Multifunctional mRNA-Based CAR T Cells Display Promising Antitumor Activity Against Glioblastoma

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ABSTRACT

Purpose: Most chimeric antigen receptor (CAR) T-cell strategies against glioblastoma have demonstrated only modest therapeutic activity and are based on persistent gene modification strategies that have limited transgene capacity, long manufacturing processes, and the risk for uncontrollable off-tumor toxicities. mRNA-based T-cell modifications are an emerging safe, rapid, and cost-effective alternative to overcome these challenges, but are underexplored against glioblastoma.

Experimental Design: We generated mouse and human mRNA-based multifunctional T cells coexpressing a multitargeting CAR based on the natural killer group 2D (NKG2D) receptor and the proinflammatory cytokines IL12 and IFNα2 and assessed their antiglioma activity *in vitro* and *in vivo*.

Results: Compared with T cells that either expressed the CAR or cytokines alone, multifunctional CAR T cells demonstrated increased antiglioma activity *in vitro* and *in vivo* in three orthotopic immunocompetent mouse glioma models without signs of toxicity. Mechanistically, the coexpression of IL12 and IFNα2 in addition to the CAR promoted a proinflammatory tumor microenvironment and reduced T-cell exhaustion as demonstrated by *ex vivo* immune phenotyping, cytokine profiling, and RNA sequencing. The translational potential was demonstrated by image-based single-cell analyses of mRNA-modified T cells in patient glioblastoma samples with a complex cellular microenvironment. This revealed strong antiglioma activity of human mRNA-based multifunctional NKG2D CAR T cells coexpressing IL12 and IFNα2 whereas T cells that expressed either the CAR or cytokines alone did not demonstrate comparable antiglioma activity.

Conclusions: These data provide a robust rationale for future clinical studies with mRNA-based multifunctional CAR T cells to treat malignant brain tumors.

Introduction

Glioblastoma is the most common and most aggressive primary brain tumor in adults (1). It is an inevitably fatal disease with an urgent need for more effective therapies than the current standard of care comprising surgery followed by radiochemotherapy with temozolomide (2). Chimeric antigen receptor (CAR) T-cell therapy has led to impressive clinical responses in hematologic malignancies (3, 4) and is also explored against glioblastoma. However, apart from a single case report (5), the antitumor activity of CAR T cells in patients with glioblastoma has only been modest (6, 7). Challenges in current clinical trials with CAR T cells against glioblastoma are heterogeneously expressed single-target antigens such as EGFR variant III (EGFRvIII), epidermal growth factor 2 (Her2), or IL13 receptor alpha 2 (IL13Rα2) as well as an immunosuppressive microenvironment averting CAR T-cell activity. Multitargeting strategies based on unconventional CAR designs such as natural killer group 2D (NKG2D) receptor-based CAR T cells (8, 9) or tandem CARs (10) based on multiple single-chain variable fragments (scFV) as well as cytokine-armored CAR constructs that coexpress a CAR and proinflammatory cytokines such as IL12 (11) or type I IFN (12) are promising emerging strategies to overcome these challenges.

However, the majority of CAR T-cell approaches against glioblastoma use retroviral vectors or nonviral transposon-transposase systems to stably integrate transgenes encoding the CAR and potentially also cytokines. These enable long-term expression but have limitations and safety concerns such as a limited transgene capacity, long production processes with the risk of treatment delays, the risk of genomic alterations that could lead to malignant transformation of T-cell clones, and persistent CAR and/or cytokine expression with the risk for off-tumor toxicities (13, 14). Consequently, regulatory hurdles associated with genetically engineered cell therapies are strict, which delays the clinical translation of innovative CAR T-cell strategies against glioblastoma (15). RNA-modified T cells are an emerging alternative. The transient nature and high transfection efficiency of mRNA-based modifications allow for the generation of multifunctional T cells that express the CAR and cytokines with a better safety profile than stably engineered cells (16). So far, the only mRNA-based CAR T-cell approach against glioblastoma was an *in vitro* study showing cytolytic activity of human T cells electroporated with mRNA encoding a CAR-targeting EGFR against long-term cultured human glioblastoma cell lines (17)

Here, we provide a preclinical evaluation of multifunctional mRNA-based CAR T cells that express a multitargeting CAR based on the

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Clin Cancer Res 2022;28:4747–56

doi: 10.1158/1078-0432.CCR-21-4384

Translational Relevance

Current chimeric antigen receptor (CAR) T-cell approaches against glioblastoma are based on persistent gene modification strategies that are associated with regulatory hurdles. This limits the timely translation of innovative CAR T-cell approaches against this fatal disease. In this work, we provide a comprehensive preclinical and translational characterization of mRNA-based multifunctional CAR T cells against glioblastoma using orthotopic immunocompetent glioma mouse models and an innovative profiling platform to functionally characterize mRNA-modified T cells in complex glioblastoma patient samples. This provides a basis and strong rationale for future clinical studies using mRNA-based multifunctional CAR T cells to treat malignant brain tumors.

NKG2D receptor and two proinflammatory cytokines in immunocompetent orthotopic mouse models of glioma and translate this therapy to glioblastoma patient samples with a complex cellular microenvironment.

Materials and Methods

Culture of glioma cell lines and murine or human T cells

GL-261 cells were obtained from the NCI, SMA-560 cells were obtained from Dr. D. Bigner (Duke University Medical Center, Durham, North Carolina), and CT-2A cells were purchased from Millipore. Glioma cell lines were cultured as described previously (8) and regularly tested negative for *Mycoplasma* by PCR. Murine T cells derived from splenocytes or human T cells derived from peripheral blood mononuclear cells (PBMC) were cultured in RPMI1640 (Gibco Life Technologies) supplemented with 10% FCS, 2 mmol/L L-glutamine (both purchased from Gibco), 100 U/mL penicillin-streptomycin (Sigma-Aldrich), and 50 U/mL murine or human IL2 (both PeproTech). For murine T cells, 55 μmol/L 2-mercaptoethanol (Gibco) was added.

In vitro transcription of mRNA

The murine and human NKG2D-based CAR constructs have been described previously (18, 19). The mRNAs encoding for the murine or human NKG2D CAR or mIFNα2 were generated by *in vitro* transcription at the mRNA platform of Zurich as described previously (20) and mRNAs encoding mIL12, hIL12, und hIFNα2 were obtained from BioNTech. The functionality of synthetic mRNAs was confirmed by transfection of lymphocytes and subsequent detection of the respective protein by flow cytometry.

Generation of multifunctional CAR T cells

For the generation of murine multifunctional CAR T cells, splenocytes from C57BL/6 mice were activated for 48 hours using immobilized anti-CD3 and anti-CD28 antibodies (both BioXCell) at 1 mg/mL and 5 mg/mL, respectively. Subsequently, between days 4 and 7 following isolation, the cells were electroporated with mRNA encoding either the mNKG2D CAR (CAR) or mIL12 and mIFNα2 (Cyt) or all three mRNAs (CAR + Cyt) using 2.5 μg mRNA for the NKG2D CAR and 0.5 μg mRNA for each cytokine per million cells.

Human multifunctional CAR T cells were produced by activation of PBMCs using Dynabeads (Thermo Fisher Scientific) for 72 hours and electroporation of the mRNA as indicated between days 4 and 12 after activation.

Electroporation was performed using a NEON transfection system (Invitrogen) with electroporation parameters set to a voltage of 1,600 mV and three pulses of 10-ms pulse width. Mock-electroporated cells served as control (ctrl.) and transfection of mRNA encoding for the fluorescent protein ZsGreen was used as a control for transfection efficiency. Following electroporation, the cells were kept in medium without antibiotics and used for experiments within a few hours. Stable retrovirally transduced NKG2D CAR T cells (CH) were generated as described previously (19) and subsequently electroporated with mRNAs encoding for mIL12 and mIFNα2 (CH + Cyt) or mock electroporated on day 5.

Antibodies and flow cytometry

For flow cytometry, the following mAbs were used: anti-CD3-PerCP-Cy5.5, anti-CD4-BV650, anti-CD8-BV786, anti-CD11b-APC-Cy7, anti-CD45.1-AF488, anti-CD45.2-PE, anti-IFNγ-BV421 and anti-NKp46-APC (BioLegend), anti-RAE-1-FITC, and anti-MULT-1-PE (R&D Systems Europe). To prevent unspecific staining, all samples were preincubated with anti-mouse CD16/CD32 (BD Biosciences). Zombie Aqua (BioLegend) was used for viability staining and isotype-matched antibodies from Sigma-Aldrich served as controls.

Measurements were acquired on a FACSVerse or LSR II Fortessa and cell sorting was performed on a FACSAria III (all from BD). Data were processed in FlowJo (Tree Star). The isolation and analysis of tumor-infiltrating immune cells was performed as described previously (21).

Immunofluorescence and IHC

Immunofluorescence and IHC was performed as described previously (21) using anti-CD3, anti-MICA, anti-ULBP2 from Sino Biological, and anti-RAE1 from Novus Biologicals. Images were analyzed in an unsupervised and blinded fashion using TMARKER, a software toolkit for histopathologic staining estimation (22).

In vitro cytotoxicity and IFNγ expression of CAR T cells

Glioma cells as target cells were labeled with PKH26 (Sigma-Aldrich) and cocultured with mRNA-based or virally transduced CAR T cells or respective control T cells with or without coexpression of mIL12 and mIFNα2 for 36–40 hours at various effector:target ratios.

Target cell lysis was determined by flow cytometry as the percentage of death in the population of labeled target cells after subtraction of background lysis.

For the assessment of T cell–specific IFNγ expression, a protein transport inhibitor cocktail (Invitrogen) was added after 18 hours of coculture and incubated for 6 hours. Samples were subsequently stained for CD4, CD8, and intracellular IFNγ and analyzed by flow cytometry.

Pharmacoscopy

All studies including patient samples were conducted in accordance with the Declaration of Helsinki and approved by the Institutional Review Board and ethical committee (KEK-StV-Nr.19/08, BASEC number 2008-02002). Upon written informed consent, newly diagnosed glioblastoma samples were obtained from the Department of Neurosurgery at the University Hospital Zurich. Tumor dissociation was performed as described previously (23) and dissociated patient cells were seeded at 0.25–1.5 × 10^4 cells/well into clear-bottom, tissue culture–treated CellCarrier-384 Ultra Microplates (PerkinElmer). As indicated, 1 × 10^4 control or mRNA-modified T cells were plated on top of patient cells in 25 μL/well of RPMI1640 media supplemented with 10% FBS and cultured at 37°C, 5% CO_2 for 24 hours. Each

condition had 6–8 corresponding replicate wells. Subsequently, cells were fixed with 4% PFA (Sigma-Aldrich), blocked with PBS containing 5% FBS and 0.1% Triton overnight, and stained with the following antibodies: Alexa Fluor 488 anti-S100 beta (Abcam), PE anti-Nestin (BioLegend), Alexa Fluor 488 anti-CD3 (BioLegend), Alexa Fluor 647 anti-CD45 (BioLegend), and DAPI (BioLegend).

Imaging of the 384-well plates was performed with an Opera Phenix automated spinning-disk confocal microscope at 20× magnification (PerkinElmer). Single cells were segmented on the basis of their nuclei (DAPI channel) using CellProfiler 2.2.0. Downstream image analysis was performed with MATLAB R2020a. Marker-positive cell counts for each condition were derived on the basis of a linear threshold of the histograms of each channel and/or marker intensity measurements across both plates. Marker-positive cancer cell counts were averaged across each well and/or condition and compared between each treatment group.

T-cell morphology deep learning

The original convolutional neural network (CNN) used for transfer learning was trained using a manually curated dataset of 16,171 conventional T cells (T_{CON}) and 9,599 activated T cells (T_{ACT}), utilizing a 39-layer CNN with an adapted ResNet architecture (24). A dataset of 50×50 pixel, 3-channel (DAPI, Brightfield, Alexa Fluor 488) images of T cells was manually curated into T_{CON} and T_{ACT} morphologic classes, generating a total of 5,564 T_{CON} and 4,269 T_{ACT} cells. Curated images of CAR T cells were rescaled to 48×48 pixels before training, and training and validation datasets were split 4:1 to evaluate overfitting of the CNN both during and after training. Before CNN training, the weights and biases of the original network were transferred, except for the last convolutional layer and final fully connected layer, which were reset and randomly initialized. To improve the learning rate of the new layers compared with the transferred layers, the weight and bias learn rate factors of the new layers were set to 10. The network was trained for 20 epochs implementing the adaptive learning rate optimization "ADAM," with an initial learning rate of 0.001 which was lowered with a factor of 0.1 every 5 epochs. A mini batch size of 256 images and L2 regularization with 0.001 was applied. In each training iteration, images were randomly rotated by 45 degrees and mirrored vertically or horizontally per iteration to limit orientation biases towards cellular features. Performance of the network was assessed with a separate test dataset composed of 854 T_{ACT} and 1,113 T_{CON} cells.

RNA sequencing

$CD45.1^+$ cells were isolated from tumor-bearing hemispheres by FACS and immediately fixed in TRIzol reagent (Invitrogen). Subsequently, extraction of total RNA was performed using the RNeasy MinElute Cleanup Kit (Qiagen). Library prep was performed using the RNA Prep with Enrichment (L) Tagmentation' Kit (Ilumina), following manufacturer's instructions. Resulting pooled library was sequenced with a NextSeq 500/550 High Output Kit v2.5 (75 Cycles) at a final concentration of 1.8 pmol/L. Generated Fastq files were aligned using the STAR aligner.

Mice and animal experiments

All experiments were done following the guidelines of the Swiss federal law on animal protection and approved by the cantonal veterinary office (ZH073-2018). $C57BL/6^{CD45.2}$ mice were purchased from Charles River Laboratories. $C57BL/6^{CD45.1}$ were bred in pathogen-free facilities at the University of Zurich (Zurich, Switzerland). For all experiments, mice of 6 to 12 weeks of age were used and stereotactic tumor implantation of 2×10^4 GL-261 or 75×10^3 CT-2A cells and monitoring of mice has been described previously (21).

Multicytokine ELISA

Tumor-bearing hemispheres were dissociated in 2 mL PBS. Subsequently, cytokines were measured in a 100 μL aliquot of the supernatant using a multianalyte ELISArray Kit (Qiagen).

MRI

On day 15 after tumor implantation, coronal T2-weighted image sequences of the mouse brains were recorded using a 4.7 T MRI and Paravision 6.0 (Bruker Biospin).

Fluorescence molecular tomography

Murine T cells were labeled with CellBriteTM NIR790 (Biotium), electroporated and administered to tumor-bearing mice as indicated. For the *in vivo* tracking of adoptively transferred cells, we used the 790 nm laser channel of a FMT2500 system and images were analyzed using TrueQuant 3.1 (PerkinElmer).

Statistical analysis

Data are presented as means ± SD. Where not indicated differently, experiments were repeated at least three times. GraphPad Prism was employed for statistical analysis using two-way ANOVA and correcting for multiple comparisons using the two-stage step-up method of Benjamini, Krieger, and Yekutieli. Kaplan–Meier survival analysis was performed to assess survival differences among the treatment groups and *P* values were calculated with the log-rank test. RNA-sequencing reads were normalized and adjusted for differential gene expression using DESeq. Significance was concluded at *, $P < 0.05$ and **, $P < 0.01$ throughout all figures.

Data availability

The data generated in this study are available within the article and its Supplementary Data. Raw data are available upon request from the corresponding author.

Results

Multifunctional NKG2D CAR T cells coexpressing mIL12 and mIFNα2 exert enhanced antitumor activity against syngeneic murine glioma cell lines *in vitro*

We first established the optimal parameters for mRNA electroporation into mouse T cells using mRNA encoding for the fluorescent protein ZsGreen. A voltage of 1,600 mV applied in three pulses of 10-ms pulse width led to transfection efficiency with preserved viability of cells (Supplementary Fig. S1A). The transgene expression was titratable with increasing concentrations of mRNA (Supplementary Fig. S1B) and stable for up to 5 days upon electroporation (Supplementary Fig. S1C and S1D). We further investigated whether simultaneous transfection of multiple mRNAs impaired transfection efficiency, which was not the case (Supplementary Fig. S1E).

Next, we electroporated murine T cells with mRNA to express either the proinflammatory cytokines mIL12 and mIFNα2 (Cyt) or the NKG2D CAR (CAR) or all three transgenes (CAR + Cyt) and used these cells as effector cells in cocultures with the murine glioma cell lines GL-261 or CT-2A. NKG2D CAR T cells that coexpressed mIL12 and mIFNα2 had the highest cytolytic activity (**Fig. 1A** and **C**). The coexpression of mIL12 and mIFNα2 in addition to the CAR was

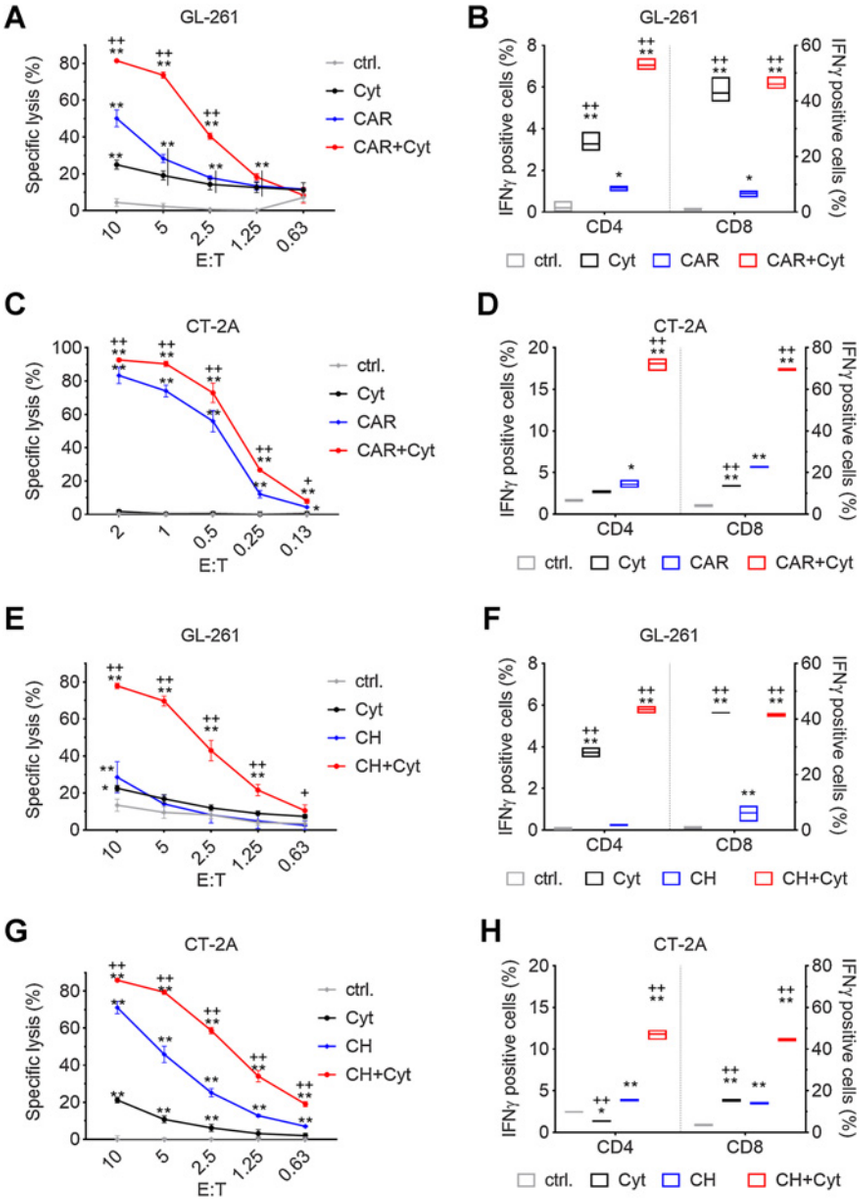

Figure 1.

Multifunctional murine NKG2D CAR T cells that coexpress mIL12 and mIFNα2 have enhanced antitumor activity against syngeneic glioma cells *in vitro*. **A,** Murine T cells were mock electroporated (ctrl.), or with two mRNAs encoding for mIL12 or mIFNα2 (Cyt), or an mRNA encoding for the NKG2D CAR (CAR) or all three mRNAs (CAR + Cyt). Subsequently, they were used as effector cells in coculture with GL-261 glioma cells at different effector:target ratios. **B,** Modified T cells as described in **A** were cocultured for 18 hours in a 2.5:1 effector:target ratio with GL-261 glioma cells and intracellular IFNγ expression was determined by flow cytometry in $CD4^+$ or $CD8^+$ cells. **C** and **D,** Same setup as in **A** and **B** but CT-2A glioma cells were used as target cells. **E–H,** Same setup as in **A–D**, but the CAR was retrovirally transduced (CH). Data are presented as mean ± SD ($^*/^+$, $P < 0.05$; $^{**}/^{++}$, $P < 0.01$; *, compared with ctrl.; $^+$, compared with CAR/CH).

also superior to combination of the CAR with either cytokine alone (Supplementary Fig. S1F and S1G). Therefore, we only focused on the coexpression of all three transgenes for all subsequent experiments. Coexpression of the CAR and the cytokines also led to the highest IFNγ expression in CD4 T cells against both GL-261 and CT-2A glioma cells, whereas for CD8 T cells the effect differed according to the target cell line: against CT-2A the CAR and cytokine-expressing CD8 T cells had the highest IFNγ expression, whereas against GL-261 already the cytokines alone increased the level of IFNγ to a similar level compared with the coexpression of the CAR and the cytokines (**Fig. 1B** and **D**).

As many CAR T approaches rely on the viral transduction of a CAR construct, we investigated whether the activity of those cells also can be boosted by cotransfection of mRNA encoding for proinflammatory cytokines. Thus, we generated stable NKG2D CAR T cells by retroviral transduction (CH), which were subsequently electroporated with mRNAs encoding for mIL12 and mIFNα2. This further improved their cytolytic activity and intracellular IFNγ expression in coculture with GL-261 or CT-2A glioma cells (**Fig. 1E–H**).

Multifunctional NKG2D CAR T cells coexpressing mIL12 and mIFNα2 have antitumor activity in immunocompetent orthotopic glioma mouse models upon intravenous and local administration

We first characterized the tumor homing of mRNA-based murine T cells expressing either mIL12 and mIFNα2 or the NKG2D CAR or all three proteins upon intravenous administration. Fluorescence molecular tomography (FMT) imaging demonstrated a similar accumulation of the different modified T cells in the tumor upon intravenous injection in immunocompetent orthotopic GL-261 glioma-bearing mice (**Fig. 2A** and **B**). Subsequently, we investigated the antitumor activity. Intravenously administered multifunctional mRNA-based NKG2D CAR T cells that coexpressed mIL12 and mIFNα2 conferred

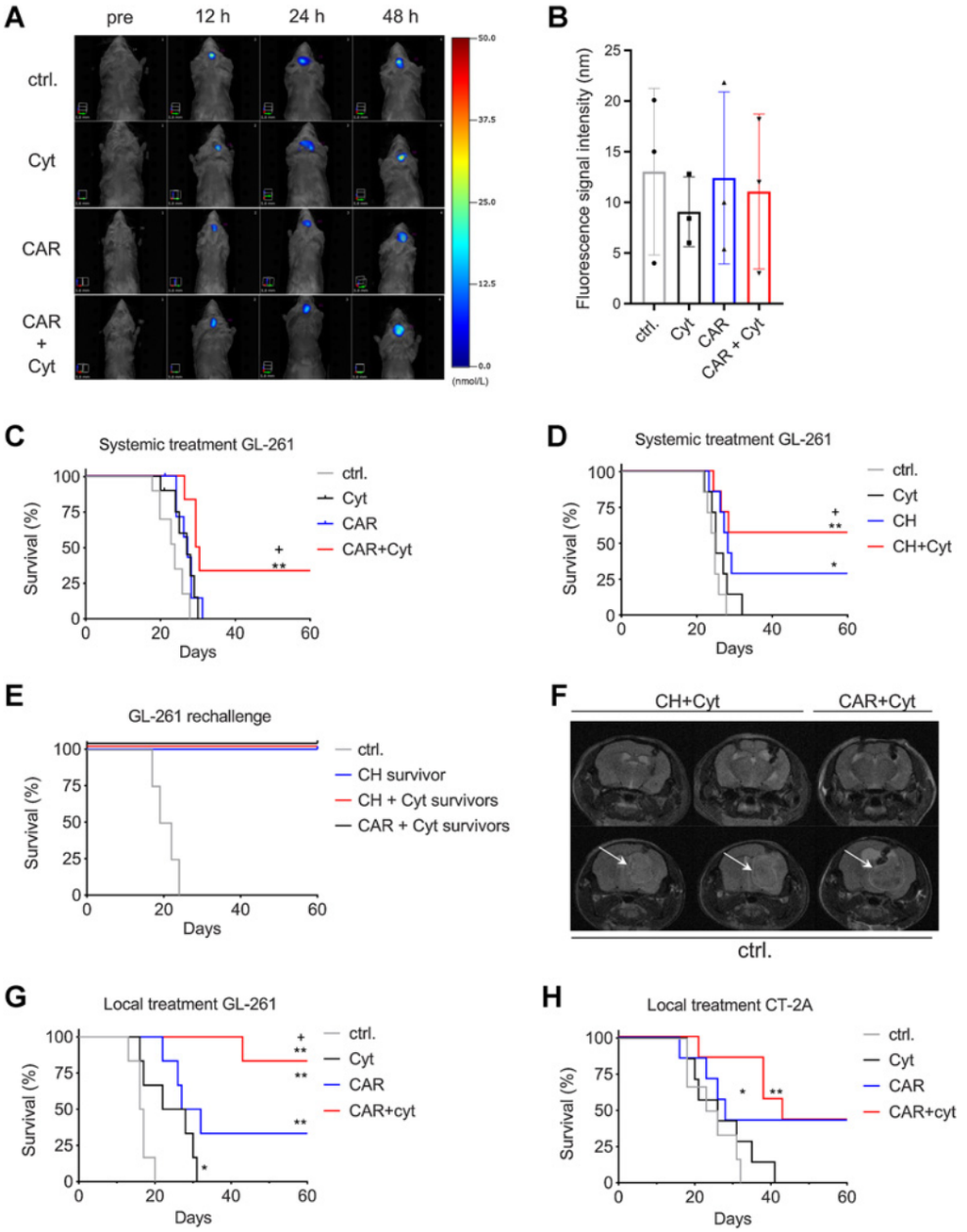

Figure 2.

Multifunctional mRNA-based NKG2D CAR T cells have promising antitumor activity in orthotopic immunocompetent murine glioma models upon intravenous and intratumoral administration. **A** and **B,** Murine T cells were mock-electroporated (ctrl.) or transfected with mRNAs encoding mIL12 and mIFNα2 (Cyt), the NKG2D CAR (CAR), or all three proteins. Subsequently, the cells were labeled with CellBrite790 and 5×10^6 cells were intravenously injected at days 4, 7, 10, and 13 after brain inoculation of GL-261 cells. The fluorescence signal from labeled T cells at the tumor site was detected by FMT at 12, 24, or 48 hours after the first injection. One representative mouse per group is shown in **A** and quantification from 3 mice per group is shown in **B**. **C,** Same setup as in **A** but Kaplan–Meier curves are shown. **D,** Same setup as in **A**, but the NKG2D CAR was retrovirally transduced (CH). **E** and **F,** GL-261 cells were implanted in naïve control mice or the contralateral hemisphere of the long-term surviving mice from **C** and **D** 6 months after the initial implantation. T2w MRI scans at day 15 after tumor implantation are shown. GL-261 (**G**) or CT-2A (**H**) tumor-bearing mice received two intratumoral injections of 2×10^6 modified T cells as described in **A** at days 7 and 12 after tumor implantation. Kaplan–Meier curves are shown and *P* values were calculated with log-rank test ($^{*}/^{+}$, $P < 0.05$; $^{**}/^{++}$, $P < 0.01$; *,compared with ctrl; $^{+}$, compared with CAR/CH).

a survival benefit and led to a fraction of long-term surviving mice whereas mRNA-modified T cells that expressed either the cytokines or the NKG2D CAR did not confer a survival benefit (**Fig. 2C**). The treatment was well tolerated and we did not observe signs of toxicity at the level of mouse behavior or bodyweight (Supplementary Fig. S2A). The antiglioma activity of intravenously administered multifunctional cytokine-expressing CAR T cells could also be confirmed in the less immunogenic CT-2A glioma model (Supplementary Fig. S2B) and we also observed a significant survival benefit but without long-term surviving mice when the treatment started at a late timepoint (Supplementary Fig. S2C). Furthermore, the coelectroporation of mRNAs encoding for mIL12 and mIFNα2 also improved the antitumor activity of intravenously administered viral vector-based NKG2D CAR T cells and increased the number of long-term surviving mice (**Fig. 2D**) without signs of toxicity at the level of bodyweight measurements (Supplementary Fig. S2D). Long-term surviving mice did not develop tumors upon rechallenge with GL-261 glioma cells into the contralateral hemisphere 6 months after the initial treatment, suggesting a long-lasting antitumor effect (**Fig. 2E** and **F**). For all subsequent experiments, we focused exclusively on the mRNA-modified T cells due to their potential for translation because of faster manufacturing processes and a potentially better safety profile compared with virally transduced T cells due to the transient nature. We also investigated the local intratumoral administration of multifunctional mRNA-based CAR T cells. This was also well tolerated in orthotopic GL-261 glioma-bearing mice without signs of toxicity at the level of behavior and bodyweight (Supplementary Fig. S2E) and further improved the therapeutic efficacy compared with intravenous administration. Two intratumoral injections of mRNA-based multifunctional NKG2D CAR T cells that coexpressed mIL12 and mIFNα2 cured 84% of GL-261 glioma-bearing mice (**Fig. 2G**). The promising antiglioma activity and tolerability of intratumorally administered mRNA-based mIL12- and mIFNα2-expressing NKG2D CAR T cells was also confirmed in two additional immunocompetent, orthoptic glioma models using CT-2A or SMA-560 glioma cells (**Fig. 2H**; Supplementary Fig. SF and SG). These models are less immunogenic and express also less NKG2D ligands compared with GL-261 (Supplementary Fig. S2H and S2I).

Multifunctional mRNA-based NKG2D CAR T cells coexpressing mIL12 and mIFNα2 increase bystander T cells and proinflammatory cytokines in the tumor microenvironment and are less exhausted

To investigate the functional mechanisms underlying the increased antitumor activity of multifunctional mRNA-based NKG2D CAR T cells that coexpress mIL12 and mIFNα2, we characterized adoptively transferred mRNA-modified T cells, bystander tumor-infiltrating immune cells and cytokines in the tumor microenvironment upon local intratumoral treatment in immunocompetent orthotopic glioma-bearing mice. Coexpression of mIL12 and mIFNα2 in addition to the NKG2D CAR led to the strongest increase in the abundance of adoptively transferred CD45.1$^+$ T cells within the tumor microenvironment. Furthermore, CAR T cells as well as multifunctional cytokine-expressing CAR T cells increased the abundance of CD45.2$^+$ bystander immune cells within the tumor microenvironment (**Fig. 3A**). The vast majority of the adoptively transferred cells were CD8$^+$, independent of the modification (**Fig. 3B**). Multifunctional cytokine- and CAR-expressing T cells increased the fraction of both tumor-infiltrating CD45.2$^+$ CD4 and CD8 T cells, whereas the cytokine- or CAR-only expressing T cells mainly increased the fraction of bystander CD4 T cells (**Fig. 3C**). *Ex vivo* cytokine profiling demonstrated increased IL12 and IFNγ levels in the tumor microenvironment upon treatment with multifunctional CAR T cells, whereas CAR-only expressing T cells mainly led to an upregulation of IL6 (**Fig. 3D**). We did not detect differences in immune cell populations in the blood and spleen suggesting a locally confined effect at the tumor site (Supplementary Fig. S3). To characterize the molecular state of the adoptively transferred cells, we FACS-sorted tumor-infiltrating CD45.1$^+$ and performed RNA sequencing. Principal component analysis showed a clear separation of the four different transfected T-cell populations (**Fig. 3E**) and we identified distinct gene expression signatures based on the 30 most abundant genes (**Fig. 3F**). We performed gene set enrichment analysis using previously reported gene sets associated with T-cell exhaustion or activation, respectively (25). This revealed that genes associated with T-cell exhaustion were predominantly enriched in control T cells and T cells that expressed the CAR only whereas genes that are associated with T-cell activation showed the highest enrichment in T cells that coexpressed the CAR and mIL12 and mIFNα2 (**Fig. 3G**).

In line with these findings, genes involved in the T-cell response to the immunosuppressive cytokine TGFβ, which has been associated with T-cell exhaustion in previous studies (26), showed the lowest expression in multifunctional CAR T cells coexpressing mIL12 and mIFNα2. Genes associated with an immunosuppressive IL10 response were enriched in CAR T cells but not upon coexpression of mIL12 and mIFNα2 (**Fig. 3H**). Accordingly, multifunctional CAR T cells coexpressing mIL12 and mIFNα2 displayed the strongest overall T-cell activation state (**Fig. 3I**).

Human mRNA-based multifunctional CAR T cells coexpressing hIL12 and hIFNα2 have antitumor activity in glioblastoma patient samples with a complex microenvironment

To determine the translational potential of multifunctional mRNA-based CAR T cells for the treatment of glioblastoma, we generated PBMC-derived mRNA-modified T cells encoding either the human NKG2D CAR or hIL12 and hIFNα2 or all three proteins. We obtained tissue samples directly from surgery of patients with newly diagnosed glioblastoma, dissociated the samples, and exposed the complex cell mixtures comprising glioma cells and cells from the tumor microenvironment to the different modified T cells. After 24 hours, we used an image-based platform (27) to determine the antitumor activity of the different engineered T cells on a single cell level (**Fig. 4A**). Markers associated with glial lineage and stemness (S100B, NESTIN) were used to identify S100B$^+$CD45$^-$ or NESTIN$^+$CD45$^-$ cancer cells whereas CD45 was used as a pan-immune cell counterstain. T cells were identified by the coexpression of CD3 and CD45 (**Fig. 4B**; Supplementary Fig. S4A–S4C). This single-cell phenotypic read-out from 10 glioblastoma samples demonstrated that despite interpatient heterogeneity, multifunctional mRNA-based CAR T cells coexpressing hIL12 and hIFNα2 most efficiently reduced the number of cancer cells (**Fig. 4B** and **D**). In contrast, T cells that only expressed either the NKG2D CAR or the proinflammatory cytokines did not consistently reduce the number of glioma cells. In all patients except for patient 8, which showed very low target antigen expression (Supplementary Fig. S4D), multifunctional CAR T cells reduced the number of glioma cells by around 50%. To determine whether specific T-cell phenotypes were associated with increased anti-glioma activity, we assessed T-cell activation through deep learning–based morphologic profiling (**Fig. 4C** and **D**). We trained a convolutional neural network (TNet) to classify T cells as either activated (T_{ACT}) or nonactivated (T_{CON}), based on whether they displayed rounded or polarized morphologies.

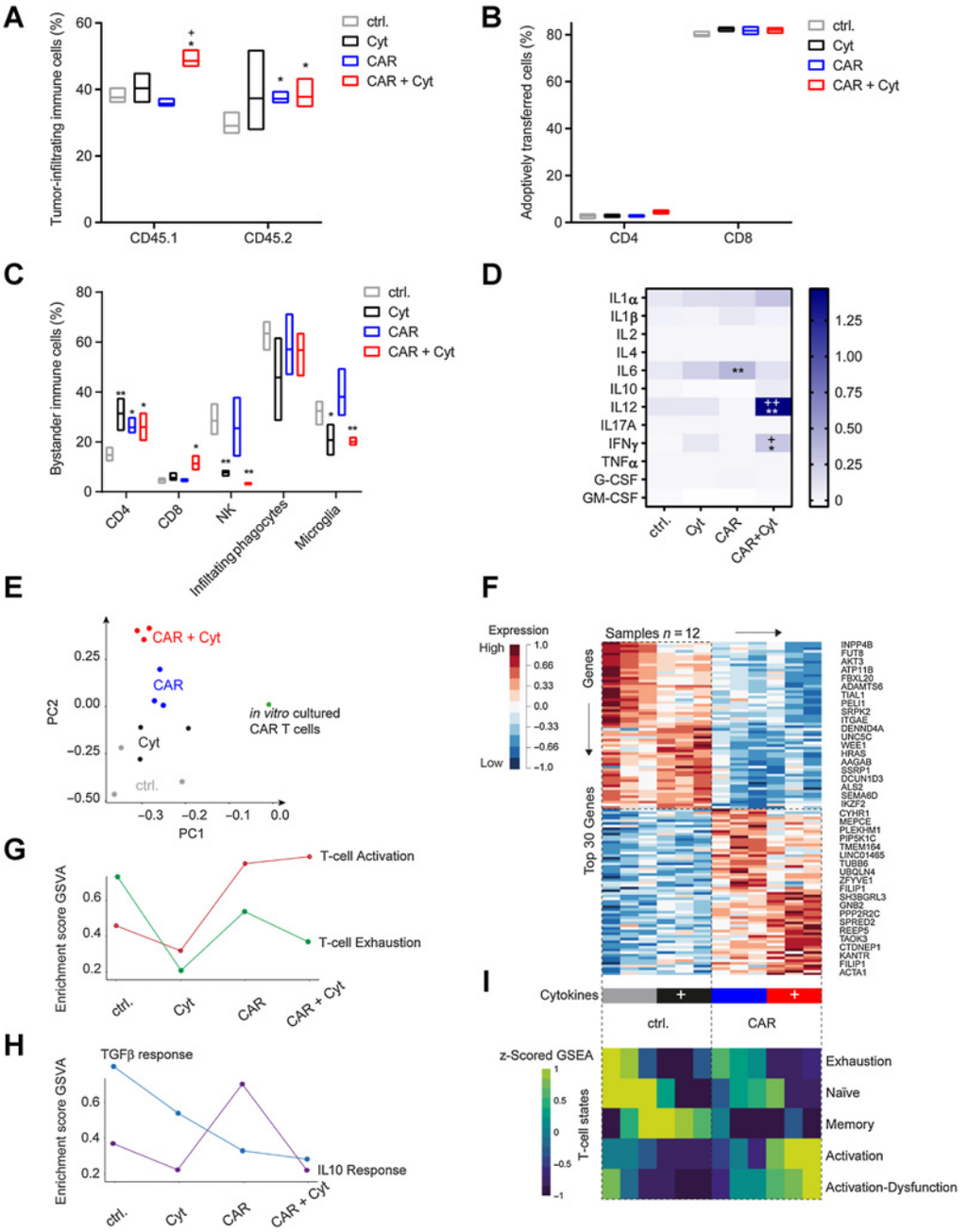

Figure 3.

Multifunctional mRNA-based NKG2D CAR T cells coexpressing mIL12 and mIFNα2 increase bystander T cells and proinflammatory cytokines in the tumor microenvironment and are less exhausted. **A–C,** Murine CD45.1$^+$ T cells were electroporated (ctrl.) or transfected with mRNAs encoding for mIL12 and mIFNα2 (Cyt), the NKG2D CAR (CAR), or all three transgenes and injected intratumorally into GL-261 glioma-bearing CD45.2$^+$ mice at days 7 and 12 after tumor implantation. At day 14 after tumor implantation, tumor-infiltrating CD45.1$^+$ (**A**, **B**) and CD45.2$^+$ (**C**) immune cells were isolated and analyzed by flow cytometry. Data are presented as mean ± SD ($^*/^+$, $P < 0.05$; $^{**}/^{++}$, $P < 0.01$; *, compared with ctrl.; $^+$, compared with CAR). **D,** Same experimental setup as in **A–C**, but cytokines were analyzed from dissociated tumor-bearing hemispheres by ELISA. The mean cytokine level is shown ($^*/^+$, $P < 0.05$; $^{**}/^{++}$, $P < 0.01$; *, compared with ctrl.; $^+$, compared with CAR). **E–I,** GL-261 glioma-bearing CD45.2$^+$ mice were treated intratumorally at day 12 after tumor implantation with modified murine CD45.1$^+$ T cells as indicated in **A–C**. A total of 48 hours later, FACS-sorted CD45.1$^+$ T cells were characterized by RNA sequencing. Principal component analysis and expression level of the most abundant 30 genes are shown in **E** and **F**, respectively. Gene enrichment scores in the different modified T cells are shown for genes associated with T-cell activation or exhaustion (**G**), TGFβ response or IL10 response (**H**), or global T-cell states comprising exhaustive, naïve, memory, activated, or activated-dysfunctional cell states (**I**).

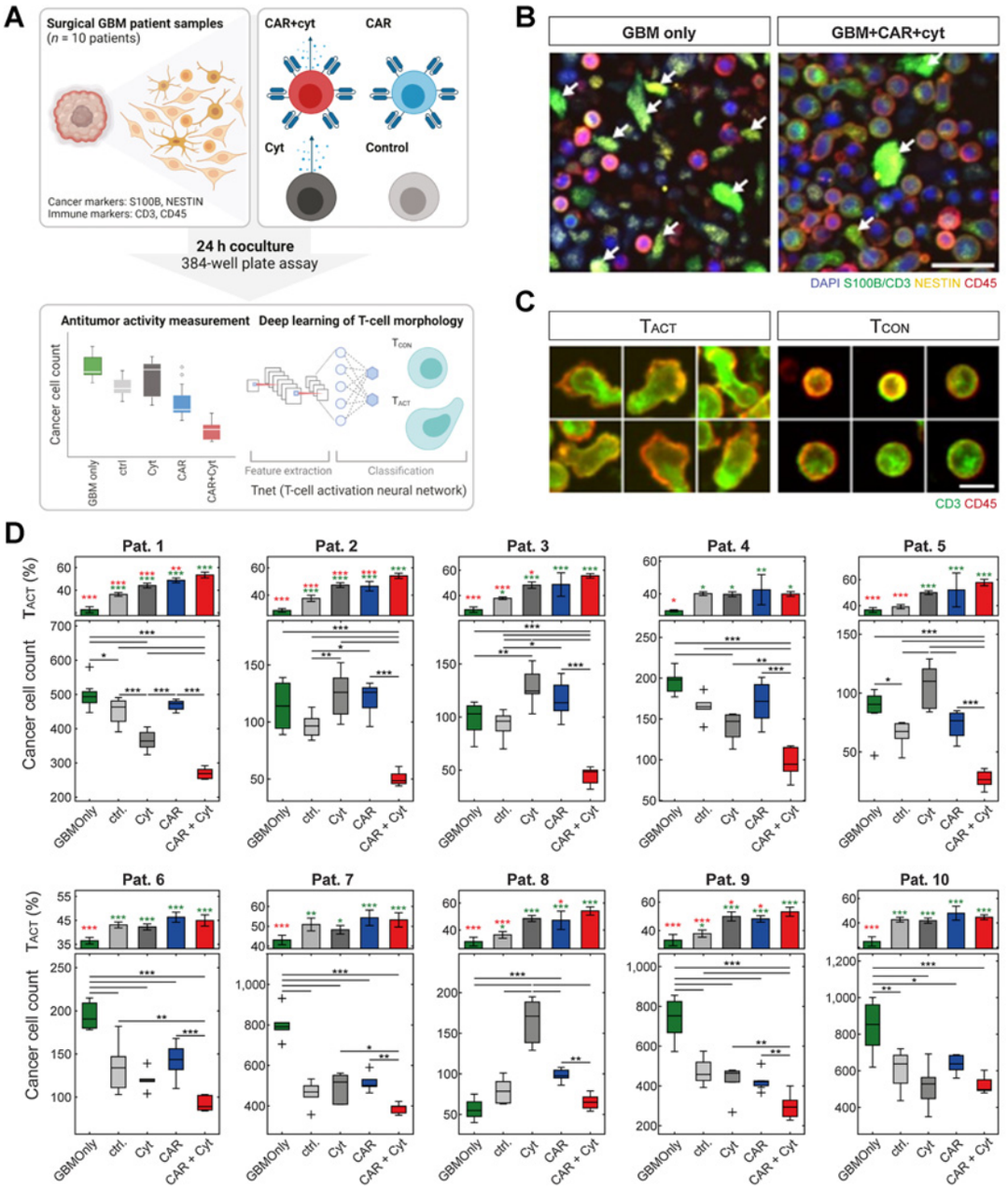

Figure 4.

Human mRNA-based multifunctional CAR T cells coexpressing hIL12 and hIFNα2 have promising antitumor activity in complex glioblastoma patient samples with an intact microenvironment. **A,** Scheme of *ex vivo* cocultures of glioblastoma (GBM) patient samples with different mRNA-modified T cells. Surgically derived patient glioblastoma samples ($n = 10$) were dissociated and cocultured for 24 hours with mock-transfected T cells (ctrl.), hIL12- and hIFNα2-expressing T cells (Cyt), hNKG2D CAR T cells (CAR), or multifunctional CAR T cells coexpressing hNKG2D CAR as well as hIL12 and hIFNα2 (CAR + Cyt). **B,** Representative immunofluorescence images of one glioblastoma patient sample (Pat. 1) in the absence of modified T cells (left) and the presence of multifunctional CAR T cells coexpressing hIL12 and hIFNα2 (right). White arrows denote cancer cells. Scale bar, 30 μm. **C,** Representative single-cell crops of T cells classified by TNet according to their morphology as either activated (T_{ACT}) or nonactivated (T_{CON}). Scale bar, 10 μm. **D,** Cocultures from 10 dissociated glioblastoma patient samples and different modified T cells were set up as indicated in **A**. Number of glioma cells (bottom) and T-cell activation states (top) in the different cocultures for each patient were obtained on a single-cell resolution from the image analyses. The number of cancer cells is shown as box plots and the fraction of T cells adopting an activated morphology (T_{ACT}%) is shown as bar plots with mean ± SD. Black asterisks (all comparisons), green asterisks (compared with GBM only), red asterisks (compared with CAR + Cyt). *, $P_{adj} < 0.05$; **, $P_{adj} < 0.01$; ***, $P_{adj} < 0.001$.

In 6 of 10 patients (60%), T cells that coexpressed the CAR and hIL12/hIFNα2 had the highest fraction of T_{ACT} cells, 4 of which were significantly higher than the NKG2D CAR T cells alone, suggesting higher levels of activation.

Discussion

Glioblastoma is a prototypic solid tumor with an immunosuppressive microenvironment and there is a need for efficient and safe engineering strategies to improve CAR T-cell activity against this deadly tumor. However, current vector-based CAR T-cell strategies against glioblastoma have limited transgene capacity, lengthy manufacturing processes, and the integration into the genome and continuous transgene expression pose the risk for malignant transformation and uncontrollable off-tumor toxicities (14, 15).

The feasibility of mRNA-based T-cell engineering has been demonstrated in other diseases (28) and is an appealing alternative to overcome these challenges and to facilitate clinical translation of innovative CAR T-cell strategies (16). We demonstrated promising antiglioma activity of mRNA-based multifunctional CAR T cells in several immunocompetent orthotopic glioma models (**Figs. 1** and **2**). We used a CAR based on the NKG2D receptor, which is attractive against glioblastoma as it allows multivalent targeting of antigens expressed on glioma cells (8) including cells with stem-like properties (9), tumor-associated immunosuppressive myeloid-derived suppressor cells, and regulatory T cells (29) as well as tumor-associated blood vessels (30). Furthermore, it has been translated to patients without signs of toxicity (18). To enhance the antitumor activity of NKG2D CAR T cells, we coelectroporated mRNA encoding for IFNα2 and IL12, which improved CAR T effector functions *in vitro* (**Fig. 1**) and the antiglioma effect *in vivo* (**Fig. 2**). Type I IFNs have immune-modulatory functions in glioma (31) and can potentially also directly inhibit the growth of stem-like glioma cells (32, 33). However, a recent clinical trial failed to confirm activity of intravenously administered IFNb in combination with standard of care treatment in newly diagnosed glioblastoma (34). IL12 is a potent proinflammatory cytokine with promising antiglioma activity that stimulates lymphoid and myeloid cells. It cannot be administered systemically at therapeutically active doses due to systemic toxicities and apart from a recent report in which we used an antibody-IL12 fusion protein (35), it has been investigated against glioblastoma mainly as a local treatment (36–38). In line with our findings, a recent study demonstrated the potential of an intratumorally administered IL12-FC fusion protein as an adjuvant for EGFRvIII-targeting CAR T cells in a preclinical glioma model (37). We did not observe signs of toxicity, which is in line with a study from Pohl-Guimaraes and colleagues, who showed that RNA-modified T cells that overexpress a proinflammatory cytokine did not cause toxicities in mice with intracranially implanted melanoma (39). A chimeric approach, to virally transduce the CAR for long-term persistence but transiently augment the activity of these CAR T cells by mRNA-based modifications (**Fig. 2D**) is another strategy to increase the activity of current CAR T-cell approaches against glioblastoma.

To investigate the translational potential, we used an image-based cell profiling technology that enables functional single-cell readouts in complex primary tumor samples with an intact microenvironment (27). This demonstrated that despite interpatient heterogeneity, multifunctional mRNA-based NKG2D CAR T cells coexpressing hIL12/hIFNα2 had the strongest antiglioma activity in this setup (**Fig. 4**).

The limitation of the mRNA-based T-cell modifications is the transient nature, which is advantageous in case of off-tumor toxicities, but would require repeated administrations. Clinical studies are required to demonstrate safety and efficacy of mRNA-based multifunctional CAR T cells in patients with glioblastoma and the current study provides a strong rationale for such investigations. Furthermore, additional studies are required to investigate in more depth mechanisms of action and resistance. This would enable to select the best combination of mRNAs encoding for cytokines, tumor-homing molecules, extracellular matrix remodeling proteins, and to further enhance the antitumor activity. Other future studies should investigate the combination of multifunctional CAR T cells with other immunotherapeutic strategies such as immune checkpoint inhibition or immunovirotherapy. We anticipate that mRNA-based multifunctional NKG2D CAR T cells coexpressing IL12 and IFNα2 could be a promising treatment for patients with glioblastoma.

Authors' Disclosures

P. Roth reports grants from Swiss National Science Foundation, Swiss Cancer Research, and University of Zurich for the CRPP ImmunoCure during the conduct of the study. P. Roth also reports personal fees from Bristol Myers Squibb, Boehringer Ingelheim, Debiopharm, QED, and Roche; grants from Merck Sharp and Dohme; and grants and personal fees from Novocure outside the submitted work. S. Pascolo reports that a patent application has been filed pending. U. Sahin reports personal fees from BioNTech Germany during the conduct of the study; in addition, U. Sahin has a patent on mRNA modifications issued to BioNTech. C.L. Sentman reports personal fees from Bellicum Pharmaceuticals, CytomX Therapeutics, Javelin Oncology, and Celdara Medical outside the submitted work. In addition, C.L. Sentman has a patent for NKG2D CAR pending, issued, licensed, and with royalties paid from Celyad; a patent for TCR deficient T cells pending, issued, licensed, and with royalties paid from Celyad; a patent for B7H6CAR pending, issued, licensed, and with royalties paid from Celyad; a patent for high affinity B7H6 specific mAbs and scFv pending and issued; a patent for novel signaling platforms for CAR T cells pending; a patent for coexpression of CARs and transcription factors pending; and a patent for MICA specific scFv pending, issued, licensed, and with royalties paid from Artiva Biotherapeutics. M. Weller reports grants from University of Zurich during the conduct of the study; M. Weller also reports grants from AbbVie, Adastra, Apogenix, Merck Sharp & Dohme, Merck (EMD), Novocure, and Quercis, as well as personal fees from AbbVie, Adastra, Bristol Myers Squibb, Celgene, Medac, Merck Sharp & Dohme, Merck (EMD), Nerviano Medical Sciences, Novartis, Orbus, Philogen, Roche, Tocagen, and Y-mAbs outside the submitted work. T. Weiss reports grants from University of Zurich, Swiss Cancer Research, Betty and David Koetser Foundation, Promedica Foundation, and Helmut Horten Foundation during the conduct of the study, as well as personal fees from Philogen outside the submitted work; in addition, T. Weiss has a patent filed. No disclosures were reported by the other authors.

Authors' Contributions

H. Meister: Visualization, methodology, project administration, writing–review and editing. **T. Look:** Data curation, formal analysis, investigation, methodology, writing–original draft. **P. Roth:** Funding acquisition, writing–review and editing. **S. Pascolo:** Conceptualization, writing–review and editing. **U. Sahin:** Conceptualization, writing–review and editing. **S. Lee:** Visualization, methodology, writing–original draft. **B.D. Hale:** Visualization, methodology, writing–original draft. **B. Snijder:** Visualization, methodology, writing–original draft. **L. Regli:** Resources, writing–original draft. **V.M. Ravi:** Visualization, methodology, writing–original draft. **D.H. Heiland:** Visualization, methodology, writing–original draft. **C.L. Sentman:** Writing–review and editing. **M. Weller:** Writing–original draft. **T. Weiss:** Conceptualization, supervision, visualization, methodology, writing–original draft.

Acknowledgments

This study was supported by the Highly Specialized Medicine (HSM) Program of the Canton of Zurich, the Clinical Research Priority Program (CRPP) of the University of Zurich for the CRPP ImmunoCure, the URPP of the University of Zurich for Translational Cancer Research and grants from the Swiss National Science Foundation (SNF 310030_170027) to P. Roth and Swiss Cancer Research (KFS-4544-08-2018-R) to P. Roth and T. Weiss. In addition, T. Weiss was supported by the Forschungskredit of the University of Zurich, grant no FK-18-054, the Betty and

David Koetser Foundation for Brain Research, the Promedica Foundation and the Helmut Horten Stiftung.

The publication costs of this article were defrayed in part by the payment of publication fees. Therefore, and solely to indicate this fact, this article is hereby marked "advertisement" in accordance with 18 USC section 1734.

Note

Supplementary data for this article are available at Clinical Cancer Research Online (http://clincancerres.aacrjournals.org/).

Received December 10, 2021; revised May 31, 2022; accepted August 25, 2022; published first August 29, 2022.

References

1. Ostrom QT, Patil N, Cioffi G, Waite K, Kruchko C, Barnholtz-Sloan JS. CBTRUS statistical report: primary brain and other central nervous system tumors diagnosed in the United States in 2013–2017. Neuro Oncol 2020;22:iv1–iv96.
2. Weller M, van den Bent M, Preusser M, Le Rhun E, Tonn JC, Minniti G, et al. EANO guidelines on the diagnosis and treatment of diffuse gliomas of adulthood. Nat Rev Clin Oncol 2021;18:170–86.
3. Maude SL, Laetsch TW, Buechner J, Rives S, Boyer M, Bittencourt H, et al. Tisagenlecleucel in children and young adults with B-cell lymphoblastic leukemia. N Engl J Med 2018;378:439–48.
4. Park JH, Riviere I, Gonen M, Wang X, Senechal B, Curran KJ, et al. Long-term follow-up of CD19 CAR therapy in acute lymphoblastic leukemia. N Engl J Med 2018;378:449–59.
5. Brown CE, Alizadeh D, Starr R, Weng L, Wagner JR, Naranjo A, et al. Regression of glioblastoma after chimeric antigen receptor T-cell therapy. N Engl J Med 2016;375:2561–9.
6. Ahmed N, Brawley V, Hegde M, Bielamowicz K, Kalra M, Landi D, et al. HER2-specific chimeric antigen receptor-modified virus-specific T cells for progressive glioblastoma: a phase 1 dose-escalation trial. JAMA Oncol 2017;3:1094–101.
7. O'Rourke DM, Nasrallah MP, Desai A, Melenhorst JJ, Mansfield K, Morrissette JJD, et al. 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;9:eaaa0984.
8. Weiss T, Weller M, Guckenberger M, Sentman CL, Roth P. NKG2D-based CAR T cells and radiotherapy exert synergistic efficacy in glioblastoma. Cancer Res 2018;78:1031–43.
9. Yang D, Sun B, Dai H, Li W, Shi L, Zhang P, et al. T cells expressing NKG2D chimeric antigen receptors efficiently eliminate glioblastoma and cancer stem cells. J Immunother Cancer 2019;7:171.
10. Ponterio E, De Maria R, Haas TL. Identification of targets to redirect CAR T cells in glioblastoma and colorectal cancer: an arduous venture. Front Immunol 2020;11:565631.
11. Yeku OO, Purdon TJ, Koneru M, Spriggs D, Brentjens RJ. Armored CAR T cells enhance antitumor efficacy and overcome the tumor microenvironment. Sci Rep 2017;7:10541.
12. Katlinski KV, Gui J, Katlinskaya YV, Ortiz A, Chakraborty R, Bhattacharya S, et al. Inactivation of interferon receptor promotes the establishment of immune privileged tumor microenvironment. Cancer Cell 2017;31:194–207.
13. Lamers CH, Sleijfer S, Vulto AG, Kruit WH, Kliffen M, Debets R, et al. Treatment of metastatic renal cell carcinoma with autologous T-lymphocytes genetically retargeted against carbonic anhydrase IX: first clinical experience. J Clin Oncol 2006;24:e20–2.
14. Neelapu SS, Tummala S, Kebriaei P, Wierda W, Gutierrez C, Locke FL, et al. Chimeric antigen receptor T-cell therapy - assessment and management of toxicities. Nat Rev Clin Oncol 2018;15:47–62.
15. Rafiq S, Hackett CS, Brentjens RJ. Engineering strategies to overcome the current roadblocks in CAR T cell therapy. Nat Rev Clin Oncol 2020;17:147–67.
16. Beck JD, Reidenbach D, Salomon N, Sahin U, Türeci Ö, Vormehr M, et al. mRNA therapeutics in cancer immunotherapy. Mol Cancer 2021;20:69.
17. Caruso HG, Torikai H, Zhang L, Maiti S, Dai J, Do KA, et al. Redirecting T-cell specificity to EGFR using mRNA to self-limit expression of chimeric antigen receptor. J Immunother 2016;39:205–17.
18. Baumeister SH, Murad J, Werner L, Daley H, Trebeden-Negre H, Gicobi JK, et al. Phase I trial of autologous CAR T cells targeting NKG2D ligands in patients with AML/MDS and multiple myeloma. Cancer Immunol Res 2019;7:100–12.
19. Zhang T, Lemoi BA, Sentman CL. Chimeric NK-receptor-bearing T cells mediate antitumor immunotherapy. Blood 2005;106:1544–51.
20. Tusup M, French LE, De Matos M, Gatfield D, Kundig T, Pascolo S. Design of *in vitro* transcribed mRNA vectors for research and therapy. Chimia 2019;73:391–4.
21. Weiss T, Schneider H, Silginer M, Steinle A, Pruschy M, Polic B, et al. NKG2D-dependent antitumor effects of chemotherapy and radiotherapy against glioblastoma. Clin Cancer Res 2018;24:882–95.
22. Schuffler PJ, Fuchs TJ, Ong CS, Wild PJ, Rupp NJ, Buhmann JM. TMARKER: a free software toolkit for histopathological cell counting and staining estimation. J Pathol Inform 2013;4:S2.
23. Friebel E, Kapolou K, Unger S, Núñez NG, Utz S, Rushing EJ, et al. Single-cell mapping of human brain cancer reveals tumor-specific instruction of tissue-invading leukocytes. Cell 2020;181:1626–42.
24. He K, Zhang X, Ren S, Sun J. Deep residual learning for image recognition. Proceedings of the 2016 IEEE Conference on Computer Vision and Pattern Recognition (CVPR); 2016. p. 770–8.
25. Singer M, Wang C, Cong L, Marjanovic ND, Kowalczyk MS, Zhang H, et al. A distinct gene module for dysfunction uncoupled from activation in tumor-infiltrating T cells. Cell 2016;166:1500–11.
26. Gunderson AJ, Yamazaki T, McCarty K, Fox N, Phillips M, Alice A, et al. TGFβ suppresses CD8(+) T cell expression of CXCR3 and tumor trafficking. Nat Commun 2020;11:1749.
27. Snijder B, Vladimer GI, Krall N, Miura K, Schmolke AS, Kornauth C, et al. Image-based *ex-vivo* drug screening for patients with aggressive haematological malignancies: interim results from a single-arm, open-label, pilot study. Lancet Haematol 2017;4:e595–606.
28. Etxeberria I, Bolaños E, Quetglas JI, Gros A, Villanueva A, Palomero J, et al. Intratumor adoptive transfer of IL-12 mRNA transiently engineered antitumor CD8(+) T cells. Cancer Cell 2019;36:613–29.
29. Barber A, Rynda A, Sentman CL. Chimeric NKG2D expressing T cells eliminate immunosuppression and activate immunity within the ovarian tumor microenvironment. J Immunol 2009;183:6939–47.
30. Zhang T, Sentman CL. Mouse tumor vasculature expresses NKG2D ligands and can be targeted by chimeric NKG2D-modified T cells. J Immunol 2013;190:2455–63.
31. Fujita M, Scheurer ME, Decker SA, McDonald HA, Kohanbash G, Kastenhuber ER, et al. Role of type 1 IFNs in antiglioma immunosurveillance–using mouse studies to guide examination of novel prognostic markers in humans. Clin Cancer Res 2010;16:3409–19.
32. Happold C, Roth P, Silginer M, Florea AM, Lamszus K, Frei K, et al. Interferon-β induces loss of spherogenicity and overcomes therapy resistance of glioblastoma stem cells. Mol Cancer Ther 2014;13:948–61.
33. Lohmann B, Silginer M, Picard D, Schneider H, Remke M, Roth P, et al. Interferon-β exposure induces a fragile glioblastoma stem cell phenotype with a transcriptional profile of reduced migratory and MAPK pathway activity. Neurooncol Adv 2020;2:vdaa043.
34. Wakabayashi T, Natsume A, Mizusawa J, Katayama H, Fukuda H, Sumi M, et al. JCOG0911 INTEGRA study: a randomized screening phase II trial of interferonβ plus temozolomide in comparison with temozolomide alone for newly diagnosed glioblastoma. J Neurooncol 2018;138:627–36.
35. Weiss T, Puca E, Silginer M, Hemmerle T, Pazahr S, Bink A, et al. Immunocytokines are a promising immunotherapeutic approach against glioblastoma. Sci Transl Med 2020;12:eabb2311.
36. Agliardi G, Liuzzi AR, Hotblack A, De Feo D, Núñez N, Stowe CL, et al. Intratumoral IL-12 delivery empowers CAR-T cell immunotherapy in a pre-clinical model of glioblastoma. Nat Commun 2021;12:444.
37. Chiocca EA, Yu JS, Lukas RV, Solomon IH, Ligon KL, Nakashima H, et al. Regulatable interleukin-12 gene therapy in patients with recurrent high-grade glioma: results of a phase 1 trial. Sci Transl Med 2019;11:eaaw5680.
38. Vom Berg J, Vrohlings M, Haller S, Haimovici A, Kulig P, Sledzinska A, et al. Intratumoral IL-12 combined with CTLA-4 blockade elicits T cell-mediated glioma rejection. J Exp Med 2013;210:2803–11.
39. Pohl-Guimaraes F, Yang C, Dyson KA, Wildes TJ, Drake J, Huang J, et al. RNA-modified T cells mediate effective delivery of immunomodulatory cytokines to brain tumors. Mol Ther 2019;27:837–49.