RESEARCH ARTICLE

EGFR⁺ GLIOBLASTOMA STEM CELLS TARGETING BY CD16^{158V}-CHIMERIC RECEPTOR T CELLS AND CETUXIMAB

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ABSTRACT

Glioblastoma multiforme (GBM) is the deadliest human brain tumor with a median survival following diagnosis of 14–16 months. Innovative therapeutic approaches are urgently needed. Cancer stem cells (CSC) from GBM resist current chemo- and radiotherapies and can generate recurrent and aggressive tumors. To envisage innovative therapeutic approaches of potential clinical use, we engineered T cells with Fcγ-chimeric receptors (CRs) to elicit antibody-dependent cellular cytotoxicity (ADCC) in the presence of mAbs specific for tumor associated an-

tigens (TAA). Indeed, in previous studies, we successfully redirected CD16^{158V}-CR T cells against KRAS-mutated colorectal carcinoma cells. Since surface overexpression of epidermal growth factor receptor (EGFR) is frequently detectable in GBM, we assessed, *in vitro*, the anti-GBM potential of polymorphic CD16-CR T cells, in combination with anti-EGFR mAbs, on GBM-derived EGFR⁺ CSC. Our results indicate that CD16^{158V}, but not CD16^{158F}-CR engineered T cells incubated with cetuximab, but not panitumumab, induced the elimination of GBM-derived CSC through

a caspase-3 dependent mechanism, and produced high amounts of TNF α and IFN γ upon recognition of target cells. These data pave the way towards

pre-clinical development of innovative GBM treatments, taking advantage of CD16^{158V}-CR engineered T cells and therapeutic antibodies.

ABBREVIATIONS

ADCC: antibody-dependent cellular cytotoxicity; **CAR**: chimeric antigen receptor; **CR**: chimeric receptor; **CSC**: cancer stem cells; **EGFR**: epidermal growth factor receptor; **Fc γ R**: IgG constant fragment receptor; **FGF2**: fibroblast growth receptor 2; **FITC**: Fluorescein isothiocyanate; **GBM**: glioblastoma multiforme; **IL-7**: interleukin-7; **IL-15**: interleukin-15; **INF**: interferon-gamma; mAb: monoclonal antibody; NK: natural killer; **PBMC**: peripheral blood mononuclear cells; **PDGFR**: platelet-derived growth factor receptor; **PE**: Phycoerythrin; **TNF α** : tumor necrosis factor-alpha.

KEY WORDS

CD16-chimeric receptors; ADCC; cetuximab; panitumumab; EGFR; Glioblastoma; cancer stem cells.

IMPACT STATEMENT

We present an innovative therapeutic strategy in which anti-EGFR monoclonal antibody, cetuximab redirects CD16^{158V}-chimeric receptor T cells against GBM stem cells, these findings may support a potential role of CD16-CR T cell-based immunotherapy in the management of EGFR+ GBM.

INTRODUCTION

Glioblastoma multiforme (GBM), high-grade glioma, has an annual incidence of 3-4 and 7 cases per 100,000 people in Europe and the USA, respectively (1). Median onset age is of 63 years and median survival after clinical diagnosis is of 14–16 months. In many cancer types, the bulk of tumor cells derive from small populations of cancer stem cells (CSC), also known as tumor-initiating cells (2). CSC are characterized by their ability to successfully seed new tumors when implanted in low numbers into experimental animals. Such cells may persist in recurrent GBM due to their enhanced resistance to chemo- and radiotherapy (2-6). In previous studies, we identified two types of CSC in several human GBM clinical specimens, hereafter referred to as core GBM (c-CSC) and peritumor tissue-derived CSC (p-CSC) (3-5). C-CSC are characterized by a higher proliferative potential *in vitro*, correlating with a higher tumor-initiating ability *in vivo*, as compared to p-CSC (5). WHO 2016 classification, highlighting a large number of genetic alterations associated with specific GBM phenotypes, has improved the classical histological classification (6, 7). The Cancer Genome Atlas (TCGA), subdivides these tumors into three subclasses based on the pattern of expression and genetic alterations: classical/EGFR+, proneural/PDGFR+ and mesenchymal/NF1+ classes (4). EGFR wild type (wt) is overexpressed in almost 50% of GBM, whilst its

activating mutation (EGFRvIII) has an overall prevalence of almost 60% in patients whose tumors show amplification of EGFR wt. Enhanced EGFR activity leads to activation of downstream signaling pathways such as Raf/MEK/Erk and PI3K/Akt pathways, which are responsible for the malignant phenotype of glioma (8). Another subset of gliomas, the PDGFR subclass account for 25-30% of GBM, and is characterized by dysregulation of PDGFR activity, which in some cases is due to amplification and rearrangements of the PDGFR α gene locus, and in others to overexpression of the PDGF ligands (9). In previous studies, we showed that EGFR and PDGFR targeting decreases GBM invasiveness (3, 4) whereas shRNA inhibition of either PDGFR α or PDGFR β signaling induces apoptosis of GBM stem cells (3, 10). In the last decade monoclonal antibodies (mAbs), targeting tumor markers and immunological checkpoints inhibitors, have successfully entered clinical practice. As a consequence, they are now included in standard treatment protocols (6, 7). Moreover, adoptive transfer of chimeric antigen receptor (CAR) transduced T cells, recognizing markers expressed on tumor surfaces, is being increasingly utilized (11). Currently, CAR-T cell therapy is approved for B-cell lymphoma and leukemia treatment and its potential relevance for GBM is actively being investigated (12-14).

Fcy-chimeric receptors (Fcy-CRs) may share the same transmembrane (TM) and intracellular chimeric signaling domains of “conventional” CAR-T cells. However, while the latter express extracellular domains including a single-chain variable fragment (ScFv) specific for a marker located on the surface of tumor cells, Fcy-CRs express the extracellular portion of the FcyRs (15, 16). Fcy-CR T cell-based immunotherapy has been designed to transfer antibody-dependent cellular cytotoxicity (ADCC) function of innate immune cells including NK cells to T lymphocytes (17-19). The rationale of using Fcy-CR T cells rather than NK cells is based on evidence indicating that: 1) T cells can be easily expanded *in vitro*, and effectively infiltrate the tumor microenvironment (TME); 2) tumor infiltration by T lymphocytes is usually associated with a favorable prognosis (20); and 3) upon conjugation with cancer cells, NK cells undergo apoptosis (21) and CD16 and NK cell activating receptor down-regulation (11, 22). In contrast, the role of NK cells in solid tumor is unclear since they barely infiltrate the TME and may not be directly associated with favorable prognosis (23).

FcyRs are classified into three major groups: FcyRI (CD64), FcyRII (CD32), and FcyRIII (CD16), widely distributed on the surface of innate immune cells. CD64 is the only high-affinity receptor binding monomeric IgG molecules, whereas CD32 and CD16 are low-affinity receptors that bind weakly to monomeric IgG. CD16 polymorphisms do influence their binding affinity to IgG (18, 19). Based on this background, here we assessed the ability of T cells expressing polymorphic CD16-CRs in combination with EGFR-specific therapeutic mAbs to prevent the expansion of GBM-derived EGFR⁺ c-CSC.

MATERIALS AND METHODS

Antibodies and reagents

Fluorescein isothiocyanate (FITC)-conjugated mouse anti-human CD3, allophycocyanin (APC)-conjugated mouse anti-human CD3, phycoerythrin (PE)-conjugated mouse anti-human CD16, FITC-conjugated mouse anti-human CD107a, PE-conjugated mouse anti-human EGFR, mouse anti-human CD3, and mouse anti-human CD28 were purchased from BD Bioscience (San Jose, CA). Rabbit anti-EGFR and rabbit anti-caspase-3 were purchased from Cell Signaling Technology (Leiden, The Netherlands). Rabbit anti-PARP-1 was from

Santa Cruz Biotechnology (Santa Cruz, CA), and mouse anti- β -actin from SIGMA-Aldrich (Saint Louis, MO). Rabbit anti-mouse and donkey-anti-rabbit peroxidase-conjugated secondary antibodies were purchased from Jackson ImmunoResearch Laboratories (West Grove, PA). Cetuximab (Erbix 5 mg/mL, Merck, Darmstadt, Germany) and panitumumab (Vectibix 20 mg/mL, Amgen, Thousand Oaks, CA) were commercially available. Chemiluminescence HRP substrate was purchased from Millipore (Burlington, MA), while Retronectin (Recombinant Human Fibronectin) was obtained from Takara Bio (Saint-Germain-en-Laye, France). Monensin sodium salt was purchased from SIGMA-Aldrich (Saint Louis, MO). Human recombinant interleukin-7 (IL-7), interleukin-15 (IL-15), epidermal growth factor (EGF), and fibroblast growth factor 2 (FGF2), were purchased from PeproTech (London, UK). Interferon-gamma (INF γ) and tumor necrosis factor- α (TNF α) were obtained from Thermo Fisher Scientific (Waltham, MA) while 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was obtained from SIGMA-Aldrich (Saint Louis, MO). Bio-Rad Protein Assay was obtained from Bio-Rad (München, Germany), NuPAGE Bis-Tris gels from Invitrogen, (Carlsbad CA), Hybond-P Extra membrane from Amersham Biosciences (GE Healthcare Life Science-Buckinghamshire, UK), and Z-VAD-FMX (Biomol) from Enzo Life Science (Farmingdale NY). Nonidet-P40 (NP-40), sodium dodecyl sulfate (SDS), Tris-base, NaCl, EDTA, sodium orthovanadate (Na₃VO₄), and protease inhibitors cocktail, were all purchased from SIGMA-Aldrich (Saint Louis MO). Iscove Modified Dulbecco Media (IMDM), Fetal Bovine Serum (FBS), RPMI 1640 medium, L-glutamine, and penicillin/streptomycin were obtained from Thermo Fisher Scientific (Waltham, MA). A 1:1 mixture of 10X Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 (DMEM/F12) was obtained from Invitrogen (Carlsbad, CA) and matrigel from SIGMA-Aldrich (Saint Louis, MO). Mycoplasma detection kit was purchased from Minerva Biolabs (Berlin, Germany), and Accutase and GeneJuice Transfection Reagent from Millipore (Burlington, MA).

Cell cultures

Primary T cells were expanded in RPMI 1640 supplemented with 10% FBS, IL-7 (10 ng/mL), and IL-15 (5 ng/mL). The generation and growth of GBM-derived c-CSC cultures were described previously (3-5). Briefly, c-CSC were grown as floating tumor spheres in serum-free DMEM/F12 (1:1), supple-

mented with 20 ng/mL EGF, 10 ng/mL FGF2 and containing L glutamine 2 mM, glucose 0.6%, putrescine 9.6 µg/mL, progesterone 0.025 mg/mL, sodium selenite 5.2 ng/mL, insulin 0.025 mg/mL, apo-transferrin sodium salt 0.1 mg/mL, sodium bicarbonate 3 mM, Hepes 5 mM, BSA 4 mg/mL, heparin 4 µg/mL (all purchased by Sigma-Aldrich). For flow cytometry, cytotoxicity, MTT assays and Western blots, medium-sized tumor spheres were dissociated in single-cell suspensions by Accutase (Millipore, Burlington, MA) at 37 °C and plated onto matrigel pre-coated dishes. The GBM-derived c-CSC cultures (# 2, 3, 4) are primary cells with a limited life span. First, following 30 passages, the proliferation rate of the cells is increasingly reduced ending up to cell cycle arrest. Second, small tumor spheres display a necrotic phenotype. These cells are kept in culture only for the time necessary to perform the experiments. The HEK293T packaging cell line (RRID: CVCL_0063) grown in IMDM supplemented with 10% FBS, was used to produce helper-free retrovirus for T cell transduction. All experiments were performed with mycoplasma-free cells.

Retrovirus production and T cells transduction

The method of transfection of HEK293T cells was reported previously (18, 19). Briefly, HEK293T packaging cells were transfected using GeneJuice Transfection Reagent with a Peg-Pam vector containing the Moloney murine leukemia virus (MoMLV) gag-pol genes, the RDF vector carrying RD114 envelope, and the SFG retroviral vector harboring CD16^{158F}-CR or CD16^{158V}-CR.

Peripheral blood mononuclear cells (PBMC) at a 0.5×10^6 cells/mL concentration were activated in 24-wells plates pre-coated with mouse anti-human CD3 and anti-CD28 mAbs. The viral supernatant was loaded on retronectin-coated 24-wells plates and activated PBMC were then seeded on the retrovirus loaded-plate for 72 hours at 37 °C under 5% CO₂.

MTT viability assay

The MTT viability assay was performed as described previously (18, 19). Briefly, the viability of GBM-derived c-CSC incubated with CD16^{158F}-CR or CD16^{158V}-CR T cells and cetuximab (C) or panitumumab (P) was assessed at 48 hours. Non-transduced T cells (NT) were used as experimental control. c-CSC (10^4 cells/well) were plated on matrigel pre-coated 96-well flat-bottom plates the day before. CD16^{158F}-CR T cells were added to target cells at a 4:1 effector/target (E/T) and CD16^{158V}-CR T cells at a 2:1 E/T cell ratio,

with or without mAbs (3 or 6 µg/ml). Five independent experiments were performed in quadruplicates.

Flow cytometry

CD16-CR transduction efficiency on T cells was assessed by flow cytometry with PE-conjugated mouse anti-human CD16 and FITC-conjugated mouse anti-human CD3. Analysis of EGFR expression on c-CSC was carried out with PE-conjugated mouse anti-human EGFR. Cells were analyzed by a 2-laser BD FACSCalibur (Becton Dickinson, S. Jose, CA) flow cytometer, and results were analyzed by utilizing the Tree Star, Inc. (San Carlos CA) FlowJo software.

Cytotoxicity assay

To assess the cytotoxic potential of transduced lymphocytes, c-CSC (#3, 4) seeded as single cells (2×10^5) onto matrigel pre-coated 24-well dishes, were left 1 day in proliferation medium before to be incubated for 4 hours at 37 °C under 5% CO₂ in the presence or absence of CD16^{158V}-CR T cell at a 2:1 E/T cell ratio, with or without cetuximab (3 µg/mL), while NT were used as control. Z-VAD-FMX pan-caspase inhibitor was used at 50 µM and added to target cells 1 hour before starting the cytotoxicity assay. After removal of supernatants and washing with PBS, c-CSC were detached with Trypsin-EDTA solution. Subsequently, cells were resuspended with Annexin V binding buffer (500 µL), for each tube, 500 µL solution was splitted in 250 µL for blank tube and 250 µL for the tube with FITC-conjugated-Annexin V, PI, APC-conjugated anti-human CD3. Finally, cells were incubated 30 minutes at room temperature and analyzed using flow cytometry by gates posted on FSC-H high cells and CD3 negative.

Cytotoxic degranulation assay

To assess the secretion of lytic granules following CD16^{158V}-CR T cells cross-linking with target cells, CD107a expression was investigated on the membrane of CD16-CR T cells by flow cytometry. c-CSC (#3, 4) were plated as single cells (5×10^4) onto matrigel pre-coated 96-well dishes, and CD16^{158V}-CR T cells were added at 2:1 E/T cell ratio with or without cetuximab and 5 µL FITC-conjugated anti-CD107a. After 1 h incubation at 37 °C under 5% CO₂, 2 µM Monensin was added for 4 h. After incubation, plates were centrifuged at 1.200 rpm for 5 min, supernatants gently discarded and 100 µL of PE-conjugated-anti-CD16 added. After 30 min incubation, cells were washed and fixed with 150 µL of 1% paraformaldehyde (PFA)-PBS directly added on the dishes. As posi-

tive control of secretion of lytic granules, CD16^{158V}-CR T cells were cross-linked with plastic bound human anti-CD3. Finally, CD16^{158V}-CR T cells were collected and transferred in tubes for assessing CD16 and CD107a expression by cytometric analyses.

ELISA

Cell culture supernatants of c-CSC (#2, 3, 4), incubated with CD16^{158F}-CR T cells or CD16^{158V}-CR T cells with or without cetuximab (C) or panitumumab (P) were collected at 48 hours, as described in MTT assay. INF γ and TNF α secretion levels were measured by ELISA according to the manufacturer's instructions.

Western blots analysis

GBM-derived EGFR⁺ c-CSC seeded as single cells (5×10^5) onto matrigel pre-coated well dishes were left 1 day in proliferation medium prior to a 4 hours incubation with CD16^{158V}-CR T cells or NT at a 2:1 E/T cell ratio, with or without cetuximab. Afterwards, T cells were removed and c-CSC were collected in 200 μ l of lysis buffer (1% NP-40, 0.01% SDS, 20 mM Tris-HCl pH 7.4, 300 mM NaCl, 1 mM EDTA, 1 mM Na₃VO₄ and a protease inhibitors cocktail, including AEBSF, Aprotinin, Bestatin, Leupeptin, and Pepstatin A. Cells were then sonicated with two pulses of 5 sec at 50% of amplitude (Sonics and Materials, Newtown, CT). Equal amounts (30 μ g/lane) of total protein extracts, determined by Bio-Rad Protein Assay, were loaded on NuPAGE Bis-Tris gels and transferred on Hybond-P Extra membrane. Filters were immunoblotted using the following primary antibodies: rabbit anti-EGFR, rabbit anti-caspase-3, rabbit anti-PARP-1, and mouse anti- β -actin. After three washings with TBS-Tween buffer, immuno-reactive proteins were detected using rabbit anti-mouse or donkey-anti-rabbit peroxidase-conjugated secondary antibodies directed to the appropriate primary antibodies. Proteins were then visualized using the chemiluminescence HRP substrate. Gels and Images acquisition was done by HP Photosmart Essential Ver. 1.12 and Adobe Photoshop CS5 respectively. The densitometric analyses of protein bands normalized against to β -actin protein levels were performed using the ImageJ software (NIH, USA).

Statistical analysis

Statistical analysis was performed with Prism5 (Graph Pad) and Microsoft Office Excel 2019. Data shown are representative of results obtained from three or five independent experiments carried out in quadruplicates, as detailed in the specific sec-

tions. The results were analyzed by two-way ANOVA and Bonferroni's post-tests. Data are expressed as mean $\pm$ standard deviation (SD) and P values $\leq$ 0.05 were considered statistically significant.

RESULTS

We first assessed T cell transduction efficiency of CD16^{158F}- and CD16^{158V}-CRs over time by flow cytometry. As reported in **figure 1 a**, 9.49%, 18.5%, and 23.0% of T cells were efficiently transduced with CD16^{158F}-CR at day 6, 9 and 16, respectively, as compared to 20.7%, 30.5%, and 43.2% for CD16^{158V}-CR transduced T cells. Cumulative data referred to transduced T lymphocytes derived from five different healthy donors are reported in **figure 1 b**. Considering the different transduction efficiency of the two viral vectors, to reliably evaluate, in a comparative manner, the effector potential of either transduced T cell populations, cytotoxicity assays were carried out using two effector/target (E/T) cell ratios: 4:1 and 2:1 for CD16^{158F}- and CD16^{158V}-CR T cells, respectively.

The cell surface expression of the EGFR on the three c-CSC (# 2, 3, and 4), under investigation, was analyzed by flow cytometry (**figure 1 c**). Although all cells expressed EGFR, a lower MFI (26.0) was observed for c-CSC 2, as compared to c-CSC 3 (56.6) and c-CSC 4 (44.5). These results are consistent with previously reported gene expression and Western blot data (3).

The anti-tumor potential of transduced T cells against c-CSC targets, in the presence or absence of therapeutic mAbs, was comparatively assessed following 48 hours co-culture by using the MTT viability assay (**figure 2 a-c**). CD16^{158V}-CR T cells significantly decreased the viability of all target c-CSC in the presence of IgG1 mAb cetuximab. In contrast, IgG2 mAb panitumumab was unable to mediate cytotoxic activity and CD16^{158F}-CR T cells were similarly ineffective in the presence or absence of either anti EGFR reagent. In parallel experiments, we observed that amounts of INF γ (**figure 2 d-f**) and TNF α (**figure 2 g-i**) released in culture supernatants, were significantly higher in cultures performed in the presence of CD16^{158V}-CR T cells and cetuximab, as compared with those from cultures including CD16^{158F}-CR and cetuximab, or NT cells. IgG2 mAb panitumumab was ineffective in triggering cytokines release by transduced T cells (**figure 2 d-i**).

To corroborate these data, the induction of apoptosis in target cells, following four hours incu-

Figure 1. Efficiency of CD16-CR expression on transduced T lymphocytes and EGFR surface expression on GBM-derived c-CSC.

a. A representative experiment of CD16^{158F}-CR (upper panel) and CD16^{158V}-CR (lower panel) surface expression in transduced T cells, as observed in a time-course experiment at 6, 9, 16 days after PBMC activation. Percentages of CD3+/CD16+ T lymphocytes are reported in the upper right quadrants of the contour plots. The mean fluorescence intensity (MFI), indicating the average density of CD16 surface expression, is reported for each time point. Cells were double stained with PE-conjugated mouse anti-human CD16 and FITC-conjugated mouse anti-human CD3. CD16 staining was carried out on transduced T cells at least three times with similar results. **b.** The histogram shows the cumulative percentage of positive T lymphocytes for CD16^{158F}- and CD16^{158V}-CR 16 days after viral infection of cells from five healthy donors; ***P value ≤ 0.001 is highly significant. **c.** EGFR surface expression on GBM-derived c-CSC. Data refer to a representative experiment assessing % of EGFR expression on c-CSC 2 (94.4%), c-CSC 3 (96.1%) and c-CSC 4 (99.0%) plated on pre-coated matrigel dishes. Cells are visualized with PE-conjugated mouse anti-human EGFR versus isotype-stained cells. EGFR staining was carried out at least three times with similar results. The MFI is displayed below the (%) EGFR positivity for each GBM-derived c-CSC.

bation in the presence of effector cells and mAbs, was also evaluated by flow-cytometry. Cultures including c-CSC 3 or c-CSC 4 target cells were harvested and stained with APC-anti-CD3, FITC-annexin V, and Propidium Iodide (PI). Among c-CSC 3 (**figure 3 a**) and c-CSC 4 (**figure 3 b**) we observed 33.1% and 16.7% of Annexin V⁺ cells, respectively, following incubation with CD16^{158V}-CR and cetuximab. ADCC was caspase-dependent since it was inhibited by the pan-caspase inhibitor Z-VAD-FMX (**figure 3 a, b**). Accordingly, Western blot experiments documented caspase-3 and PARP1 cleavage in both c-CSC 3 and c-CSC 4 target cells (**figures 3 c, d**), following incubation with CD16^{158V}-CR T cells and cetuximab, whereas EGFR expression was not modified (**figure 3 c, d**).

In order to estimate the levels of cleaved caspase-3 and PARP1 proteins, a quantitative densitometric analysis of the proteins bands by using ImageJ software was performed. We reported more than 10 and 5 fold increase of caspase-3 and PARP1 cleavage relative to β-actin levels in c-CSC3, respectively (**figure 3 d**). Similar results were observed in c-CSC 4 with 5 fold increase of caspase-3 and PARP1. No signal was quantified in the lanes with the application of pan-caspase inhibitor Z-VAD-FMX (**figure 3 d**). Variations observed in EGFR protein levels were not statistically significant.

To demonstrate that CD16^{158V}-CR T cells induced target cells cytotoxicity by exocytosis of perforin and granzyme-containing granules, we assessed the expression of CD107a, a sensitive marker of CD8

Figure 2. Co-culture of CD16-CR engineered T cells and GBM-derived c-CSC in combination with cetuximab reduced c-CSC viability and induced significantly INF γ and TNF α secretion.

a, b, c. Histograms show viability, as measured by MTT assay, of c-CSC (#2, 3, 4) cells, following 48 hours incubation with CD16^{158F/V}-CR T cells in the presence or absence of anti-EGFR mAbs at different concentrations (3, 6 μ g/mL). No effect on c-CSC survival was recorded using non-transduced T cells (NT) in the presence or absence of cetuximab (C) or panitumumab (P). 4:1 and 2:1 E/T cell ratios were used for assays with CD16^{158F}-CR and CD16^{158V}-CR T cells, respectively. The figure reports cumulative data, with mean $\pm$ (SD) and ***P values $\leq$ 0.001, **P values $\leq$ 0.01 related to c-CSC (#2, 3, 4) viability, as observed by using effector cells from five different donors in independent experiments. **d, e, f.** CD16^{158V}-CR engineered T cells in combination with cetuximab induce significant INF γ and TNF α secretion. Histograms refer to INF γ release, as detected in the culture supernatants of c-CSC (#2, 3, 4) after 48 hours incubation with CD16^{158F}-CR- and CD16^{158V}-CR T cells or NT in the presence or absence of cetuximab (C) or panitumumab (P) (3, 6 μ g/mL). **g, h, i** Histograms refer to TNF α release in culture supernatants of c-CSC (#2, 3, 4) after 48 hours incubation with CD16^{158F}-CR- and CD16^{158V}-CR T cells or NT in the presence or absence of cetuximab (C) or panitumumab (P). 4:1 and 2:1 E/T cell ratios were used for assays with CD16^{158F}-CR and CD16^{158V}-CR T cells, respectively. The figure reports cumulative data, with mean $\pm$ (SD) and ***P values $\leq$ 0.001, from ELISA assays from experiments performed by using effector cells from five different donors in independent experiments.

positive T cell degranulation following activation. Flow cytometry analysis did not detect any CD107a surface expression by CD16^{158V}-CR T cells upon conjugation with target cells and cetuximab (**figure 4**, middle and lower panel). In contrast, activation of CD16^{158V}-CR T with plastic bound anti-CD3 antibody, promoted mobilization of CD107a on T cells surface membrane (**figure 4**, right upper panel).

DISCUSSION

Recent studies support the safety and effectiveness of intraventricular and intra-tumoral CAR-T administration for the treatment of CNS malignancies (24-26). Donovan *et al.*, evaluated the efficacy of the loco-regional administration into the cerebrospinal fluid of EPHA2, HER2 and IL13R α 2 CAR-T cells (24).

This therapeutic approach was validated in pre-clinical models for the treatment of ependymoma and primary, metastatic and recurrent medulloblastoma. These results provided the basis for extending the same treatment modality in humans. Brown *et al.*, evaluated the safety and efficacy of CAR-T directed against IL13R α 2 for the treatment of recurrent GBM through a loco-regional intraventricular administration (27). A primary goal for the success of CAR-T based-immunotherapy in brain tumors and in solid tumors should envisage strategies for regulating T lymphocytes trafficking in the parenchyma. Baron *et al.* in the early 1993, have showed as T cells expressing alpha 4 integrin are able to leave blood and enter the brain parenchyma, overcoming the blood-brain barrier (28). More recently, Zhu *et al.* reported that T cells infiltration into the brain parenchyma may occur in the pres-

Figure 3. CD16^{158V}-CR engineered T cells in combination with cetuximab induce ADCC of c-CSC by caspase-3 activation.

a, b. These panels report flow cytometry data of a representative experiment of cytotoxicity using c-CSC 3 (**a**) and c-CSC 4 (**b**) target cells in the presence of CD16^{158V}-CR T cells with 3 µg/mL of cetuximab (C). After 4-5 hours incubation, cells were harvested, stained with APC-anti-CD3, FITC-annexin V and propidium iodide (PI), and analyzed by flow cytometry. Data are representative of three experiments independently performed and show a significant increase of apoptotic cells in the presence of cetuximab, as indicated (%) in the lower right quadrants. Cell death is blocked by 50 µM of pan-caspase inhibitor Z-VAD. No effect was recorded using non-transduced cells (NT) in the presence or absence of cetuximab. A 2:1 E/T cell ratio was used. **c.** CD16^{158V}-CR engineered T cells in combination with cetuximab (C) induce apoptosis of c-CSC 3 and c-CSC 4. C-CSC were incubated with CD16^{158V}-CR T cells plus cetuximab (C); after 4 hours incubation, cells were harvested and protein extracts immunoblotted with anti-caspase-3, anti-PARP1, and anti-EGFR antibodies. Western blots analyses show caspase-3 activation and PARP-1 cleavage induced by CD16^{158V}-CR T cells in the presence of cetuximab. The pan-caspase inhibitor Z-VAD blocks caspase-3 and PARP1 cleavages, but EGFR expression is not affected. No effect was recorded using NT in the presence or absence of cetuximab. A 2:1 E/T cell ratio was used. **d.** Histograms report a quantitative protein analysis of Caspase-3, PARP-1 and EGFR relative to β-actin protein levels in c-CSC 3 and c-CSC 4. Data shown are representative of three experiments independently performed. ****P value ≤ 0.0001 is highly significant.

ence of an altered TME such as the induction of the chemokine CXCL10 in the glioma site in IFN-α and IFNγ dependent manner. The blockade of CXCL10 with a specific mAb abrogated the efficient glioma homing of cytotoxic T lymphocytes (CTL) (29). Importantly, overexpression and/or enhanced activity of EGFR and its variant (EGFRvIII) have frequently been reported in human GBM (4, 26). Since high EGFR/EGFRvIII expression designates an aggressive GBM subtype, these markers do represent attractive targets for immunotherapeutic approaches (30). In previous work, we have generated CD16^{158F/V}-CR DNA constructs expressing phenylalanine (F) or valine (V) at position 158, efficiently promoting effector functions upon transduction into T lymphocytes on breast cancer and colorectal carcinoma cells (18,19). Here, we have addressed the possibility to target

GBM-derived EGFR⁺ c-CSC by using CD16^{158F/V}-CR engineered T cells and specific therapeutic mAbs. Cetuximab is a human-murine chimeric IgG1, instead panitumumab is a fully human IgG2. Several reports have described, both *in vitro* and *in vivo*, how these mAbs are able to elicit ADCC (17-19). The effects of the ligation of the Fc portion of the mAbs with the Fc receptors on the cells depend on the specificity of the Fc receptors for a given IgG class (31). For instance, CD16-CR T cells bind cetuximab (IgG1) upon recognition of the ligand on target cells, but do not bind soluble mAbs (18, 19). Thus, GBM-derived c-CSC are targeted by CD16^{158V}-CR T cells and cetuximab treatment, with timing and effectiveness comparable to those observed for long term-established tumor cell lines (18). In contrast, panitumumab mAb (IgG2) preferably binds to CD32-CR rather

Figure 4. Cross-linking of CD16^{158V}-CR engineered T cells with c-CSC and cetuximab does not induce T cells degranulation.

The panel reports the mobilization of CD107a on engineered T cells surface assessed by flow cytometry. c-CSC3 and c-CSC4 target cells were incubated with CD16^{158V}-CR T cells in presence or without 3 µg/mL of cetuximab (C) and anti-CD107a-FITC. After 5 hours incubation, CD16^{158V}-CR T cells were further incubated with anti-CD16-PE. The upper panel (right upper quadrant) reports the positive control as displayed by CD107a expression (19.1%) on CD16^{158V}-CR engineered T cells activated with plastic bound anti-CD3 antibody. The middle and lower panel show the lacking of positivity for CD107a in the presence of cetuximab (C) after incubation of effector cells with c-CSC3 or c-CSC4 (right upper quadrants). A 2:1 E/T cell ratio was used. Data shown are representative of five experiments independently performed.

than CD16-CR T cells, and therefore no target cells cytotoxicity was recorded in our context in presence of panitumumab. mAb subclasses critically impacted on the effectiveness of CD16-CR T cells mediated-ADCC (16, 18). Our results are in line with what it has been shown by other investigators where Fcγ-CRs polymorphisms influenced the binding of engineered T cells to tumor cells coated with IgG (16, 18, 19).

Here, we demonstrate the lacking of CD107a expression accounting for defective degranulation, (32, 33) but CD16^{158V}-CR T cells generate fully competent immune response including INFγ and TNFα secretion and target cells cytotoxicity as reported here and elsewhere (17). This led us to assume the engagement of cell death surface receptors such as Fas/Fas ligand and TRAIL-R/TRAIL in mediating target cells elimination (17). Therefore, in our future investigations we aim to clarify more thoroughly the mechanisms underlying the GBM cancer stem cells elimination triggered by CD16^{158V}-CR T cells with cetuximab.

CONCLUSIONS

GBM is characterized by dismal prognosis, and there is an urgent need for innovative therapeutic approaches. Current GBM treatment is based on the use of temozolomide, a DNA alkylating agent. However, resistant tumor cell subsets promoting recurrence, rapidly emerge. Although these cells, presenting CSC features, do frequently express EGFR, treatment with small molecules targeting the EGFR signal transduction pathway has proven largely disappointing, thus suggesting that direct immune-mediated targeting of this marker could be more effective (34). Our data indicate that, indeed, expansion of GBM-derived c-CSC may be prevented by ADCC mediated by CD16^{158V}-CR T cells and anti-EGFR IgG1 mAb.

While the *in vitro* nature of our study represents an obvious limitation, these findings nevertheless underline the high potential relevance of this therapeutic approach and pave the way towards *in vivo* pre-clinical investigations.

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ETHICS

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Conflict of interests

The authors have declared no conflict of interests.

Availability of data and material

N/A

Authors' contribution

GS conceived this study, supervised the experiments and edited the manuscript; CC performed the MTT viability assays, drafted and edited the manuscript; SC and AO contributed to the execution of the transfection and infection experiments, and Western blots; GL performed the flow cytometry for CD16/CD107a and EGFR. TS assisted to do the ELI-

SA; RA and DL performed the cytotoxicity assays by flow cytometry; GI, GCS, AV, MR contributed to the final editing of the manuscript; HEM collected the data and revised the statistical analysis. All authors read and approved the final edited manuscript.

Ethical approval and consent to participate

Procedures for collection of adult human GBM-derived CSC were approved by the Ethical Committee of the Catholic University of Rome, as reported previously (4, 5). Informed consent was obtained, and all patients were fully aware of the aims of this work. The ethical principles of the declaration of Helsinki were strictly followed.

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