation at 37°C in TrypLE Express (#12604-021, Gibco, USA). Cells were washed in PBS, counted, seeded at 5×10^4 cells per well in a 96-well flat-bottom plate (#353072, Corning, USA), and incubated for 48 h to allow attachment. CAR T cells were counted and diluted to 1×10^6 cells per mL in X VIVO 15. Tumor cells were washed in PBS, dissociated, and counted. All cell counts were performed by Trypan Blue exclusion. U251vIII cells were diluted to 1×10^6 cells per mL in IMDM. U87 cells were stained with 62.5×10^{-6} M CellTracker Green (#C2925, Thermo Scientific, USA) diluted in IMDM (#12440-053, Gibco, USA) for 30 min at 37°C at a density of 1×10^6 cells per mL. After two washes with PBS, stained cells were counted and adjusted to 1×10^6 cells per mL in IMDM. U87 and U251vIII cells were then mixed in a 1:1 ratio. The tumor cell mixture (1×10^5 cells) and CAR T cells (5×10^4 cells) were added to each well containing macrophages. The contents were resuspended in IMDM and incubated at 37°C for 3 h. After incubation, cells were detached using TrypLE, transferred to a 96-well U-bottom plate, and washed twice in PBS. Viability staining was carried out by incubating cells with a Zombie UV Viability kit (#423107, BioLegend, USA) for 20 min at 4°C in the dark. Fc-block was performed by incubating cells in a dilution of Human TruStain FcX (#101320, BioLegend, USA) for 10 min at 4°C. Antibody mastermixes (full-stains and FMOs) were prepared freshly in autoMACS Running Buffer. Cells were stained with surface marker antibody mastermixes for 25 min at 4°C in the dark and then washed twice in autoMACS Running Buffer. Cells were fixed by incubation for 20 min at RT using a Cyto-Fast Fix/Perm Buffer set (#426803, BioLegend, USA). After antibody staining, samples were washed twice with autoMACS Running Buffer, resuspended in 200 µL MACS, and acquired on a Cytex Aurora 5-Laser Spectral Analyzer (Cytex Biosciences, USA), using standard, daily quality-controlled, Cytex-

Assay-Settings. All conditions were performed with donor-matched CAR T cells and macrophages from 3 HDs.

Animal experimentation

All animal handling, surveillance and experimentation were performed according to the guidelines of the Swiss Federal Veterinary Office (SFVO) and the Cantonal Veterinary Office (CVO) of Basel-Stadt, Switzerland. GBM model experiments were executed under license #2929_31795 and lymphoma model experiments under licenses #2929_31795 and #3176_35274. Animals were maintained at the local animal facility in pathogen-free, ventilated HEPA-filtered cages under stable housing conditions of 45-65% humidity, a temperature of 21-25°C and a gradual light cycle from 7 am to 5 pm. Animals were provided standard food and water without restrictions.

Mouse strains and humane endpoint assessment

NOD.Cg-Prkdc^{scid} Il2rg/SzJ (NSG) mice with identifier RRID:IMSR_JAX:005557 were obtained from in-house breedings or externally (Janvier Labs, France) under protocols approved by the SFVO and CVO of Basel-Stadt. Co-housed animals were assigned to treatment or control groups using a randomized approach and euthanized upon reaching the humane endpoint, including significant reduction of locomotion, significant weight loss and mild-to-severe neurologic symptoms. Tumor cell implantation was set as day 0 and the survival time was set as the day of euthanasia.

GBM mouse models and survival assessment

All experiments were performed on NSG mice of the male sex, aged 7-12 weeks at the time of tumor implantation. To assess the efficacy of anti-EGFRvIII CAR T cell and anti-CD47 antibody monotherapies, mice were injected intracranially (i.c.) with 5×10^4 U251vIII-NLuc cells. To test our proposed combination therapy, mice were injected i.c. with a total of 5×10^4 GBM cells consisting of 2.5×10^4 U87-Luc2 and 2.5×10^4 U251-NLuc, resulting in EGFRvIII-mosaic tumors. In both models, the animals were anesthetized in an induction chamber with $2.0 \pm 0.5\%$ isoflurane in an O₂ atmosphere immediately before the tumor injections. Anesthesia on the stereotactic frame (Neurostar, Germany) was maintained at $2.0 \pm 0.5\%$ isoflurane delivered through a nose/mouth adaptor. General analgesia (Buprenorphine; Bupaq-P, Streuli

Tiergesundheits, Switzerland) was given subcutaneously (s.c.) immediately before surgery at 0.05 mg per kg and local analgesia was applied s.c. under the scalp. Eye gel (Lacrinorm, Bausch+Lomb Swiss AG, Switzerland) was applied to prevent drying of the eyes during surgery. The scalp was briefly swabbed with povidone-iodine solution, and a midline incision was made. The scalp was briefly swabbed with povidone-iodine solution and a midline incision was made. A burr hole was manually drilled 2 mm lateral from the cranial midline and 1 mm posterior of the bregma suture using a microdrill. A digitally-controlled injection was performed with the Stereodrive Software (Neurostar, Germany) using a 10 μ L Microliter Syringe (#80300, Hamilton, USA). The syringe was lowered into the burr hole to a depth of 3 mm below the surface of the dura and retracted by 0.5 mm to form a small reservoir in the cortex. A volume of 4 μ L of single-cell suspension was injected at 1 μ L per min. The needle was left in place for at least 1 min and carefully retracted by 0.5 mm every 30 sec. After injection, the incision was sutured (#D7585, Ethicon, USA). Buprenorphine analgesia (Bupaq-P, Streuli Tiergesundheits, Switzerland) was given intraperitoneally (i.p.) at 0.05 mg per kg immediately post-op. The following treatments and controls were administered intratumorally (i.t.) in a volume of 4 μ L on days 7 and 14: Vehicle (PBS), antibody Isotype (InVivoMAb mouse IgG1 isotype control, clone MOPC-21; 5 μ g), aCD47 (InVivoMAb anti-human CD47, clone B6.H12; 5 μ g), aCD19 CAR (5×10^5 cells), aCD19-SGRP CAR (5×10^5 cells), aEGFRvIII CAR (5×10^5 cells), aEGFRvIII CAR + aCD47 (5×10^5 cells and 5 μ g, respectively) or aEGFRvIII-SGRP CAR (5×10^5 cells). In EGFRvIII-mosaic survival experiments, aCD47 and aEGFRvIII CAR + aCD47 treatment groups received additional doses of anti-CD47 (100 μ g) administered i.p. in a volume of 100 μ L on days 19, 22, 26 and 29. In the EGFRvIII-mosaic, CCL3 blockade survival experiment, CAR T cells were applied as described above and 50 ng of antibody per mouse (aCCL3 or isotype) were administered i.p. in a volume of 100 μ L on days 8, 10, 13, 15, 17, 20, 22, 24, 27, 29, 31, 34, 36, 38, and 41. Mice were monitored for clinical signs until day 90, upon which all remaining survivors were either euthanized or assigned to a tumor rechallenge experiment. Animals assigned to a rechallenge experiment were injected with the same mix of tumor cells using the same procedure described above. Tumor-rechallenged animals received no treatment and a historic Vehicle group was used as a control. Kaplan–Meier survival comparison was performed using a Log-rank (Mantel-Cox) test with GraphPad Prism v10 Software.

In vivo GBM bioluminescence imaging

GBM engraftment and growth were monitored by bioluminescence imaging (BLi). Mice implanted i.c. with EGFRvIII-mosaic tumors were subjected to dual BLi with specific substrates to separately monitor the growth of the EGFRvIII⁺ and EGFRvIII⁻ tumor fractions.

- 5 The animals' clinical scores and luminescence images were taken weekly, after i.p. injection of 150 mg kg⁻¹ of D-luciferin or intravenous (i.v.) injection of 0.325 μmol of fluorofurimazine per mouse. Subsequently, mice were anesthetized by isoflurane inhalation and imaged on a Newton 7.0 instrument (Vilber, France). Photon flux over a defined ROI (brain surface) was quantified with NEWTON v7 Software (Vilber, France).

10 Olink proteomics of mouse plasma

- Peripheral blood samples were collected approximately 24 h after each treatment dose (days 8 and 15 after tumor implantation). Unanesthetized mice were briefly put under a heat lamp and then placed in a cylindrical restrainer. A small puncture was made on the tail to allow the dripping of approximately 100 μL of blood into lithium heparin-coated Microvette 100 capillary
15 blood collection tubes (#20.1282.100, Sarstedt, Germany) and kept at RT. Blood samples were centrifuged at 4500 rpm for 15 min at RT and the top layer of plasma was transferred into sterile 0.5 μL Eppendorf tubes. Samples were immediately frozen at -80°C until analysis. 15 samples were analyzed by proximity extension assay technology using a standard Olink Target 96 Immuno-Oncology panel (Olink Holding, Sweden). Samples were analyzed across 2 plates, 16
20 samples from the first run were included in the second in order to perform a reference sample normalization. This bridging procedure consisted in calculating the median of the paired normalized protein expression levels (NPX) differences per protein between the overlapping samples to determine the adjustment factors to be applied between the 2 datasets. For the overlapping samples, the mean value was kept. A cyclic loess normalization was applied. Since
25 data below the limit of detection may be non-linear, the differential expression for the contrasts of interest was assessed by a Mann-Whitney U test with Benjamini-Hochberg correction.

Lymphoma mouse model and survival assessment

- To test the efficacy of the CAR constructs in a subcutaneous (s.c.) tumor model, NSG mice of
30 the female sex, aged 8-12 weeks were anesthetized with isoflurane and injected with 5×10^5

CD19-expressing Raji cells s.c. in the right flank. Raji cells were suspended in BD Matrigel Basement Membrane Matrix High Concentration (#354248, BD Biosciences) diluted 1:1 in phenol red-free DMEM without additives in a total volume of 100 μ L. Three days after tumor inoculation, each mouse received 8×10^5 CAR T cells suspended in 200 μ L of PBS or PBS alone, i.v. via the tail vein. Tumor-bearing mice injected with untargeted CAR T cells or PBS were used as controls. Tumor size was measured 3 times per week using a caliper. Animals were sacrificed before reaching a tumor volume of 1500 mm³ or when reaching an exclusion criterion (ulceration, severe weight loss, severe infection or bite wounds). Tumor volume was calculated according to the following formula: Tumor volume (mm³) = (d² $\times$ D)/2 with D and d being the longest and shortest tumor parameter in mm, respectively.

Mouse tissue collection and processing

For spectral FC analyses, mice were euthanized by CO₂ suffocation, and brain regions were immediately harvested into ice-cold HBSS. Depending on the experiment, the tumor-injected hemisphere, contralateral hemisphere or brain meninges were carefully dissected and manually minced using razor blades and enzymatically dissociated at 37°C for 30 min with 1 mg/mL collagenase type IV (#LS004188, Worthington Biochemical Corporation, USA) and 250 U/mL DNase 1 (#10104159001, Roche, Switzerland) in a buffer containing HBSS with Ca²⁺/Mg²⁺ (#14205-050, Gibco, USA), 1% MEM NEAA, 1 mM sodium pyruvate, 44 mM sodium bicarbonate (#25080-060, Gibco, USA), 25 mM HEPES (#H0887, Gibco, USA), 1% GlutaMAX-I and 1% antibiotic-antimycotic (#15240062, Gibco, USA). Extraction of the meninges was performed as described earlier (Roussel-Queval A, Rebejac J, Eme-Scolan E, Paroutaud LA, Rua R. Flow cytometry and immunohistochemistry of the mouse dural meninges for immunological and virological assessments. STAR Protoc. 2023 Mar 17;4(1):102119. doi: 10.1016/j.xpro.2023.102119. Epub 2023 Feb 14. PMID: 36853673; PMCID: PMC9958090.). The resulting cell suspensions were filtered through a 70 μ m strainer and centrifuged in a density gradient using debris removal solution (#130-109-398, Miltenyi Biotec, Germany) according to the manufacturer's protocol to remove myelin and cell debris. Erythrocytes were removed using ACK lysing solution (#A1049201, ThermoFisher Scientific, USA), and cell suspensions were washed with PBS and kept on ice until spectral flow staining. For IHC, quantification of brain tumor area, IF brain analysis, and spleen size measurement, mice were anesthetized i.p. with a mix of 80 mg per kg of ketamine (Ketanarkon, Streuli Tiergesundheit, Switzerland) and 16 mg per kg of xylazine (Rompun, Elanco, USA), and transcardially perfused

with ice-cold PBS. Brains and spleens were dissected and immediately fixed in formalin at 4°C for 72 h before paraffin embedding.

Mouse brain immunohistochemistry

5 Stainings

Formalin-fixed brains were embedded in paraffin and 5 µm sections were made using a microtome. Slides were stained according to the standard H&E protocol using the automated Gemini AS Slide Stainer (ThermoFisher Scientific, USA) and covered with Permount Mounting Medium (#SP15-100, ThermoFisher Scientific, USA). For CD3 and CD68 IHC stainings, FFPE brain sections were stained with a Ventana DISCOVERY ULTRA automated slide preparation system (Ventana Medical Systems Inc., USA) using a Histofine Simple Stain MAX PO anti-rabbit (#414142F, Nichere Biosciences Inc., Japan) as a detection reagent. For myelin staining, brain sections were deparaffinized and rehydrated before incubation in Luxol solution (#1B 389, Mediate, Switzerland) at 60 °C for 2 h. After cooling, sections were washed with 96% ethanol and flowing tap water for 5 min, and then distilled water. Differentiation was performed with 0.1% lithium carbonate solution (#62470, Sigma-Aldrich, USA) for 10 sec. After washing the sections in distilled water, nuclei were stained using 1% cresyl violet (#1.05235, Merck, Germany) in 96% ethanol for 5 sec until the background became colorless. Sections were then dehydrated in 100% ethanol, Xylol (#253-VI53TE, Biosystems, Switzerland), and embedded (#41-4012-00, HistoLab, Sweden).

Acquisition

Slides were acquired using a Nanozoomer S60 digital slide scanner (Hamamatsu, Japan) with a 40x objective.

Histological brain tumor size measurement

25 To calculate tumor size, a pixel classifier was trained to detect tumors in mouse brain histological sections in QuPath³⁷. The Random Trees Classifier was used with the following parameters: Pixel size 3.53 µm; Channels: red, green and blue; Features: Gaussian and Laplacian of Gaussian; Scales: 4.0 and 8.0; no normalization; classification was done based on

example annotations to distinguish brain tissue, tumor and background (ignore*). Results were visually verified.

CD3 and CD68 quantification

Cells were segmented based on Hematoxylin staining with the StarDist2D³⁸ plugin within
5 QuPath³⁷ using the following parameters: Probability threshold: 0.2; Pixel size: 0.2; Cell
expansion: 2.0; using a pre-trained model 'he_heavy_augment.pb'. Cells were classified for
CD3 and CD68 positivity based on mean DAB staining in the nucleus with a threshold of 0.12
and 0.2, respectively.

Immunofluorescence multiplex staining and imaging

10 The protocol was adapted from Gut and colleagues³⁹. FFPE brains were sectioned in 5 µm-thick
sections using a microtome. Slides were deparaffinized 3 times for 5 min with RotiHistol (Roth,
#6640) and 30 s with 100% EtOH, 95% EtOH, 70% EtOH, 50% EtOH and dH₂O. For antigen
retrieval, we used pre-heated citrate buffer (Merck, #C9999) in a microwave for 20 min,
followed by a washing step with PBS for 10 min. Tissue sections were permeabilized in PBS
15 with 0.2% Tween-20 (Sigma-Aldrich, #P5927) for 2 x 15 min. Thereafter, the slides were
washed 2 x 5 min with PBS followed by blocking with 10% donkey serum (Jackson
ImmunoResearch, #017-000-121) in PBS and incubation for 1 h in a dark humid chamber.
Afterward, sections were stained in different cycles overnight at 4 °C with the following
antibodies: anti-EGVRvIII (1:1000, Rabbit, ThermoFisher, #RM419), anti-Ki-67 (1:500, Rat,
20 ThermoFisher, #14-5698-82), anti-Iba-1 (1:500, Goat, Novus Biological, #NB100-1028), anti-
CD69 (1:500, R&D systems, #AF2359-SP), anti-TMEM119 (1:500, Rabbit, Abcam,
#ab209064), anti-CD3 (1:100, Rat, BioRad, #MCA1477), anti-CD206 (1:200, Rabbit,
BioConcept, #24595s). Subsequently, slides were washed for 3 x 5 min with PBS, and stained
with fluorescent secondary antibody (Donkey-anti-rabbit 1:300, Jackson ImmunoResearch,
25 #711-605-152, donkey anti-rat 1:1000, Invitrogen, #2474993; donkey anti-goat 1:300, Jackson
Immunoresearch, #705-545-147) and DAPI (1:1000), and incubated for 1 h in a humid chamber
at RT. Slides were washed 3 x 5 min with PBS and mounted with imaging buffer at pH 7.4 (700
mM N-Acetyl-Cysteine (NAC, ThermoScientific, #160280250) in ddH₂O + 20% HEPES
(Sigma, #H0887)). After each cycle, slides were treated with 3 x 15 min elution buffer
30 containing 0.5 M L-Glycine (Roth, #3790.2), 1.2 M Urea (Sigma, #U5378), 3 M Guanidium

chloride (Sigma, #G3272), and freshly added 70 mM TCEP-HCl (Sigma, #C4706) followed by ddH₂O, and re-stained.

Acquisition

Images were acquired using a Nikon Eclipse Ni upright microscope equipped with 395, 470, 561 and 640 nm lasers with a Prior PL-200 robotic slide loader equipped with Microscan MiniHawk (Omron Microscan Systems, Inc.) and the Photometrics Prior 95B camera. The acquisition was done using JOBS automation in NIS-Elements software (version 5.11.00). 4x Plan Apo NA 0.2 (Nikon) objective was used to make a slide overview and GA3 (general analysis3) with Otsu threshold was used to detect the tissue. The focus surface was created by performing software autofocus every 2 points based on the DAPI signal with Plan Apo λ 20x NA 0.8 and the tissue was scanned with the same objective. Slides were scanned after each staining cycle, with blank (=unstained) cycles used to evaluate autofluorescence. The exposure time of each channel was constant for all cycles and corresponding blank channels (GFP, Cy3 and Cy5) were subtracted from the signal cycle.

Preprocessing

Fiji⁴⁰ was used for image preprocessing. Single tiles were stitched using Grid/Collection Stitching⁴¹. Stitched tissues were registered based on consecutive cycles based on DAPI (4',6-diamidino-2-phenylindole) channel using MultiStackReg plugin⁴² and the same correction was propagated on the remaining channels. Images were saved as a pyramidal file using the Kheops⁴³ plugin, available under an open-source license: <https://github.com/BIOP/ijp-kheops/releases>.

Cell segmentation and quantification

Cells were segmented based on DAPI staining with the StarDist2D³⁸ plugin within QuPath³⁷ using the following parameters: Probability threshold: 0.5; Pixel size: 0.5; Cell expansion: 2.0; using a pre-trained model 'dsb2018_heavy_augment.pb'. Object classification in QuPath was used to define cell positivity for CD3, CD206, EGFRvIII and Iba1. All mouse brain IHC and IF images were prepared using OMERO.web app (www.openmicroscopy.org/omero/figure/).

Post-therapy toxicity monitoring

Spleen size measurement

The coronal oblique length of FFPE spleen cross-sections was measured and plotted as the ratio of spleen length to mouse bodyweight at the spleen collection time point.

5 Mouse weight monitoring

Animals were weighed weekly, starting from the tumor-implantation day until endpoint.

Plasma IL6 and CRP

- Plasma IL6 was assessed from the Olink proteomics dataset described in the “Olink proteomics of mouse plasma” section. Plasma mouse CRP levels were investigated with a Mouse C-Reactive Protein ELISA kit (#41-CRPMS-E01, ALPCO Diagnostics, USA), following the manufacturer’s instructions. The chromogenic reactions were stopped by adding 50 μ L per well of 10% sulfuric acid (H_2SO_4), and absorbance values were measured by a Synergy H1 Hybrid microplate reader (BioTek, USA). Absolute CRP concentrations in test samples were interpolated from a standard curve and represented using GraphPad Prism v10 Software.
- 10

15 Automated hematology analysis

- Longitudinal analysis of hematological parameters was performed after a single CAR treatment using 20 μ L of mouse blood collected from the tail vein into 1.5 mL Eppendorf tubes prefilled with 120 μ L of 1 mM EDTA diluted in PBS. Blood samples from multiple time points (1, 3, 6, 13, and 20 days after treatment) were immediately acquired on a XN-1000 benchtop hemocytometer (Sysmex, Japan). Values were normalized and scaled to allow multiple time point comparisons.
- 20

Myelin quantification

- Brain sample collection and processing and the myelin staining procedure were described above in the “Mouse brain immunohistochemistry” section. Images were color-deconvolved (Ruifrok AC, Johnston DA. Quantification of histochemical staining by color deconvolution. Anal Quant Cytol Histol. 2001 Aug;23(4):291-9. PMID: 11531144.) to separate luxol and cresyl violet staining using QuPath v0.5.0 (Bankhead P, Loughrey MB, Fernández JA, Dombrowski Y, McArt DG, Dunne PD, McQuaid S, Gray RT, Murray LJ, Coleman HG, James JA, Salto-Tellez
- 25

M, Hamilton PW. QuPath: Open source software for digital pathology image analysis. Sci Rep. 2017 Dec 4;7(1):16878. doi: 10.1038/s41598-017-17204-5. PMID: 29203879; PMCID: PMC5715110.). The Random Trees Classifier was trained to detect brain tissue and myelin area with the following parameters: Pixel size: 7.07 μm ; Channels: luxol and cresyl violet; Features: Gaussian, structure_tensor_eigenvalue_max, and hessian_determinant"; Scales: 1.0 and 4.0; no normalization. The positive area of myelin (luxol staining) was quantified within brain tissue as an area above the threshold. Results were visually verified. The relative myelin area per sample was plotted using GraphPad Prism v10 Software.

Spectral flow cytometry

Freshly dissociated cells were resuspended in 400 μL PBS. 200 μL per well was distributed into a 96-well plate (200 μL for FS, 100 μL for US and 100 μL for FMO). Primary mouse and human cells were isolated as described above and stained with Zombie fixable viability dye for 20 min at RT (#423102, BioLegend, USA) and pre-incubated with anti-mouse Fc block at 10 mg mL⁻¹ (#101320, BioLegend, USA). Surface markers were stained with appropriate antibodies for 20 min at 4°C. For intracellular staining, cells were fixed and permeabilized using Cyto-Fast Fix/Perm Buffer Set (#426803, BioLegend, USA), prior to staining for 20 min at RT in the dark. For intracellular cytokine staining, cells were fixed, permeabilized and washed using True-Nuclear Transcription Factor Buffer Set (#424401, BioLegend, USA) according to the manufacturer's protocol. Followed by staining with intracellular antibodies for 30 min at RT in the dark. After the respective staining protocol, cells were washed twice and resuspended in FACS buffer. For FC, either a Cytoflex S (Beckman Coulter) flow cytometer or an Aurora spectral flow cytometer was used for cell acquisition and FlowJo software (v.10.8.1, TreeStar) for data analysis. Cell sorting was performed using a BD FACSAria III or BD FACSMelody (BD Bioscience). We performed compensation using Ultracomp beads Compensation Beads (#01-2222-42, Invitrogen, USA), which were stained with appropriate antibodies and analyzed on the same voltage and settings. Gates were drawn by using Fluorescent Minus One (FMO) controls. Either the percentage of cell population of interest or Median Fluorescent Intensity (MFI) is reported. Panel information: **Table 5** below.

Table 5

Surface marker	Fluorophore	Clone	Catalog reference	Lot	Company	Final dilution
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AXL	AF488	175128	FAB8541 G	AEWU04 22121	R&D Systems	1:200
CD4	BUV496	GK1.5	612952	2272360	BD Bioscienc es	1:800
CD8a	BV650	53-6.7	100742	B368585	BioLegen d	1:200
CD11b	BV510	M1/70	101245	B376886	BioLegen d	1:1600
CD11c	PE/Cy7	N418	117317	B346714	BioLegen d	1:3200
CD45	BUV737	30-F11	748371	23222187	BD Bioscienc es	1:6000
CD86	PE/Cy5	GL-1	105016	B240898	BioLegen d	1:1600
CD206	AF700	C068C2	141734	B384250	BioLegen d	1:100
EGFRvIII	PerCP	DH8.3	NBP2- 50599PC P	D139305	Novus Biological s	1:200
F4/80	BV605	BM8	123133	B386425	BioLegen d	1:50
LY6c	BV785	HK1.4	128041	B371649	BioLegen d	1:12000
LY6g	BUV563	1A8	612921	2343097	BD Bioscienc es	1:2500
MERTK	BV711	2B10C42	151515	B354635	BioLegen d	1:100
MHC-II	NF610- 70S	M5/114.1 52	M024T02 B06	2511116	ThermoFi sher Scientific	1:1600
P2RY12	APC- Fire810	S16007D	848013	B343968	BioLegen d	1:50
Siglec-H	APC	551	129612	B286621	BioLegen d	1:150
XCR1	BV421	ZET	148216	B377550	BioLegen d	1:100
Intracellu lar marker	Fluoroph ore	Clone	Catalog reference	Lot	Compan y	Final dilution
TNF	PE	MP6- XT22	506305	B336284	BioLegen d	1:100
Fc- blocking antibody	Target	Clone	Catalog reference	Lot	Compan y	Final dilution

Human TruStain FcX	CD16/CD32/CD64	Unknown	422302	B369982	BioLegend	1:50
TruStain FcX	CD16/CD32	93	101320	B380119	BioLegend	1:50

FlowSOM analysis

Data were manually pre-gated to remove the debris and select for CD45⁺, live, single cells using FlowJo v10 Software. The analysis was subsequently performed in R (version 4.3.1). Data was transformed by asinh transformation using variance stabilizing cofactors for each channel (*estParamFlowVS* and *transFlowVS* functions from the FlowVS package). Pre-processing QC (using *PeacoQC* function from the PeacoQC package) was performed to remove outliers and unstable events (IT_limit was set at 0.55 and MAD at 6). Clustering was performed using FlowSOM and ConsensusClusterPlus using the wrapper function *cluster* from the CATALYS package (xdim = 10, ydim = 10, maxK = 15, seed = 1234). The resulting clusters were manually annotated. Differential testing was performed using the diffcyt package (diffcyt-DA-edgeR for differential abundance and diffcyt-DS-limma for differential state).

Human GBM samples and patient information

Freshly resected primary tumor tissue samples from patients with a pathology-confirmed GBM diagnosis were obtained from the Neurosurgical Clinic of the University Hospital Basel, Switzerland, following the Swiss Human Research Act and institutional ethics commission (EKNZ 02019-02358). The study was conducted following the ethical principles of the Declaration of Helsinki, regulatory requirements, and the Code of Good Clinical Practice. All patients gave written informed consent for tumor biopsy collection and signed a declaration enabling the use of their biopsy specimens in scientific research (Req-2019-00553) with all identifying information removed. All patients were treatment-naïve at the time of tissue collection. Clinical sample information is summarized in **Table 6 below**:

Table 6

I D	Sex	Age	First diagn osis	IDH status	MGMT status	Preexisting conditions	Steroids during surgery	Additional information
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6 7 6	M	71	08/21	Wt	Methylated	Arterial hypertension, structural epilepsy, deep vein thrombosis	None	
6 9 1	M	63	11/21	Wt	Non methylated	Arterial hypertension	None	Early postoperative meningitis
6 3 5	M	64	06/20	Wt	Methylated	Chronic alcoholism, depression	None	
6 8 5	M	63	08/21	Wt	Non methylated	Atrial fibrillation	12mg fortecortin	
7 2 8	M	61	09/22	Wt	Methylated	None	None	
6 3 9	W	55	07/20	Wt	Methylated	None	10mg fortecortin	Postoperative wound infection
7 1 6	M	82	06/22	Wt	Non methylated	Prostata carcinoma, arterial hypertension, diabetes mellitus	4mg fortecortin	
6 9 2	M	80	11/21	Wt	Methylated	Prostata carcinoma, atrial fibrillation	4mg fortecortin	
7 2 5	M	69	08/22	Wt	Methylated	Coronary heart disease	8mg fortecortin	

7	M	59	11/22	Wt	Methlyated	Chronic obstructive pulmonary disease	None	
3								
9								

Human GBM tissue processing

GBM tissue samples were transported on ice and transferred to the laboratory for dissociation into single-cell suspensions within 2-3 h after surgical resection. Human brain tissue was mechanically minced using razor blades and enzymatically dissociated as described above (under “Spectral Flow Cytometry”) except that debris and myelin were removed by a 0.9 M sucrose (#84100, Sigma-Aldrich, USA) density gradient centrifugation. After ACK-lysis, the single-cell suspensions were washed with PBS, resuspended in Bambanker at an approximate density of 2×10^6 live cells per mL and stored long-term in LN₂.

Real-time quantitative PCR

The total RNA contents of approximately 1×10^6 cells from human CAR T cells were extracted with TRIzol Reagent (#15596026, Invitrogen, USA) and an AllPrep DNA/RNA/Protein Mini kit (#80004, QIAGEN, USA). cDNA was synthesized with an iScript cDNA Synthesis kit (#1708891, Bio-Rad, USA) using 500 ng of input RNA in a 20 μ L reaction. The reaction included priming for 5 min at 25°C, reverse transcription for 20 min at 46°C, and inactivation for 1 min at 95°C. RT-qPCR reactions were performed with a SsoFast EvaGreen supermix (#172-5201, Bio-Rad, USA) using 2 μ L of input cDNA in a 20 μ L reaction. The total RNA contents of approximately 2×10^6 cells from culture-dissociated tumor cells or human GBM single-cell suspensions were extracted with TRIzol Reagent and a Direct-zol RNA Miniprep Plus kit (#R2070, Zymo Research, USA). cDNA was synthesized with a SuperScript VILO cDNA Synthesis kit (#11754-050, ThermoFisher Scientific, USA) using 20 ng of input RNA in a 20 μ L reaction. The reaction included priming for 10 min at 25°C, reverse transcription for 60 min at 42°C, and inactivation for 5 min at 85°C. RT-qPCR reactions were performed with a SsoFast EvaGreen supermix using 2 μ L of input cDNA in a 20 μ L reaction. RT-qPCR primers were used at a final concentration of 1 μ M and are listed in **Table 7** below. Thermal cycling was performed using a CFX96 Touch Real-Time PCR Detection System (Bio-Rad). It included an initial step of enzyme activation for 30 sec at 95°C, followed by 40 cycles of denaturation

for 5 sec at 95°C and annealing/extension for 5 sec at 60°C. Relative mCherry, SGRP, EGFR, and EGFRvIII expression was calculated with the $\Delta\Delta C_t$ method, using TBP or GAPDH Ct to normalize signal expression.

5 **Table 7**

Primer	SEQ ID NO:	Template strand (5'-3')	Primer length	Product length
EGFRvIII Forward	43	CTGCTGGCTGCGCTCTG	17	72
EGFRvIII Reverse	44	GTGATCTGTCACCACATAATTACCTTTC	28	
EGFRwt Forward	45	TATGTCCTCATTGCCCTCAACA	22	62
EGFRwt Reverse	46	CTGATGATCTGCAGGTTTCCA	22	

Pharmacoscopy

Frozen patient single-cell suspensions were thawed and resuspended in DMEM medium supplemented with 10% FBS, 25 mM HEPES and 1% pen strep and were seeded at 8×10^3 cells per well in 50 μ L per well into clear-bottom, tissue-culture treated, CellCarrier-384 Ultra Microplates (#50-209-8071, Perkin Elmer, USA). mCherry-labeled CAR T cells were added and co-cultured with the patient single cell suspensions for 48 h at 37°C, 5% CO₂. Every CAR T cell co-incubation condition was tested with five technical replicate wells and six technical replicate wells for the PBS control. After the co-incubation period, the cells were fixed with 4% PFA, blocked with PBS containing 5% FBS, 0.1% Triton-X and 4 μ g/mL DAPI (#422801, Biolegend, USA) for 1 h at RT and stained with two staining panels: (1) Alexa Fluor 488 anti-human CD3 (1:300, #300415, Biolegend, USA) and Alexa Fluor 647 anti-human CD14 (1:300, #325612, Biolegend, USA) and (2) primary antibodies mouse anti-human nestin (1:150, #656802, Biolegend, USA) and rabbit anti-human EGFRvIII (1:150, #MA5-36216, ThermoFisher Scientific, USA) overnight at 4°C. The wells were washed, and the cells that were stained with primary antibodies were incubated with the respective secondary antibodies, Alexa Fluor 488 goat anti-mouse IgG (1:500, #A32723TR, Invitrogen, USA) and Alexa Fluor 647 goat anti-rabbit IgG (1:500, #A32733, Invitrogen, USA) for 1 h at RT in the dark. The plates were imaged with an Opera Phenix automated spinning-disk confocal microscope at 20X magnification (Perkin Elmer, USA). Single cells were segmented based on nuclear DAPI

staining with CellProfiler v2.2.0 Software. Downstream image analysis was performed with MATLAB R2021b. Marker-positive cell counts for each condition were identified based on a linear threshold of each channel, were averaged across each well and compared between CAR T cell co-incubation conditions.

5 Statistical analyses

Data analysis and visualization were performed using Excel version 16.14.1 (Microsoft) and Prism 8.4 (GraphPad). Graphs represent either group mean values $\pm$ s.d. (for in vitro experiments) or $\pm$ s.e.m. (for in vivo experiments) or individual values. For in vitro studies, statistical comparisons were made with either unpaired *t*-tests when comparing two groups or one-way ANOVA with multiple comparison corrections when comparing more than two groups. For in vivo studies, survival curves were compared with the log-rank test; tumor growth was compared with repeated-measures ANOVA and the Mann–Whitney test was used to compare two groups. $P < 0.05$ was considered statistically significant. Significance is shown with *P* or *adjusted P* values. In some Figures, *P* values are denoted with asterisks: * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; and **** $P < 0.0001$.

Results

Engineering CAR T cells that secrete an optimized SIRP γ -derived blocker of the CD47-SIRP α phagocytosis axis

Antigen escape is a significant mechanism of anti-EGFRvIII CAR T cell therapy resistance (O'Rourke DM, Nasrallah MP, Desai A, Melenhorst JJ, Mansfield K, Morrisette JJD, Martinez-Lage M, Brem S, Maloney E, Shen A, Isaacs R, Mohan S, Plesa G, Lacey SF, Navenot JM, Zheng Z, Levine BL, Okada H, June CH, Brogdon JL, Maus MV. A single dose of peripherally infused EGFRvIII-directed CAR T cells mediates antigen loss and induces adaptive resistance in patients with recurrent glioblastoma. *Sci Transl Med*. 2017 Jul 19;9(399):eaaa0984. doi: 10.1126/scitranslmed.aaa0984. PMID: 28724573; PMCID: PMC5762203.) (Fig. 1). We propose a fourth-generation CAR design, whereby anti-EGFRvIII CAR T cells constitutively release a SIRP γ -related protein (SGRP) with high affinity to CD47 (Fig. 1). The reported dissociation constant (K_D) of SIRP α binding to human CD47 is 279 nM (Weiskopf K, Ring AM, Ho CC, Volkmer JP, Levin AM, Volkmer AK, Ozkan E, Fernhoff NB,

van de Rijn M, Weissman IL, Garcia KC. Engineered SIRP α variants as immunotherapeutic adjuvants to anticancer antibodies. *Science*. 2013 Jul 5;341(6141):88-91. doi: 10.1126/science.1238856. Epub 2013 May 30. PMID: 23722425; PMCID: PMC3810306.). A SIRP α analog, CV1, recently used in CAR constructs for peripheral tumors, has a K_D of 11.1 pM (Dacek MM, Kurtz KG, Wallisch P, Pierre SA, Khayat S, Bourne CM, Gardner TJ, Vogt KC, Aquino N, Younes A, Scheinberg DA. Potentiating antibody-dependent killing of cancers with CAR T cells secreting CD47-SIRP α checkpoint blocker. *Blood*. 2023 Apr 20;141(16):2003-2015. doi: 10.1182/blood.2022016101. PMID: 36696633; PMCID: PMC10163312.). In comparison, SGRP used in the present study, has a reported K_D of 92 pM (US20160340397A1), far outcompeting endogenous human phagocyte-expressed SIRP α and SIRP α analogs in the literature. To design a CAR T cell that concomitantly targets GBM cells via EGFRvIII recognition and reprograms GAMs by blocking a major regulatory pathway of phagocytosis, we armed anti-EGFRvIII-BBz CAR T cells (3C10.BBz³³, termed aEGFRvIII CAR) with a secretable SGRP by adding an IL2 signal peptide to the SGRP sequence (**Fig. 2**), resulting in aEGFRvIII-SGRP CAR). Synthetic SGRP shares a strong identity with the endogenous SIRP γ binding domain (SIRP γ -V1) amino acid (AA) sequence (**Table 1**) and binds to human CD47 in a similar manner as competitive human and murine SIRP α (**Fig. 3**). Specifically, we generated *EF1A*-driven polycistronic constructs encoding overall identical CAR structures targeting either EGFRvIII or control antigen CD19 (FMC63.BBz³⁵; termed aCD19 CAR), a mCherry (mC) fluorescent reporter, and a secretable SGRP. The resulting polyproteins were generated by flanking T2A or P2A self-cleaving peptide sequences (**Fig. 4** and **Table 2**) and were incorporated into lentiviral expression vectors (**Fig. 5**). *EF1A*-driven polycistronic constructs encoding overall identical CAR structures targeting either EGFRvIII or control antigen CD19 (FMC63.BBz³⁵; termed aCD19 CAR), and a secretable SGRP and a secretable SGRP (without a mCherry (mC) fluorescent reporter) are shown in SEQ ID NO: 1 (nucleotide sequence of CD19 construct) and SEQ ID NO: 2 (nucleotide sequence of EGFRvIII construct) (**Table 2**). The corresponding amino acid sequences are shown in SEQ ID NO: 3 (nucleotide sequence of CD19 construct) and SEQ ID NO: 4 (nucleotide sequence of EGFRvIII construct).

CAR T cells were produced using healthy donor (HD) peripheral blood mononuclear cells (PBMCs), depleted for non-T cells and activated with a cocktail of IL2, IL7, IL15, IL21 and anti-CD3/CD28 microbeads for 48 h⁴⁴. Activated T cells were stably transduced with lentiviral supernatants and T cell cultures were expanded in the presence of IL2 for 5 to 7 days,

subsequently enriched for mCherry-expression by cell sorting, and further expanded for downstream applications (Fig. 6, 7). As intended, aCD19 and aEGFRvIII CARs showed specific binding affinity to their respective targets, but not to mismatched targets (Fig. 8) or wild type (wt) EGFR proteins (Fig. 9).

- 5 We then confirmed the presence of similar amounts of mCherry mRNA in aEGFRvIII CAR and aEGFRvIII-SGRP CAR, and SGRP mRNA specifically in aEGFRvIII-SGRP CAR T cells from four T cell donors (Fig. 10). In conditioned media from 24 h-rested, antigen-naïve, CAR T cell cultures from 2 donors, SGRP was the most differentially enriched protein in the secretome of aEGFRvIII-SGRP CAR compared to aEGFRvIII CAR, detected by LC-MS (Fig. 11, 12). The identified peptide sequences included SGRP-specific AA modifications, confirming specific SGRP detection rather than contamination with peptides of highly conserved SIRP-family proteins (Fig. 13).

CAR T cell effector function against GBM is unaffected by SGRP-mediated CD47 blockade *in vitro*

- 15 To validate CAR and CAR-SGRP T cells, we profiled surface target expression in four GBM cell lines (U251, U87, U251vIII with transgenic (tg) overexpression of EGFRvIII, BS153 with endogenous EGFRvIII expression), one Burkitt's lymphoma cell line (Raji) and one normal neural stem cell (NSC) line (NSC197). We used aCD19 CAR and aCD19-SGRP CAR as controls, and Raji as CD19⁺ target cells throughout the study (Fig. 14, left plot). We confirmed the EGFRvIII⁺ status of GBM cell lines by surface staining of U251vIII and BS153 (99.3% and 28.9%, respectively), while U251 and U87 were EGFRvIII-neutral (16.4% and 23.5%, respectively), exhibiting smaller fractions of cells EGFRvIII-expressing cells (Fig. 14, center plot). Prior to *in vitro* and *in vivo* experiments, U251vIII and BS153 were enriched for EGFRvIII by cell sorting, while U251 and U87 were enriched for their EGFRvIII⁻ fractions ensuring EGFRvIII negativity during experimental validation. Furthermore, we also confirmed CD47 overexpression in all aforementioned tumor cell lines (U251vIII: 56.5%, U251: 51.4%, U87: 53.5%, BS153: 80.6% and Raji: 58.4%), compared to normal NSCs (Fig. 14, right plot). We then established co-cultures of mCherry⁺ CAR or CAR-SGRP T cells with GBM cells expressing a nuclear-restricted EGFP (nEGFP) as a fluorescence viability reporter. A decline of the nEGFP signal in the co-cultures meant a reduction of tumor viability, as previously validated by puromycin selection (Fig. 15). Time lapse imaging revealed the specific cytotoxic capacity of aEGFRvIII CAR and aEGFRvIII-SGRP CAR against U251vIII cells (Fig. 16).

aEGFRvIII CAR also showed similar efficacy against BS153 cells (**Fig. 17**). By contrast, no measurable off-target cytotoxicity was observed against U251, an EGFRvIII-negative GBM cell line (**Fig. 18**). At various time points and effector-target (E:T) ratios, targeted CAR T cells displayed a dose-dependent effect against EGFRvIII^{ts} GBM cells (**Fig. 19**). Moreover, CAR T cell cytotoxic capacity remained unaffected by SGRP secretion in co-cultures with U251vIII (**Fig. 19**) or U251 (**Fig. 20**), as shown by the largely identical responses of aEGFRvIII CAR and aEGFRvIII-SGRP CAR in co-cultures with GBM cells in the absence of tumor-associated myeloid cells. Notably, we observed a natural enrichment of CD4⁺ T cells in CAR T cell products with our expansion protocol (**Fig. 21**).

We further examined the degranulating and cytokine-producing capacity of CAR T cells in co-cultures with GBM cell lines of varying EGFRvIII status. FC analysis of CAR T cells in 24 h co-cultures showed that targeted CAR T cells specifically expressed higher levels of CD107a in response to EGFRvIII⁺ GBM cell lines (**Fig. 22**). We also detected significantly higher concentrations of IFN γ in co-cultures of targeted CAR T cells with EGFRvIII⁺, but not with EGFRvIII⁻ GBM cell lines (**Fig. 23**).

To determine the CD47-blocking capacity of T cell-secreted SGRP compared to anti-human CD47 antibody (aCD47) *in vitro*, we pre-treated BS153 tumor cells with CAR T cell-conditioned media (aEGFRvIII-SGRP CAR vs aEGFRvIII CAR), or antibody dilutions (anti-CD47, clone B6.H12 vs. IgG1 isotype, clone MOPC-21). In a second step, BS153 cells were exposed to biotinylated SIRP α (bt-SIRP α) that bound to the remaining available CD47 on tumor cells. Cell-bound bt-SIRP α was detected by labeling with fluorescence-conjugated streptavidin (SA). aCD47 treatment induced a potent blockade of the CD47-SIRP α interaction (**Fig. 24**, left dot-plot) whereas treatment with aEGFRvIII-SGRP CAR-conditioned medium slightly impaired this interaction (**Fig. 24**, right dot-plot). Since CD47 is ubiquitously expressed²¹ and SGRP has a very high affinity to CD47, we hypothesize that SGRP may be partially captured in autocrine or paracrine loops by CAR T cells. Consequently, the amount of available SGRP collected from cell culture supernatants may be reduced, contributing to the apparent lower efficacy of SGRP-mediated CD47 blockade. Importantly, CD47 expression is required for CAR T and peripheral T cell survival, as shown by studies using CD47 mutant T cells (Beckett AN, Chockley P, Pruett-Miller SM, Nguyen P, Vogel P, Sheppard H, Krenciute G, Gottschalk S, DeRenzo C. CD47 expression is critical for CAR T-cell survival in vivo. *J Immunother Cancer*. 2023 Mar;11(3):e005857. doi: 10.1136/jitc-2022-005857. PMID: 36918226; PMCID: PMC10016274; Komori S, Saito Y, Nishimura T, Respatika D, Endoh H, Yoshida H, Sugihara

R, Iida-Norita R, Afroj T, Takai T, Oduori OS, Nitta E, Kotani T, Murata Y, Kaneko Y, Nitta R, Ohnishi H, Matozaki T. CD47 promotes peripheral T cell survival by preventing dendritic cell-mediated T cell necroptosis. *Proc Natl Acad Sci U S A*. 2023 Aug 15;120(33):e2304943120. doi: 10.1073/pnas.2304943120. Epub 2023 Aug 7. PMID: 37549290; PMCID: PMC10440595.). Despite constitutive secretion of SGRP, CD47 surface expression remained unchanged on aEGFRvIII-SGRP CAR T cells (**Fig. 25**).

In an attempt to quantify the effects of CAR targeting and SGRP-mediated macrophage modulation *in vitro*, we performed a 3 h-phagocytosis assay combining a mosaic GBM model consisting of EGFRvIII⁻ U87, EGFRvIII⁺ U251vIII cells with donor-matched macrophages and CAR T cells in a 1:1:1:1 ratio (**Fig. 26**). In this short-term phagocytosis assay, we assessed: (1) macrophage polarization, by human CD163, CD206, HLA-DR, and CD86; (2) phagocytic marker expression, by human CD209; (3) antigen presentation, by human SIGLEC-1, and (4) tumor cell phagocytosis. However, in this *in vitro* system, we found no significant differences between conditions involving conventional CARs and SGRP-secreting CARs.

Nevertheless, *in vitro* models overlook crucial cell interactions in the context of the GBM TME, namely the impact of other phagocytes, e.g., microglia and dendritic cells (DCs), on *in vivo* tumor clearance. Thus, understanding the effect of T cell-secreted SGRP requires a GAM-infiltrated *in vivo* GBM model.

Anti-EGFRvIII-SGRP CAR combination therapy leads to significant survival benefit in an aggressive EGFRvIII-mosaic GBM xenograft model

Many aEGFRvIII CAR preclinical studies show effective cytotoxicity *in vitro* and *in vivo* but fail to model the heterogeneity of human GBM^{45,46}. Although preclinical aEGFRvIII CAR monotherapy has demonstrated efficacy in overall tumor control, this strategy translates poorly in the clinical setting¹⁰. To establish a baseline for combination therapies, we first tested the efficacy of aEGFRvIII CAR and aCD47 monotherapies in an EGFRvIII⁺ GBM xenograft model. For these experiments, U251vIII cells were enriched for EGFRvIII by cell sorting and orthotopically implanted in NSG mice. Tumor-bearing mice were treated with 2 doses, 7 and 14 days after tumor implantation. Clinical scores and tumor growth were monitored weekly by bioluminescence imaging until a maximum of 13 weeks (90 days) or until animals reached the humane endpoint (**Fig. 27**). The therapeutic scheme included i.t. treatments of mouse IgG1 (MOPC-21; Isotype), anti-human CD47 (B6.H12; aCD47), aCD19 CAR and aEGFRvIII CAR (**Fig. 28**). Overall survival analysis (**Fig. 29**) showed that local aCD47 monotherapy failed to

improve survival (median survival: 34.5 days) when compared to the Isotype antibody control group (median survival: 30.5 days). Conversely, aEGFRvIII CAR treatment (median survival: 65.5 days) led to 40% survival by day 90 after tumor implantation, a stark contrast to aCD19 CAR control (median survival: 29 days). Bioluminescence assessment confirmed an overall lower tumor burden in the aEGFRvIII CAR treatment group (**Fig. 30**).

To assess the efficacy of CAR-SGRP combination therapy against GBM, we established a preclinical EGFRvIII-mosaic GBM model with orthotopic tumor implantation and intracranial therapy administration in mice. First, we enriched GBM cell lines for their EGFRvIII⁺ (BS153 and U251vIII) or EGFRvIII⁻ (U87 and U251) cell subsets based on cell surface staining followed by cell sorting and then assessed their engraftment in NSG mice. We selected U251vIII and U87 for their consistent engraftment and fast progression to the onset of clinical signs. Thereafter, we co-injected U251vIII and U87 intracranially in a 1:1 ratio as single-cell suspensions and allowed them to settle for 7 days. All animals received 2 intratumoral (i.t.) treatment doses (5×10^5 CAR T cells or 5 μ g of antibody) 7 and 14 d after tumor implantation.

For better equipoise in comparing antibody-based CD47 blockade (aCD47) with constitutive CAR-mediated SGRP release, animals receiving aCD47 as monotherapy or in combination with aEGFRvIII CARs (aEGFRvIII CAR + aCD47) received 4 additional i.p. antibody doses (100 μ g of antibody) in the 2 weeks following the i.t. treatment regimen. To monitor U251vIII and U87 cell populations separately *in vivo*, we differentially labeled U251vIII with NanoLuciferase (NLuc) and U87 with Luciferase2 (Luc2) bioluminescence reporters using lentiviral transgene delivery. The experiment timeline and therapeutic setup are illustrated in **Fig. 31, 32**. Overall survival analysis (**Fig. 33**) showed that untreated animals in the Vehicle control group had a median survival of 31.5 days. Surprisingly, combined locoregional/systemic aCD47 monotherapy failed to improve survival (median survival: 34 days) when compared to an Isotype antibody control group (median survival: 32 days). We reasoned that despite combined local/systemic administration, the aCD47 treatment regimen did not maintain a sufficient blockade of CD47 to levels that could induce persistent GAM phagocytic activity. Notably, aggressive tumor models in the NSG context are difficult to treat using even higher doses of aCD47 antibodies²³. As expected, treatment with non-GBM-targeted aCD19 CARs resulted in a minor, non-significant survival benefit (median survival: 36 days). Similarly, survival after aCD19-SGRP CAR therapy (median survival: 40 days) suggested that peripherally-released SGRP without direct CAR-mediated targeting was insufficient to eliminate orthotopic xenograft tumors. As expected, aEGFRvIII CAR monotherapy was less efficacious in the

EGFRvIII-mosaic tumor model compared to the homogenous EGFRvIII⁺ tumor model. Nonetheless, aEGFRvIII CAR was able to eliminate GBM in 20% of treated animals (**Fig. 33**; tumor-free survival analysis in **Fig. 34**). However, the combination of aEGFRvIII CAR + aCD47 failed to improve survival beyond what we had observed with aEGFRvIII CAR monotherapy (20%). This pointed towards a potential elimination of grafted CD47⁺ CAR T cells via phagocytosis or insufficient continuous dosing of local anti-CD47 antibodies resulting in failure of additive efficacy in this model. Notably, clone B6.H12 was used in the experiments because of the limited availability of clone Hu5F9-G4⁴⁷. In contrast, aEGFRvIII-SGRP CAR combination therapy was highly potent in this challenging GBM model. A near complete therapeutic response was observed with 94.7% overall survival (**Fig. 33**) and 63.2% tumor-free survival (**Fig. 34**).

Differential bioluminescence monitoring of either U251vIII-NLuc (**Fig. 35**) or U87-Luc2 (**Fig. 36**) confirmed that a majority of EGFRvIII⁺ tumors were cleared after aEGFRvIII-SGRP CAR treatment. Bioluminescence plots of individual treatment groups are shown in **Fig. 37**. A direct bioluminescence imaging comparison and quantification of tumor burden of EGFRvIII⁺ and EGFRvIII⁻ tumor fractions in aEGFRvIII CAR, aEGFRvIII CAR + aCD47 and aEGFRvIII-SGRP CAR treatment groups on a representative time point (week 7 post tumor implantation) are shown in **Fig. 38, 39**.

To further elucidate the mechanism of aEGFRvIII-SGRP CAR-mediated tumor clearance, we performed an *in vivo* experiment with orthotopic EGFRvIII⁻ (U87) or EGFRvIII-mosaic (U87+U251vIII) tumor implantation and treatment with aEGFRvIII-SGRP or aCD19-SGRP CAR (**Fig. 40**). Three days after the treatment, we collected the tumor-implanted brain hemisphere to characterize condition-specific immune cell abundance (cluster definition is shown in **Fig. 41**). Treatment with aEGFRvIII-SGRP CAR resulted in higher T cell frequencies in the tumor-implanted brain hemisphere compared to aCD19-SGRP CAR, regardless of tumor type (**Fig. 42**). Notably, aEGFRvIII-SGRP CAR treatment led to a higher influx of neutrophils, moMacs, and monocyte-derived DCs (moDCs)/conventional type 1 DCs (cDC1s) and an increase in CD25^{hi}CD4⁺ CAR T cells exclusively in EGFRvIII-mosaic tumors (**Fig. 42**). This suggests that the benefit of SGRP-mediated CD47 blockade combined with CAR-mediated tumor killing relies on CAR target priming, leading to CAR T cell activation and persistence and enhanced myeloid cell infiltration to the brain.

Tumor rechallenge of aEGFRvIII-SGRP CAR-cured mice in the GBM cohort of **Fig. 33, 34** resulted in prolonged survival compared to the historic vehicle control group (**Fig. 43**).

Interestingly, tumor monitoring after rechallenge revealed preferential control of the EGFRvIII⁺ fraction (**Fig. 44**). Residual aEGFRvIII-SGRP CAR T cells in tumor-free brains 90 days post tumor implantation were few and mostly found at the brain periphery (**Fig. 45, 46**), suggesting a potential role for host innate immune cell priming, or lasting TME alterations from aEGFRvIII-SGRP CAR treatment, resulting in reduced tumor engraftment.

Anti-EGFRvIII-SGRP CAR combination therapy induces a myeloid-driven inflammatory response in the plasma of GBM-bearing mice

To elucidate potential mechanisms of innate immune activation upon locoregional CAR T cell GBM treatments, we conducted a human immuno-oncology-targeted proteomic analysis of plasma samples collected 24 h after the second CAR treatment (day 15 post tumor implantation). Using an Olink Immuno-Oncology panel, we analyzed a total of 92 proteins on 2 complementary datasets encompassing 54 plasma samples from individual mice (**Fig. 47**). Plasma from sex- and age-matched healthy control mice was used to determine baseline protein expression. We first visualized the distribution of all assessed markers with a principal component analysis which showed that aEGFRvIII CAR- and aEGFRvIII-SGRP CAR-treated plasma segregated the furthest from vehicle controls (**Fig. 48**). To examine the peripheral immune response associated with the superior aEGFRvIII-SGRP CAR treatment, we performed differential expression analysis against aEGFRvIII CAR monotherapy (**Fig. 49**), which showed significant enrichment of CCL3 and IL13 in aEGFRvIII-SGRP CAR-treated plasma (adj. $P < 0.001$ and adj. $P < 0.05$, respectively). Conversely, CD27 was significantly increased in aEGFRvIII CAR-treated plasma (adj. $P < 0.05$). To identify additional immune markers associated with aEGFRvIII CAR +/- SGRP, we looked for differences between SGRP-secreting CARs (aEGFRvIII-SGRP CAR vs aCD19-SGRP CAR) or non-SGRP-secreting CARs (aEGFRvIII CAR vs aCD19 CAR) across all 92 markers (**Fig. 50**). This analysis revealed significant differences in the expression of CCL3, CXCL1, CXCL8, GZMA, TNFRSF21, TNFSF14 and VEGFA between SGRP-secreting CAR T cell treatments. Moreover, it showed significant differences in CD5, CXCL1, GZMA, IFNG, KLRD1, PGF and TNFSF14 expression between non-SGRP-secreting CAR T cell monotherapies. Collectively, these analyses identified CCL3 as the key plasma marker associated with aEGFRvIII-SGRP CAR treatment response out of all 92 markers studied. T cell-activation markers including GZMA

and IFNG were found to be similarly highly expressed in aEGFRvIII CAR and aEGFRvIII-SGRP CAR plasma.

The myeloid chemoattractant CCL3 emerged as a significantly upregulated protein in the plasma of mice treated with aEGFRvIII-SGRP CAR, potentially shaping peripheral responses to local antitumor therapy, promoting antigen presentation in tumor-draining lymph nodes or inducing T cell proliferation and differentiation (Allen F, Rauhe P, Askew D, Tong AA, Nthale J, Eid S, Myers JT, Tong C, Huang AY. CCL3 Enhances Antitumor Immune Priming in the Lymph Node via IFN γ with Dependency on Natural Killer Cells. *Front Immunol.* 2017 Oct 23;8:1390. doi: 10.3389/fimmu.2017.01390. PMID: 29109732; PMCID: PMC5660298.). To investigate whether CCL3 plays a crucial role in aEGFRvIII-SGRP CAR efficacy, we administered a CCL3-neutralizing antibody i.p. with concomitant i.t. CAR T cell therapy (**Fig. 51**). Control groups included an isotype antibody and PBS-injected animals. Despite CCL3 blockade, we observed no reduction in the efficacy of aEGFRvIII-SGRP CAR T cells compared to control groups (**Fig. 52**). The slight reduction in survival across all treatment/control groups compared to aEGFRvIII-SGRP CAR treatment in **Fig. 33** could be attributed to overall lower activity of the T cell batch or the significant increase in frequency and duration of animal interventions.

Anti-EGFRvIII-SGRP CAR T cells elicit an early rejection of EGFRvIII-mosaic GBM without overt signs of toxicity

To better characterize the remarkable response of GBM xenografts to aEGFRvIII-SGRP CAR combination therapy, we collected spleen and brain tissue at intermediate time points and performed a histological and immunofluorescence workup of GBM-brains from animals at an intermediate treatment time point (7 days after the 2nd treatment dose, 21 days post tumor implantation; **Fig. 53**). Tumor size assessment in the 7 experimental conditions confirmed the persistence of intracerebral GBM xenografts in all but the aEGFRvIII-SGRP CAR treated animals (**Fig. 54-56**). Summed tumor areas calculated from brain histological sections of day 21 post tumor implantation showed the lowest tumor burden in anti-EGFRvIII and anti-EGFRvIII SGRP CAR T cell treatment groups (**Fig. 56**). Only minute tumor remnants at this time point in aEGFRvIII-SGRP treated animals could be discovered, precluding us from a detailed multidimensional analysis of the iTME in this condition despite whole-brain sectioning

and staining. However, assessment of CAR T cell persistence was possible in all conditions using anti-human CD3 IHC (**Fig. 57**).

Multiplexed immunofluorescence focussing on GAMs (murine Iba1, Tmem119, CD206), astrocytes (murine GFAP), CAR T cells (human CD3) and tumor surface/proliferation markers (human EGFRvIII, Ki67) confirmed the persistence of CAR T cells in aCD19 CAR, aEGFRvIII CAR and aEGFRvIII-CAR + aCD47 treated animals, albeit with a comparatively reduced number of CAR T cells in aCD19 CAR and aEGFRvIII-CAR + aCD47 tumor burdened brains (**Fig. 58**). This underscores the importance of the presence of tumor antigen in case of aCD19 CAR treatments, and hints at a potential CAR phagocytic effect in case of additional anti-CD47 treatment. The quantification of EGFRvIII surface expression confirmed selective/preferential eradication of EGFRvIII⁺ tumor cells after any aEGFRvIII-specific treatment, accompanied by the persistence of EGFRvIII⁻ tumor cells. Analysis of GAM polarization markers revealed a reduction of CD206⁺ cells in aCD47 treated animals in line with published data²⁴, while overall macrophage influx by Iba1⁺ cells tended to be higher in aEGFRvIII-CAR + aCD47 treated animals (**Fig. 58**). Alternatively, anti-mouse CD68 IHC of tumor-burdened brains showed a tendency towards a more pronounced proinflammatory macrophage activation in aEGFRvIII-SGRP-treated mice over the other CAR-treated conditions (**Fig. 59**).

Despite its efficacy, aEGFRvIII-SGRP CAR showed no signs of systemic toxicity, as evidenced by the absence of splenomegaly after two rounds of therapy (**Fig. 60**), and the normal weight fluctuation throughout the experiment (**Fig. 61**). We observed a sharp drop in the weight of a few animals after the first aEGFRvIII-SGRP CAR treatment, suggestive of acute inflammatory events that resolve on their own after the second treatment (**Fig. 61**, right plot). Moreover, we examined CAR hematotoxicity parameters (Rejeski K, Perez A, Sesques P, Hoster E, Berger C, Jentzsch L, Mougiakakos D, Frölich L, Ackermann J, Bücklein V, Blumenberg V, Schmidt C, Jallades L, Fehse B, Faul C, Karschnia P, Weigert O, Dreyling M, Locke FL, von Bergwelt-Baildon M, Mackensen A, Bethge W, Ayuk F, Bachy E, Salles G, Jain MD, Subklewe M. CAR-HEMATOTOX: a model for CAR T-cell-related hematologic toxicity in relapsed/refractory large B-cell lymphoma. *Blood*. 2021 Dec 16;138(24):2499-2513. doi: 10.1182/blood.2020010543. PMID: 34166502; PMCID: PMC8893508.: plasma IL6 and CRP, erythrocyte (RBC), platelet (PLT), and neutrophil (NEUT) counts, but found no indication of increased inflammation, anemia, thrombocytopenia or neutropenia (**Fig. 62-64**). The increase in neutrophil counts in the vehicle group is likely linked to a higher tumor burden in these

animals from day 13 on (**Fig. 64**, bottom plot). Locally, we did not detect differences in brain myelination after therapy, an additional indication of safety, particularly in a model of intracerebral CAR T cell infusion (**Fig. 65, 66**).

CAR treatments induce a proinflammatory GAM conversion, and SGRP 5 improves GAM-mediated tumor phagocytosis

To further elucidate the differential effects of aEGFRvIII-SGRP CAR treatments on the iTME during treatment, we performed a focused spectral flow cytometry analysis of myeloid cells shortly after i.t. treatment with CAR T cells. We designed and optimized a panel consisting of 17 myeloid-specific surface markers and 3 intracellular markers including the detection of
10 endogenous fluorescence by tumor cells and CAR T cells (**Fig. 67**). In comparison to vehicle treatment, both aEGFRvIII and EGFRvIII-SGRP triggered a rapid influx of CD45⁺ cells (**Fig. 68**). We confirmed the presence of mCherry⁺ CAR T cells and mTagBFP2⁺EGFRvIII⁺ tumor cells in the CD45⁺ gate, while specifically gating for murine CD45⁺ cells, and both CAR treatments reduced tumor cell burden at this stage (**Fig. 69**). We then specifically elaborated
15 on potential early time point differences between tumor-associated microglia and macrophage subsets in the host NSG mice. Using conventional analysis, two main microglia populations arose upon CAR T cell treatment: ‘resting’ microglia were characterized by P2YR12^{hi}F4/80^{int} while activated microglia were defined as P2YR12^{int}F4/80^{hi}. Other influxing myeloid cells consisted of P2YR12^{neg}F4/80^{int} monocyte-derived macrophages and P2YR12^{neg}F4/80^{neg}
20 myeloid cells, most of which were determined to be Ly6g⁺ neutrophils (**Fig. 70, 71**). Activated microglia expressed CD11c, MHCII, CD86 and Siglec-H, associated with a proinflammatory phenotype (**Fig. 72, 73**). We next assessed the intracellular signal intensity of eBFP as a surrogate for tumor cell phagocytosis. aEGFRvIII-SGRP treated tumor-associated activated microglia, microglia and macrophages displayed a significantly increased eBFP-signal intensity
25 compared to aEGFRvIII CAR T cell suggesting SGRP-mediated phagocytosis of BFP⁺ tumor cells in the early treatment phase (**Fig. 73**). Furthermore, aEGFRvIII-SGRP CAR-treated activated microglia displayed increased intracellular TNFa measurements by trend compared to EGFRvIII-CARs alone (**Fig. 74**).

We alternatively performed a multiparametric analysis combined with FlowSOM
30 metaclustering of GAMs. Initial clustering identified 15 metaclusters (TSNEs/UMAP in **Fig. 75**) that were characterized by differential lineage marker expression (**Fig. 76, 77**). Cluster frequencies between aEGFRvIII-CAR and aEGFRvIII-SGRP CAR treatment conditions were

not different (**Fig. 78**). Subsequently, these clusters were merged into 8 main subsets including microglia, activated microglia, monocyte-derived macrophages (MoMacs), monocyte-derived dendritic cells (MoDCs), monocytes, plasmacytoid dendritic cells (pDCs), neutrophils, and unknowns (TSNE plots and UMAP in **Fig. 79, 80**), which exhibited similar population frequencies among conditions (**Fig. 81, 82**). We next focussed on the overall most abundant phagocyte population, microglia, which mainly expressed P2RY12, F4/80 in varying levels as seen in the conventional analysis (**Fig. 83**). Activated microglia expressed high levels of CD11c (**Fig. 84**). Further subclustering specifically on microglia revealed 3 major populations (resting, MHCII^{high}, and activated microglia, TSNE in **Fig. 85**), characterized by marker profiles outlined in **Fig. 86**). While population frequencies between aEGFRvIII and aEGFRvIII-SGRP CAR treated animals were not different (**Fig. 87**), differential abundance analysis among conditions showed significantly increased XCR1-expression in activated microglia of aEGFRvIII-SGRP CAR treated brain tumors (expression heatmap in **Fig. 88**, individual heatmaps of all assessed markers in **Fig. 89**). XCR1 is described to be a specific classical dendritic cell marker involved in antigen presentation⁴⁸, however, in our dataset, the cluster ‘activated microglia’ is characterized by P2RY12^{int}, SiglecH^{high}, MHCII^{high}, F480^{high}, which most probably classifies them as of microglial origin.

aEGFRvIII CARs outperform aCD19 CARs in co-cultures with patient-derived single cell suspensions

To demonstrate efficacy of aEGFRvIII-SGRP in a translational setting, we co-cultured CAR T cells with EGFRvIII⁺ GBM patient-derived single cell suspensions, and performed multidimensional pharmacoscopy analysis in 5 patients (**Fig. 90**). CAR T cells and patient-derived GBM cells were not matched in this setup, raising the potential of alloimmunological phenomena. EGFRvIII status was determined by a custom qPCR assay (**Fig. 91**). Readout included assessment of Nestin⁺ tumor cells and EGFRvIII⁺ tumor cells, as well as CD14⁺ immune cell counts (**Fig. 92**). In this setting, a direct benefit of aEGFRvIII-SGRP over EGFRvIII-CARs was not appreciated. As expected, all EGFRvIII targeted CARs significantly reduced the number of EGFRvIII⁺ cells compared to all CD19-targeted control CARs (**Fig. 93**). Moreover, aCD19-SGRP CARs induced moderate antigen-independent killing of both EGFRvIII⁺ and Nestin⁺ tumor cells, presumably due to a potential SGRP-mediated effect or constitutive secretion of IFN γ (**Fig. 94**). The fact that no differential effect between aEGFRvIII and aEGFRvIII-SGRP was detected could be explained by the use of frozen single-cell

suspensions with reduced overall viability and subsequent functional deficits in myeloid cell composition.

Anti-CD19-SGRP CAR T cells have superior efficacy over conventional aCD19 CAR T cells in a peripheral lymphoma xenograft model

5 To demonstrate a potential benefit of iTME-targeted secretion of SGRP in another solid tumor context, we further assessed the capacity of aCD19-SGRP CAR T cells against CD19⁺ lymphoma xenografts. In contrast to locoregional injection as the preferred application route in brain tumors, we considered a systemic approach mirroring CAR T cell treatments currently performed against leukemia⁴⁹. CD19⁺ Raji cells were injected s.c. in the right flank of NSG
10 mice followed by a single dose of systemic CAR T cell infusion 3 days after tumor implantation. Animals were monitored for survival analysis and volumetric tumor burden quantification (**Fig. 95**). The therapeutic setup consisted of targeted CAR T cells (aCD19 CAR or aCD19-SGRP CAR) or non-targeted CAR T cells (aEGFRvIII CAR or aEGFRvIII-SGRP CAR) serving as controls (**Fig. 96**). As expected, all aCD19 CAR-treated animals had a significant survival
15 benefit compared to either Vehicle or non-targeted CAR controls (**Fig. 97**). Strikingly, aCD19-SGRP CAR treatment resulted in the longest survival benefit (20% overall survival) with two cured animals (undetectable tumor), a significantly superior response compared to conventional aCD19 CAR application ($P = 0.012$; **Fig. 97, 98**). Thus, the contribution of SGRP-mediated innate immune modulation might be of relevance and clinical applicability in other, non-
20 cerebral cancer entities, depending on efficient CAR T cell homing to the respective tumor site.

Conclusion

In conclusion, we showed significant synergy of aEGFRvIII-SGRP CARs against orthotopic and patient-derived glioblastoma models, credentialing this approach for a first-in-human clinical trial. Additionally, this work highlights the importance of paracrine, high-affinity CD47
25 blockade to relieve macrophage-mediated immunosuppression, which might not be achievable by systemic or episodic local application of anti-CD47 antibodies in the context of aggressive solid tumors. In summary, this work represents one of the first examples of GBM targeting by adoptive T cells with concurrent local paracrine GAM modulation in a single therapeutic modality.

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Claims

1. A polynucleotide molecule comprising a nucleotide sequence encoding a chimeric antigen receptor (CAR) or a fragment thereof i) comprising an extracellular domain comprising an antigen-binding region which binds to epidermal growth factor receptor variant III (EGFRvIII); or ii) comprising an extracellular domain comprising an antigen-binding region which binds to CD19; wherein the nucleotide sequence encoding the CAR is operably linked to a promoter and wherein the polynucleotide molecule further comprises a nucleotide sequence encoding a heterologous signal peptide fused to a signal regulatory protein gamma (SIRP γ)-related protein (SGRP).
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2. The polynucleotide molecule of claim 1, wherein the polynucleotide molecule further comprises a nucleotide sequence encoding a self-cleaving peptide.
3. The polynucleotide molecule of claim 2, wherein the polynucleotide molecule comprises in the following order from the 5' to the 3' end:
15
a) a promoter;
b) a nucleotide sequence encoding a chimeric antigen receptor (CAR) or a fragment thereof i) comprising an extracellular domain comprising an antigen binding region which binds to epidermal growth factor receptor variant III (EGFRvIII); or ii) comprising an extracellular domain comprising an antigen binding region which binds to CD19; wherein the nucleotide sequence encoding the chimeric antigen receptor (CAR) or a fragment thereof is operably linked to the promoter of a);
20
c) a nucleotide sequence encoding a self-cleaving peptide; and
d) a nucleotide sequence encoding a heterologous signal peptide fused to a signal regulatory protein gamma (SIRP γ)-related protein (SGRP).
25
4. The polynucleotide molecule of any one of claims 1-3, wherein the heterologous signal peptide fused to a signal regulatory protein gamma (SIRP γ)-related protein (SGRP) is a signal peptide selected from the group consisting of interleukin 2 (IL-2) signal peptide, interleukin 4 (IL-4) signal peptide, interleukin 9 (IL-9) signal peptide and interferon gamma (IFN γ) signal peptide, preferably selected from the group consisting of human
30

IL-2 signal peptide, human IL-4 signal peptide, human IL-9 signal peptide and human IFN γ signal peptide.

- 5 5. The polynucleotide molecule of any one of claims 1-3, wherein the heterologous signal peptide fused to a signal regulatory protein gamma (SIRP γ)-related protein (SGRP) is a human interleukin 2 (IL-2) signal peptide.
- 10 6. The polynucleotide molecule of any one of claims 1-5, wherein the heterologous signal peptide is fused to the N-terminal region of the signal regulatory protein gamma (SIRP γ)-related protein (SGRP).
- 15 7. The polynucleotide molecule of any one of claims 1-6, wherein the promoter which is operably linked to the nucleotide sequence encoding the chimeric antigen receptor (CAR) or a fragment thereof is the elongation factor 1 alpha (EF1A) promoter or the elongation factor 1 alpha short (EFS) promoter, preferably the EF1A promoter.
- 20 8. The polynucleotide molecule of any one of claims 1-7, wherein the chimeric antigen receptor (CAR) or a fragment thereof comprises i) an extracellular domain comprising an antigen binding region which binds to epidermal growth factor receptor variant III (EGFRvIII); or ii) an extracellular domain comprising an antigen binding region which binds to CD19; wherein the CAR or a fragment thereof further comprises a CD8 α leader, CD8 α hinge and transmembrane domains, a TNF receptor superfamily member 9 (4-1BB) costimulatory domain and a CD3 ζ signaling domain.
- 25 9. The polynucleotide molecule of claim 8, wherein i) the extracellular domain comprising an antigen binding region which binds to epidermal growth factor receptor variant III (EGFRvIII) is a single-chain variable fragment (scFv); or ii) the extracellular domain comprising an antigen binding region which binds to CD19 is a single-chain variable fragment (scFv).
- 30 10. The polynucleotide molecule of any one of claims 1-9, comprising the sequence as shown in SEQ ID NO: 1 or the sequence as shown in SEQ ID NO: 2.

11. An amino sequence comprising a chimeric antigen receptor (CAR) or a fragment thereof comprising an extracellular domain comprising an antigen-binding region which binds to epidermal growth factor receptor variant III (EGFRvIII); wherein the nucleotide sequence encoding the chimeric antigen receptor (CAR) is operably linked to a promoter and wherein the polynucleotide molecule further comprises a nucleotide sequence
5 encoding a heterologous signal peptide fused to a signal regulatory protein gamma (SIRP γ)-related protein (SGRP) comprising the sequence as shown in SEQ ID NO: 3.
12. An amino sequence comprising a chimeric antigen receptor (CAR) or a fragment thereof comprising an extracellular domain comprising an antigen-binding region which binds
10 to CD19; wherein the nucleotide sequence encoding the CAR is operably linked to a promoter and wherein the polynucleotide molecule further comprises a nucleotide sequence encoding a heterologous signal peptide fused to a signal regulatory protein gamma (SIRP γ)-related protein (SGRP) comprising the sequence as shown in SEQ ID
15 NO: 4.
13. A construct comprising the polynucleotide molecule of any one of claims 1-10.
14. A chimeric antigen receptor (CAR)-T cell comprising T cells expressing the
20 polynucleotide molecule of any one of claims 1-10 and/or the construct of claim 13.
15. The chimeric antigen receptor (CAR)-T cell of claim 14, for use in a method for the treatment of cancer in a subject suffering from an epidermal growth factor receptor (EGFR)-associated cancer or suffering from a CD19-associated cancer, the method comprising administering to the subject a therapeutically effective amount of a chimeric
25 antigen receptor (CAR)-T cell of claim 14.
- 30