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Genetically engineered T cells to target EGFRvIII expressing glioblastoma

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Abstract

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Glioblastoma remains a significant therapeutic challenge, warranting further investigation of novel therapies. We describe an immunotherapeutic strategy to treat glioblastoma based on adoptive transfer of genetically modified T-lymphocytes (T cells) redirected to kill EGFRvIII expressing gliomas. We constructed a chimeric immune receptor (CIR) specific to EGFRvIII, (MR1- ζ). After in vitro selection and expansion, MR1- ζ genetically modified primary human T-cells specifically recognized EGFRvIII-positive tumor cells as demonstrated by IFN- γ secretion and efficient tumor lysis compared to control CIRs defective in EGFRvIII binding (MRB- ζ) or signaling (MR1-del ζ). MR1- ζ expressing T cells also inhibited EGFRvIII-positive tumor growth in vivo in a xenografted mouse model. Successful targeting of EGFRvIII-positive tumors via adoptive transfer of genetically modified T cells may represent a new immunotherapy strategy with great potential for clinical applications.

Keywords

Adoptive immunotherapy; Glioma; Chimeric immune receptor; Chimeric T cell receptor; EGFRvIII; MR1

Introduction

Glioblastoma multiforme comprises up to 50% of all adult brain tumors and is currently treated with surgical resection, radiation therapy, and chemotherapy. However, despite the multimodality therapy, the majority of patients survive <2 years due to tumor recurrence, warranting continued exploration of additional new strategies to improve patient survival. In this report, we describe a novel, tumor specific, targeted immunotherapy that is based on genetic engineering of human T cells to redirect their killing specificity against EGFRvIII expressing glioblastoma cells.

EGFRvIII is a mutant, oncogenic form of the EGF receptor that is expressed on the cell surface in ~30% of malignant gliomas [1]. Because it is not expressed on normal tissues, EGFRvIII represents a potential target for immunotherapy. Currently, targeting of EGFRvIII in glioma using immunotoxin and/or vaccination strategies is being actively pursued in both preclinical studies and clinical trials in other laboratories [2,3].

The conventional approach of generating large quantities of native, antigen specific T cell lines or clones derived from patients for adoptive cancer immunotherapy is often labor intensive and time-consuming, if not impossible due to the lack of or extremely low frequency of antigen specific T cells in the patient's circulation. As an alternative, adoptive immunotherapy for cancer with genetically modified T cells has also been shown to be a promising strategy [4]. T cells can be genetically engineered via viral or plasmid vectors to express either native [4] T cell receptors (TCR; comprised of TCR alpha and beta chains) or chimeric immune receptors (CIR). CIRs (also known as chimeric T cell receptors, artificial T cell receptors or T bodies [5]) are generally comprised of a single polypeptide chain encoding a tumor associated antigen-binding domain (e.g., single chain antibody), a transmembrane linker and a cytoplasmic TCR signaling domain 1 (e.g., CD3 ζ). CIRs bind to their target antigens in an MHC independent manner, which is an advantage over native TCRs that are MHC restricted. This MHC independent recognition permits CIRs to (1) overcome the potential target recognition problem associated with the down-regulation of MHC, as significant down-regulation of MHC-I has been described in glioblastomas [6]; and (2) be applied to broader patient population for potential immunotherapy. T cells expressing such antibody based CIRs by gene modification have been shown to induce cytokine secretion [7–9], proliferation [7,9], and cytotoxicity [9, 10] against antigen-expressing targets.

We tested the hypothesis that it is possible to genetically engineer T cells to kill glioblastoma cells that express EGFRvIII. In this report, we describe the gene transfer of an EGFRvIII-specific CIR into peripheral blood mononuclear cells and a subsequent selection process for expansion and enrichment of CIR-expressing T cells. Furthermore, we outline a methodology for producing high numbers of EGFRvIII-specific immune effectors and describe the testing and efficacy of these effectors against EGFRvIII expressing glioma in vitro and in vivo. We also discuss the limitations of this approach and future directions.

Materials and methods

Anti-EGFRvIII CIR designs and construction

Three different CIRs specific to EGFRvIII were constructed by standard molecular biology techniques. The MR1- ζ CIR consists of the MR1 single chain antibody (scFv) against EGFRvIII [11], linked to the hinge and transmembrane portion of human CD8 α that is fused to the intracellular signaling domain of human CD3 zeta (ζ) chain (Fig. 1a). MRB- ζ represents a binding mutant of the original MR1- ζ CIR, which has a 228-base pair deletion in the heavy chain of MR1. This deletion includes the 33 base pair heavy chain complementarity determining region 3 (V_HCDR3) which is important for binding of the MR1 scFv to EGFRvIII [12] (Fig. 1b). MR1-del ζ is a signaling defective version of MR1- ζ (Fig. 1c). It differs from MR1- ζ in that the cytoplasmic portion of the ζ chain is deleted. The first intracellular TAC codon [Y] was substituted with a TGA stop codon as described previously [13]. A c-myc epitope tag [EIKLISEED] was inserted into each of the three CIRs between the C-terminus of the MR1 scFv and the N-terminus of the CD8 α element for monitoring cell surface expression of the CIRs. The plasmid-based CIR-expression cassettes were constructed by standard fusion polymerase chain reaction (PCR) and were ligated into the multiple cloning site of the mammalian plasmid expression vector pMG (Invivogen, San Diego, CA) that was modified to co-express hygromycin phosphotransferase-HSV thymidine kinase (HyTK) selection/suicide fusion gene [14]. The transcription of the CIRs is driven by a modified human Elongation Factor-1 α (EF1 α) promoter whereas the expression of HyTK fusion-protein is controlled by human cytomegalovirus (CMV) promoter. The pMG-HyTK expression vector was a generous gift of Dr. Michael C. Jensen (City of Hope National Medical Center, Duarte, CA).

Cell lines and cell cultures

Target cell lines—The human glioma cell line U87MG was obtained from American Type Culture Collection (ATCC, Rockville, MD). The U87vIII cell line, an EGFRvIII expression derivative of U87, was a kind gift of Dr. Xandra Breakfield (Massachusetts General Hospital, Boston, MA). The U87vIII-FFlucZeo cell line was generated in our laboratory by infecting the original U87vIII parental cell line with replication-defective lentivirus encoding for Firefly Luciferase-Zeocin fusion-protein, which was produced using a five-plasmid transfection procedure as described previously [15].

Effector cells—Human peripheral blood mononuclear cells (PBMCs) were obtained from healthy donors via Ficoll gradient separation. MR1- ζ , MRB- ζ , or MR1-del ζ CIR encoding plasmid DNA was linearized by *PacI*, (New England Biolabs, Inc, Ipswich, MA), purified and resuspended in endotoxin-free buffer TE (Qiagen Inc., Valencia, CA) at a concentration of 1.5 μ g/ μ l. A total of 10 μ g linearized plasmid DNA was nucleofected according to the manufacturer's protocol (Nucleofector I, Amaxa Biosystems, Gaithersburg, MD) into 5×10^7 freshly isolated PBMCs. Nucleofected cells were then cultured in T cell medium consisting of RPMI-1640 (Cambrex BioScience, Walkersville, MD) supplemented with 10% heat inactivated fetal bovine serum (HyClone, Logan, UT) and 100 U/ml penicillin and 100 μ g/ml streptomycin (HyClone Logan, UT) in repeated 14-day stimulation cycles up to 8 cycles.

Stimulation cycles began on day 0 with T cells activation by 30 ng/ml OKT-3 (a monoclonal antibody against human CD3, Ortho Biotech, Bridgewater, NJ) and irradiated feeder cell supplementation. The initial stimulation cycles immediately following nucleofection were done in the absence of feeder cells. 50 IU/ml recombinant human IL-2 (Chiron, Emeryville, CA) was given to the cultures every other day starting on day 1. Cultures were selected on hygromycin (Invivogen, San Diego, CA) in the presence of irradiated lymphoblastoid cell lines (LCL, 8,000 rads) and PBMC (3,500 rads) feeder cells. Cultures were split every 2–3 days to maintain $1\text{--}2 \times 10^6/\text{ml}$ approximate cell density. LCL were a generous gift of Dr. Michael C. Jensen (City of Hope National Medical Center, Duarte, CA).

Detection of gene expression

RT-PCR—Gene expression was measured by reverse transcription-PCR (RT-PCR), using primers to amplify a 496 base pairs (bp), a 886 bp and a 327 bp DNA fragments of the three CIRs (MR1- ζ , MRB- ζ and MR1-del ζ) encoding constructs respectively. Total RNA was extracted from CIR-nucleofected and non-nucleofected human PBMCs on days 10–14 of stimulation cycles 2–10, using Absolutely RNA Miniprep Kit (Stratagene, La Jolla, CA) according to the manufacturer's protocol. To eliminate residual contaminating DNA, 1 U of RNase-free DNase I (Stratagene, La Jolla, CA) was added to each sample during the RNA purification procedure. Reverse-IT One Step Kit (ABgene, Rochester, NY) was employed for RT-PCR. Each reaction contained 1.2 μg of total RNA as a template and one of specific pairs of sense and antisense primers at a concentration of 10 μM . The three pairs of primers are: for detection of MR1- ζ , sense 5'-CGT GCC TCT TAC ATT CGG TGA T-3' and antisense 5'-CCT CCG CCA TCT TAT CTT TCT G-3'; for MRB- ζ , sense 5'-CTC TCC TG GTA ACC-3' and antisense 5'-CCT CCG CCA TCT TAT CTT TCT G-3'; for MR1-del ζ sense 5'-CGT GCC TCT TAC ATT CGG TGA T-3' and antisense 5'-TAT CGC TCA GCG CGC GGG AGG CTC TGC GCT-3'. As a positive control, an RNA fragment encoding β -Actin cellular sequence was also amplified simultaneously but in separated tubes using a pair of primers, sense 5'-TGA CGG GGT CAC CCA CAC TGT GCC CAT CT-3' and antisense 5'-CGA AGC ATT GCG GTG GAC GAT GGA GC-3'. RT-PCR products were analyzed by agarose gel separation and photographing.

Flow cytometry

Target cell lines—The expression of wild-type epidermal growth factor receptor (wtEGFR) and EGFRvIII was determined on the glioma cell line U87MG and its derivatives U87vIII and U87vIIIFluc-Zeo by flow cytometry. WtEGFR and the EGFRvIII were detected by first staining the cells with anti-EGFR mouse IgG1 monoclonal antibody (Abcam, Cambridge, MA) and L8A4 anti-EGFRvIII mouse IgG1 monoclonal antibody (generous gift of Dr. Bigner, Duke University, Durham, NC), respectively, and then followed by R-phycoerythrin (R-PE) conjugated rat anti-mouse IgG1 monoclonal antibodies (BD Pharmingen, San Jose, CA) prior to flow cytometry.

Effector cells—Surface expression of the CIRs on effector T cells was investigated by detection of the embedded c-myc tag via flow cytometry. Cells were stained with mouse IgG1, κ monoclonal antibody against the c-myc epitope [9E10] (Abcam, Cambridge, MA) followed by R-PE conjugated rat anti-mouse IgG1 monoclonal secondary antibodies (BD Pharmingen, San Jose, CA). MOPC-21 mouse IgG1, κ (GeneTex, San Antonio, TX) antibody was used as an isotype control for the [9E10] antibody. The distribution of CD4⁺ and CD8⁺ T cells in the freshly isolated PBMC or cultured, CIR-transfected cell populations was determined by flow cytometry using allophycocyanin (APC) conjugated anti-human CD4 [RPA-T4] and fluorescein isothiocyanate (FITC)-conjugated anti-human CD8 [RPA-T8] monoclonal antibodies (BD Pharmingen, San Jose, CA). Samples were collected by Guava EasyCyte™

Mini using CytoSoft™ software (Guava Technologies, Inc., Hayward, CA). Data were analyzed by FlowJo® (FlowJo LLC, Ashland, OR).

Functional analysis

Cytotoxicity assay—In vitro cytolytic capacity of CIR+ T cells was measured by DELFIA europium-DTA cytotoxicity assay (PerkinElmer, Wellesley, MA). Briefly, tumor target cells were loaded with 0.1 μ l/10⁶ cells of fluorescence enhancing ligand, BATDA, for 30 min at 37°C, then washed 5 times with 0.02 mg/ml PBS-mitomycin-C (Fisher BioReagent, Fair Lawn, NJ). The labeled target cells were then added to V-bottom tissue culture plates (Corning Inc., Corning, NY) at a density of 10⁴ cells/well. CIR+ T cells were then added to each well at various effector-to-target ratios (E/T) at a final volume of 200 μ l/well. To determine the maximum and spontaneous lysis, BATDA loaded target cells were mixed with 100 μ l of 200 proof absolute ethanol (Sigma-Aldrich, Inc., St Louis, MO) or T cell medium, respectively. Following a 2-h incubation in a humidified 5% CO₂ incubator at 37°C, plates were centrifuged at 1,700 RPM for 10 min, before 20 μ l supernatant from each well was transferred to a DELFIA Microtitration Plate. 180 μ l of DELFIA europium solution was added to each well then the plates were read by a VICTOR3™ Multilabel Counter (PerkinElmer, Inc. Wellesley, MA).

Cytokine assays—A total of 2×10^5 irradiated (8,000 rads) target cells (U87MG, U87vIII, U87vIII-FFLuc-Zeo) were co-cultured with 2×10^6 CIR+ T cells in a 24-well tissue culture plate (Corning Inc., Corning, NY) containing 2 ml of T cell media. After 72 h of incubation in a humidified 5% CO₂ incubator at 37°C, culture supernatants were assayed for cytokine secretion by cytometric bead array using the Human Th1/Th2 Cytokine kit (BD Pharmingen, San Jose, CA). In short, cytokine-capture beads were labeled with PE-conjugated detection antibody and mixed with recombinant standards or cell culture supernatant. The standards and supernatants were then incubated for 3 h at room temperature and washed. Data was acquired on BD FACSCalibur™ flow cytometer using BD CellQuest™ software and analyzed with the BD™ CBA software (BD Pharmingen, San Jose, CA).

In vivo experiments

Six to eight week-old, female, NOD/NCrCrI-PrkdcSCID mice were obtained from Charles River Laboratories (Wilmington, MA). Animals were kept under the accordance of protocols approved by the Institutional Animal Care and Use Committee (IACUC) of Massachusetts General Hospital, with standard animal care requirements. On experimental day 0, mice underwent intracranial (*i.c.*) implantation of 5×10^4 U87vIIIFLuc-Zeo target and 1×10^6 effector cells in 2 μ l PBS using the stereotactic coordinates as 0.5 mm posterior, 2.5 mm lateral, and 3.5 mm intraparenchymal from the bregma. Target and effector cells were mixed in a separate microcentrifuge tube immediately before each implantation. Tumor progression was followed every 3–5 days up to 50 days when the study was terminated by bioluminescence imaging (BLI) as previously described [16]. In a second in vivo experiment, mice were implanted on Day 0 with 50,000 U87-EGFRvIII tumor cells and a single intracranial inoculation of 1 million CIR+ T cells was performed 3 days later. For BLI, the mice were injected intraperitoneally (*i.p.*) with 150 mg D-luciferin/kg (Xenogen Corp., Hopkinton, MA) 10 min prior to imaging. Photon emission (photons/sec/cm²) was recorded every 5 min until peak emission value was obtained and a decrease in emission was observed.

Statistical methods

Differences in group percentage cytotoxicity and bioluminescence were evaluated by one-way analysis of variance (ANOVA) testing.

Results

Expression of anti-EGFRvIII CIRs by T cell transfectants

Human PBMCs were transfected with linearized plasmid DNAs encoding one of the three anti-EGFRvIII CIRs (MR1- ζ , MRB- ζ and MR1-del ζ) and expanded over several biweekly cycles (up to 8 cycles) as described above. RTPCR based expression analysis starting on stimulation cycle 3 yielded a distinct fragment corresponding to the expected size from each of the three CIR-samples (Fig. 2): a 496 bp fragment from the MR1- ζ samples (Fig. 2a), a 886 bp fragment from MRB- ζ (Fig. 2b) and a 304 bp fragment from MR1-del ζ (Fig. 2c). The results confirmed the expression of the each CIRs mRNA transcripts in nucleofected T cells. We confirmed cell surface expression of each the CIRs in nucleofected and cultured T cells by flow cytometry analysis. Detection of the c-myc epitope (located extracellularly between the C-terminus of the scFv and the N-terminus of the CD8 α hinge region in each construct) by flow cytometry revealed that approximately 30–50% of the viable PBMCs expressed correspondent CIRs after day 10 between stimulation cycles 3–8 (Fig. 3). Flow cytometric analysis was not attempted in earlier stimulation cycles because of limited cell numbers. Flow cytometric analysis of CD8 expression status showed that the transduced T cells were 95–99.9% CD8+ after three stimulation cycles, without significant increase in CD8 expression after multiple stimulation cycles (data not shown).

MR1- ζ transfected T cells secreted IFN- γ upon engagement with EGFRvIII+ targets

To determine whether MR1- ζ CIR+ T cells become activated to exhibit effector functions upon engagement with EGFRvIII expressing target cells, a cytokine secretion assay was conducted. Effector cells, nucleofected with each of the three CIRs were co-incubated with irradiated EGFRvIII-negative U87MG (Fig. 4a) or EGFRvIII-positive U87vIIIffluc-Zeo (Fig. 4b) glioblastoma cell lines. The MR1- ζ CIR+ T cells secreted a detectable amount of IFN- γ in comparison to binding and signaling mutant controls upon engaging EGFRvIII+ tumor cells but not tumor cells that expressed only wtEGFR (Fig. 4c). MR1-del ζ or MRB- ζ cells had negligible IFN- γ secretion (Fig. 4c). These results suggested that both antigen-binding and signaling functions of an anti-EGFRvIII CIR are essential for cytokine (e.g., IFN- γ) secretion by CIR+ anti-EGFRvIII T cells. We did not observe significant IL-2, IL-4, TNF- α , or IL-10 production upon antigen engagement (data not shown).

In vitro cytotoxicity assay demonstrated specific recognition and effective killing of EGFRvIII-positive tumor cells by MR1- ζ + T cells

CIR+ effector cells were also tested for their capacity in killing EGFRvIII expressing target tumor cells, a critical hallmark of cytotoxic activity of anti-tumor effector cells. MR1- ζ + effector cells effectively lysed EGFRvIII expressing target cells (U87vIIIffluc-Zeo) as determined by europium release assay. In contrast to negligible lysis of wild-type EGF receptor-expressing target cells (U87MG) by MR1- ζ + effector cells (Fig. 5a), specific lysis of U87vIIIffluc-Zeo cells were observed at E/T ratios of 50:1, 25:1, and 5:1; 93, 87, and 31%, respectively (Fig. 5b). In addition, MRB- ζ + or MR1-del ζ + T cells exhibited negligible lysis activity to target cells expressing the wild-type EGF receptor or EGFRvIII at all E/T ratios tested, mentioned above (Fig. 5). These results clearly demonstrated that genetically engineered T cells expressing the MR1- ζ CIR exhibited effective and specific cytotoxic activity against EGFRvIII expressing target tumor cells.

In vivo administration of MR1- ζ + T cells delayed the propagation of EGFRvIII expressing tumor formation and prolonged survival of mice bearing tumor xenografts

We investigated the in vivo therapeutic effects of MR1- ζ + T cells in tumor-implanted on a NOD/SCID mouse model. In this model, U87-EGFRvIII + luciferase + tumor cells were

implanted and tumor growth was monitored by BLI. In an admixture experiment (Fig. 6a), we noticed significant delay in tumor propagation in the MR1- ζ + treatment group compared to both MRB- ζ + and MR1-del ζ + control groups, as detected by serial bioluminescent recordings of tumor cell photon emissions (Fig. 6a). The suppression of tumor growth was evident as early as day 1 and continued throughout the experiment. The delay of tumor growth in the MRB- ζ + group was intermediate between the MR1-del ζ + (most rapid tumor growth) and the MR1- ζ + treatment group (slowest growth) which may be explained by the fact that this receptor maintains an intact signaling domain with partial abrogation of binding capacity of the MRB- ζ binding region to the EGFRvIII protein.

In a pre-existing tumor experiment (Fig. 6b), all mice which received a T cell implantation (one million cells delivered day 3 after implantation) demonstrated some inhibition of growth compared to PBS treated animals. However, the delay in tumor growth was significantly less pronounced than observed with the co-implantation experiment and all mice succumbed to tumor.

Discussion

In this report, we have demonstrated that human T cells can be specifically redirected to lyse EGFRvIII-positive glioma cells by genetic introduction of a chimeric T cell receptor based on the MR1 scFv [11]. Upon engagement with EGFRvIII expressing target cells, the MR1- ζ transfected human T cells were able to secrete IFN- γ and specifically lysed tumor target cells. In addition, it was shown that both specific binding and intact TCR signaling was required as the full-length MR1- ζ CIR showed better cytotoxicity, cytokine secretion, and in vivo tumor growth control compared to CIR's with reduced EGFRvIII binding (MRB- ζ) or defective signaling (MR1-del ζ). However, the in vivo effect was limited after a single co-administration of CIR+ T cells with tumor or single treatment of pre-existing tumor; and all mice succumbed to disease.

Our results, that genetically engineered T cells expressing MR1- ζ exhibited efficient in vitro cytotoxicity but limited in vivo anti-tumor activity, correlate well with prior reports describing CIRs that incorporate TCR ζ signaling alone in their signaling portion. CIRs that are based on CD3 ζ signaling alone do not provide for natural co-stimulation that might occur with typical antigen presentation via MHC. T cells expressing this form of monopartite CIRs have limited antigen-mediated cytokine production [17] and survival after antigen encounter. Indeed, the relatively low level of IFN- ζ and negligible IL-2 production observed in our experiments is likely reflective of the lack of co-stimulation. Second generation receptors which incorporate co-stimulation into their signaling domain have been shown to improve IFN- ζ and IL-2 secretion [18]. While it has been reported that in vivo co-stimulation may not be required if CIR-expressing T cells are provided in sufficient numbers [19] it is likely that in clinical settings repeated dosing and cytokine support may be necessary for sustainable activity.

These pre-clinical results provide a proof of principle that we are able to genetically engineer and redirect human T cells against EGFRvIII expressing glioblastoma in vitro and in vivo. A variety of factors that may affect overall efficacy of a CIR have been identified, including the type of scFv chosen [20], the size of the extracellular region between the scFv and the signaling domain [20,21] and finally the type and combination of signaling domain(s) [18,22,23].

To improve the sustainability of this immunotherapy effect in vivo, we are currently testing new generations of the anti-EGFRvIII CIR signaling designs that consist of TCR (e.g., CD3 ζ and co-stimulatory (e.g., CD28 alone or plus OX40) signaling. Similar CIR designs consisting of bipartite (e.g., including CD28 and CD3 ζ) [18,24] or tripartite (e.g. CD28, OX40, and

TCR ζ) signaling domains [18] have been developed by others and are in preclinical testing, demonstrating an improvement in terms of survival of gene modified T cells [25].

Besides EGFRvIII, other cell surface markers have been exploited as targets for glioblastoma immunotherapy with genetically engineered T cells in adoptive transfer approaches. Kahlon et al. showed that a CIR (IL-13 zetakine) based on a mutant membrane tethered IL-13 cytokine can effectively redirect T cells to lyse IL-13R2 α expressing gliomas [26]. Human T cells expressing this IL-13 zetakine is currently being tested in clinical studies (M. Jensen, personal communication).

This report does not address the potential engraftment of an adoptively transferred T cell population into an immunocompetent or partially immunosuppressed host. Morgan et al. showed that engraftment of gene-engineered T cells in melanoma patients was best achieved by immune depletion prior to adoptive transfer [4]. They further showed that minimization of ex vivo culturing of gene-engineered T cells was critical to achieve robust and persistent engraftment of the adoptively transferred T cells. In two patients after adoptive transfer of genetically engineered, anti-MART-1, autologous peripheral blood T cells in the setting of a partial myeloablation; 20–70% of circulating T cells expressed the genetically transferred TCR at 1 year after adoptive transfer.

The exact conditions for effective, adoptive transfer of anti-EGFRvIII expressing T cells for glioma immunotherapy, such as whether to myeloablate recipient patients, provide exogenous cytokine support, or perform simultaneous EGFRvIII directed vaccination will require additional clinical investigation. Other conditions for transfer also remain to be tested such as the comparison of direct intraparenchymal transfer of gene modified T cells versus intravenous administration of these cells, and exactly when, in a patients clinical course (before or after radiation therapy; temozolamide chemotherapy, or in relation to anti-angiogenic therapy) gene modified cell transfer is best undertaken. It is known that high-grade glial tumors pose special challenges to cellular adoptive immunotherapy as both the glioma microenvironment and the patient's systemic immune status are tilted to favor immune evasion rather than detection. These immune evasion mechanisms include the secretion of TGF- β locally [27], down-regulation of MHC-I on glioblastoma cells [6], and upregulation of the immunosuppressive CD4 + CD25 + Treg component of the immune system [28]. Despite these hurdles, investigators have been able to mobilize the CD8+ effector arm against EGFRvIII+ glioma in vivo via direct EGFRvIII-derived peptide based vaccination an approach currently under Phase II/III study [29]. Jiang et al. reported that improved anti-tumor effect was observed when vaccination was combined with adoptive transfer of CIR-T cells, possibly due to the improved anti-tumor T cell expansion and trafficking [30]. The availability of EGFRvIII-based vaccine and genetically engineered anti-EGFRvIII T cells raises the possibility of testing multimodality targeted immunotherapies in an effort to maximize anti-tumor effect in glioblastoma patients.

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Figure 1.

Schematic representation of the three CIR constructs. MR1-ζ (**a**) represents the full-length anti-EGFRvIII CIR based on the MR1 scFv linked to partial extracellular domain (hinge) and transmembrane domain (TM) of CD8 α that is fused with the cytoplasmic domain (IC) of human CD3ζ. MRB-ζ (**b**) is a binding mutant variant of MR1-ζ that has a 76 amino acid-deletion in the heavy chain. MR1-delζ (**c**) represents a mutant version of MR1-ζ in which the intracellular signaling domain of human CD3ζ has been truncated

Figure 2.

RT-PCR analysis of the anti-EGFRvIII-CIRs transgene expression. RT-PCR analysis of the RNA expression of MR1- ζ (a), MRB- ζ (b), and MR1-del ζ (c) in corresponding nucleofected PBMCs indicated successful transcription of the three CIRs. Total RNA was extracted from CIR-nucleofected PBMCs, and was amplified in the presence of RNase-free DNase I with and without RT-ase. RT-PCR products were analyzed on 1% agarose gel and photographed

Figure 3.

Flow cytometry analysis of the surface expression of the anti-EGFRvIII-CIRs. Surface expression of MR1- ζ (**a**), MRB- ζ (**b**), and MR1-del ζ (**c**) on corresponding nucleofected and hygromycin selected PBMCs was detectable after two stimulation cycles. Live cells were gated for analysis. Dashed lines represent cells stained with isotype control whereas solid lines illustrate cells stained with anti-c-myc [9E10] monoclonal antibody. Results indicated that 43.4% of MR1- ζ , 49.5% of MRB- ζ , and 49.7% MR1-del ζ nucleofected PBMCs expressed the corresponding CIR on the cell surface

Figure 4.

EGFR and EGFRvIII expression in target cell lines and antigen-mediated cytokine secretion by CIR+ T cells. Cell surface expression of EGFR and EGFRvIII was determined by flow cytometry analysis in U87MG (a), U87vIII-FFlucZeo (b) and U87vIII (data not shown) cell lines. Cells were stained with anti-EGFR monoclonal antibody for wild-type receptor expression (solid lines) or with L8A4 anti-EGFRvIII monoclonal antibody for EGFRvIII expression (dashed lines). Live cells were gated for analysis. Flow cytometry analysis showed that U87MG cells expressed wild-type EGFR but not the mutant EGFRvIII, whereas U87vIII-FFlucZeo and U87vIII cells expressed both the wild-type EGFR and the mutant EGFRvIII on the cell surface. 2×10^5 irradiated target cells (U87MG, U87vIII, and U87vIII-FFlucZeo) were co-cultured with 2×10^6 CIR+ T cells for 72 h (c). Culture supernatants were harvested and assayed for cytokine secretion by cytometric bead array. Only T cells expressing the full-length MR1- ζ secreted significant amounts of IFN- γ when co-cultured with EGFRvIII-positive cell lines; U87vIIIFFluc-Zeo (213 pg/ml) and U87vIII (125 pg/ml). No significant amount of IFN-

γ was detectable when CIR⁺ T cells were co-cultured with the wild-type, EGFRvIII-negative U87MG cells. T cells expressing either forms of the mutant MR1- ζ CIR (MRB- ζ , and MR1-del ζ) did not secrete IFN- γ when co-cultured with target cells

Figure 5.

In vitro cytolytic activity of the CIR+ T cells. MR1- ζ , MRB- ζ , or MR1-del ζ CIR+ T cells were co-incubated with BATDA loaded wild-type, EGFRvIII-negative U87MG (a) or EGFRvIII-positive U87vIII-FFlucZeo (b) target cells at varying effector to target ratios for 2 h. Cytolytic activity of the CIR+ T cells was analyzed by europium release assay. Effector cells in their third stimulation cycle were used in this assay. Cytolytic capacity of the CIR+ T cells was expressed by specific lysis percentage of target cells. MR1- ζ CIR+ T cells exhibited significant cytotoxicity against EGFRvIII expressing target cells, but not against cells that only expressed the wild-type EGF receptor. MRB- ζ and MR1-del ζ expressing T cells showed no significant cytolytic activity against either the wild-type or the EGFRvIII-positive target cells as indicated by one-way ANOVA ($P < 0.001$)

Figure 6.

In vivo anti-tumor activity of anti-EGFRvIII CIR-T cells. **(a)** A total of 2.5×10^4 U87-FFLuc-EGFRvIIIZe tumor cells were admixed with CIR+ T cells in a ratio of 1:25 immediately prior to implantation in NOD/SCID mice at day 0. Cells were implanted intracranially (*i.c.*) in the right frontal lobe. Mean BLI (photons/s $\times 10^6$) is presented for each group of mice ($n = 4$ per group) at indicated time points. Mice were observed as long as 42 days; group means were calculated for the duration when no mice were lost to follow-up (through day 29) and were compared by one-way ANOVA (see results). **(b)** 5×10^4 U87-FFLuc-EGFRvIIIZe tumor cells were implanted *i.c.* in NOD/SCID mice at day 0. At day 3, a single *i.c.* inoculation of 1 million CIR+ T cells was performed. Mice were observed as long as 17 days; group means

were calculated for the duration when no mice were lost to follow-up (through day 13) and were compared by one-way ANOVA (see results). Effector cells in their third stimulation cycle were used in both assays