Bionatura Journal
Ibero-American Journal of Biotechnology and Life Sciences

Review

# CRISPR-Cas9–Enhanced CAR-T Cell Engineering for Glioblastoma: Mechanisms, Preclinical Advances, and Emerging Clinical Applications

*Saúl Emilio Guamba Cajas*[1], *Diana Estefanía Paucar Fiallos*[1], *Arianna Rafaela Castillo Guevara*[1], *Esteban Leonardo Guamba Cajas*[1]
[1] Universidad Yachay Tech/ Urcuqui/ Ecuador.

* *Correspondence: saul.guamba@yachaytech.edu.ec ; Tel.:: +593 99 895 0919*



## ABSTRACT

Glioblastoma multiforme (GBM) remains one of the most aggressive and treatment-resistant human cancers, characterized by highly infiltrative growth, extensive cellular heterogeneity, and a profoundly immunosuppressive microenvironment. Although CAR-T cell therapy has revolutionized the treatment of hematologic malignancies, its clinical impact in GBM has been limited by antigenic escape, poor tumor infiltration, T-cell exhaustion, and significant toxicity risks. Recent advances in CRISPR-Cas9 genome editing offer unprecedented opportunities to overcome these barriers by enabling precise, multiplex genetic reprogramming of T cells.

In this review, we synthesize current progress in CRISPR-enhanced CAR-T engineering for GBM, focusing on strategies to overcome immune checkpoint suppression, optimize metabolic fitness, enhance trafficking across the blood–brain barrier, reduce neuroinflammation-associated toxicities, and generate universal allogeneic CAR-T products. We also compare the genomic target spaces of candidate guide RNAs (crRNA, d10r10, X37) and highlight their predicted off-target profiles relevant to GBM therapeutic design. Preclinical studies demonstrate that CRISPR-edited CAR-T cells targeting EGFRvIII, IL-13Rα2, HER2, and B7-H3 significantly enhance survival in murine GBM models, while emerging clinical trials indicate acceptable safety and early evidence of anti-tumor activity.

We further discuss technological innovations—including base editing, prime editing, CRISPRi/a, non-viral delivery platforms, and precision-medicine–guided CAR design—as well as regulatory, ethical, and manufacturing considerations required for clinical translation. Collectively, these advances underscore the transformative potential of CRISPR-engineered CAR-T therapies to reshape GBM treatment and pave the way toward more effective and accessible cellular immunotherapies.

**Keywords.** Glioblastoma multiforme (GBM), CAR-T cell therapy, CRISPR-Cas9 genome editing, T-cell engineering, Immune checkpoint resistance, Tumor microenvironment, Guide RNA design, EGFRvIII, IL-13Rα2, Universal allogeneic CAR-T cells, Preclinical models, Precision immunotherapy

## INTRODUCTION

Glioblastoma multiforme (GBM) is the most frequent and deadliest primary brain tumor in adults, with a median overall survival that rarely exceeds 15 months despite standard multimodal therapy[1,4]. Its highly infiltrative growth pattern, marked cellular heterogeneity, and profoundly immunosuppressive tumor microenvironment pose formidable obstacles to conventional treatments[2,5].

Chimeric antigen receptor T-cell (CAR-T) immunotherapy, which has achieved remarkable success in hematologic malignancies, faces major challenges in solid tumors such as GBM. Among the key barriers are the blood–brain barrier, antigenic heterogeneity, and multiple immune escape mechanisms[3,6].

In parallel, CRISPR-Cas9 technology has emerged as a transformative tool for cellular engineering, enabling precise genetic modifications that can augment the therapeutic potency of CAR-T cells[7,8]. This review brings together recent advances in CRISPR-Cas9–mediated reprogramming of CAR-T cells, with a focus on strategies to overcome the dynamic immunosuppression of the GBM microenvironment and move towards its eradication. We discuss molecular mechanisms, preclinical evidence, challenges in clinical translation, and future perspectives, drawing on more than 100 studies published over the last 5 years.

## Immunosuppressive mechanisms in the GBM microenvironment

Glioblastoma multiforme (GBM) establishes a highly sophisticated immunosuppressive niche that effectively blunts anti-tumor immunity[9,10]. Multiple interconnected suppressive pathways shape this microenvironment.
First, there is profound suppression of cellular immune activity, as shown by the abundant infiltration of regulatory T cells (Tregs), tumor-associated macrophages (TAMs) with an M2-like phenotype, and myeloid-derived suppressor cells (MDSCs). These populations secrete immunosuppressive cytokines such as TGF-β, IL-10, and prostaglandin E2[11,12].
Second, immune checkpoint pathways are upregulated through the overexpression of inhibitory ligands such as PD-L1, galectin-3, and HLA-G. By engaging their receptors on T cells, these ligands drive functional exhaustion and anergy[13,14].
In addition, altered tumor metabolism contributes to immunosuppression by competing for essential nutrients (e.g., glucose and glutamine), acidifying the microenvironment through lactic acid production, and accumulating immunosuppressive metabolites such as adenosine[15,16].
Finally, physical barriers—including a dense extracellular matrix, complex cytokine networks, and the blood–brain barrier itself—severely restrict the infiltration of effector immune cells and the access of therapies to the tumor bed[17,18].

| Immunosuppressive mechanism | Effect on immunity | CAR-T/CRISPR strategy | Expected outcome | Ref. |
|---|---|---|---|---|
| PD-L1 expression | T-cell exhaustion | PD-1 knockout | Restoration of effector function | 35 |
| TGF-β secretion | Treg differentiation | Expression of dominant-negative TGF-βR | Resistance to suppression | 11 |
| Adenosine metabolism | Inhibition of TCR signalling | A2AR deletion | Improved activation | 39 |
| Microenvironment acidification | Impaired cytotoxicity | Expression of pH transporters | Preserved function at low pH | 40 |
| Blood–brain barrier | Physical exclusion | Expression of adhesion molecules | Enhanced tumor infiltration | 18 |

**Table 1. Main immunosuppressive mechanisms in GBM and counteracting strategies using edited CAR-T cells.**

## Development and limitations of conventional CAR-T therapies for GBM

CAR-T cells are T lymphocytes genetically modified to express chimeric receptors that combine an antigen-recognition domain—typically a single-chain variable fragment (scFv)—with intracellular signalling domains derived from native T-cell receptors and costimulatory molecules[1,9].

In GBM, several antigenic targets have been evaluated as entry points for CAR-T therapy. The most extensively studied include:

1. EGFRvIII, a tumor-specific mutation present in 20–30% of GBM cases and valued for its high tumor specificity[20].
2. HER2, a protein frequently overexpressed in solid tumors, including GBM[21].
3. IL-13Rα2, a receptor overexpressed in more than 50% of cases and associated with an invasive phenotype[22].
4. B7-H3, a coinhibitory molecule expressed on tumor cells and tumor vasculature[23].
5. GD2, a glycolipid characteristic of neuroectodermal tumors[24].

Despite the revolutionary impact of CAR-T therapies in hematologic cancers, their application to GBM still faces substantial hurdles, including:

1. Tumor antigen heterogeneity which facilitates escape of subclones that are not recognized.
2. Limited persistence of CAR-T cells within a strongly immunosuppressive tumor microenvironment;
3. Difficulty accessing the central nervous system, due to both physical and physiological barriers;
4. Associated toxicities, such as cytokine release syndrome and neurotoxicity;
5. High cost and operational complexity of manufacturing and validating the cellular product[25,27].

Taken together, these limitations help explain why clinical trials in GBM have produced only modest responses to date, driving interest in more advanced genetic engineering approaches.

## CRISPR-Cas9: principles and applications in T-cell engineering

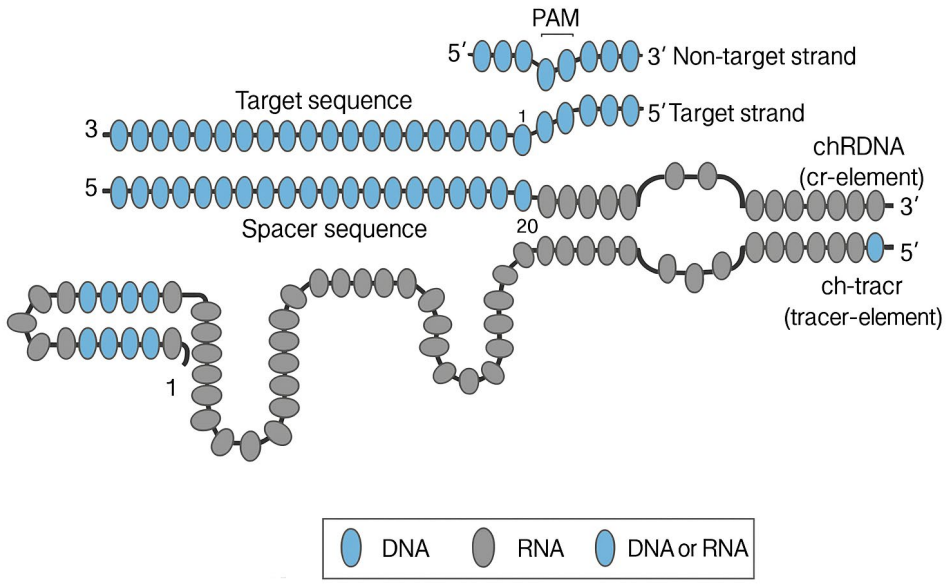


**Figure 1. Schematic representation of CRISPR-Cas9 target recognition showing the target and non-target DNA strands, PAM motif, spacer sequence, and chimeric guide RNA (chR-DNA and tracr elements). Blue beads represent DNA nucleotides, grey beads denote RNA, and dual-colored beads indicate DNA/RNA hybrids.**

The CRISPR-Cas9 system, originally described as an adaptive bacterial defense mechanism, has become a highly precise genome-editing tool[28]. It is built around two key components:

- The Cas9 endonuclease, which generates double-strand breaks in DNA, and
- a single-guide RNA (sgRNA), which directs Cas9 to specific genomic sequences that are preceded by a PAM motif[29].

Induction of a double-strand break activates endogenous DNA repair pathways. The most relevant are:

1. non-homologous end joining (NHEJ), an error-prone process that introduces insertions or deletions and often results in gene disruption; and
2. homology-directed repair (HDR), which enables precise insertion of new sequences when a donor DNA template is provided[30].

In T-cell engineering, CRISPR-Cas9 supports an expanding range of advanced strategies, including:

1. knockout of inhibitory genes such as *PD-1*, *CTLA-4*, *TIM-3* and *LAG-3* to overcome immune checkpoints[31];
2. targeted transgene insertion, for example, integrating CAR constructs into defined loci such as *TRAC* to achieve more uniform and stable expression[32];
3. multiplex editing, allowing simultaneous modification of several genes to confer polygenic traits such as resistance to immunosuppression[33];
4. generation of "universal" T cells, through deletion of the endogenous TCR and HLA molecules to create allogeneic products compatible with multiple recipients[34].

These applications have given rise to a new generation of edited CAR-T therapies designed to overcome the shortcomings of conventional approaches.

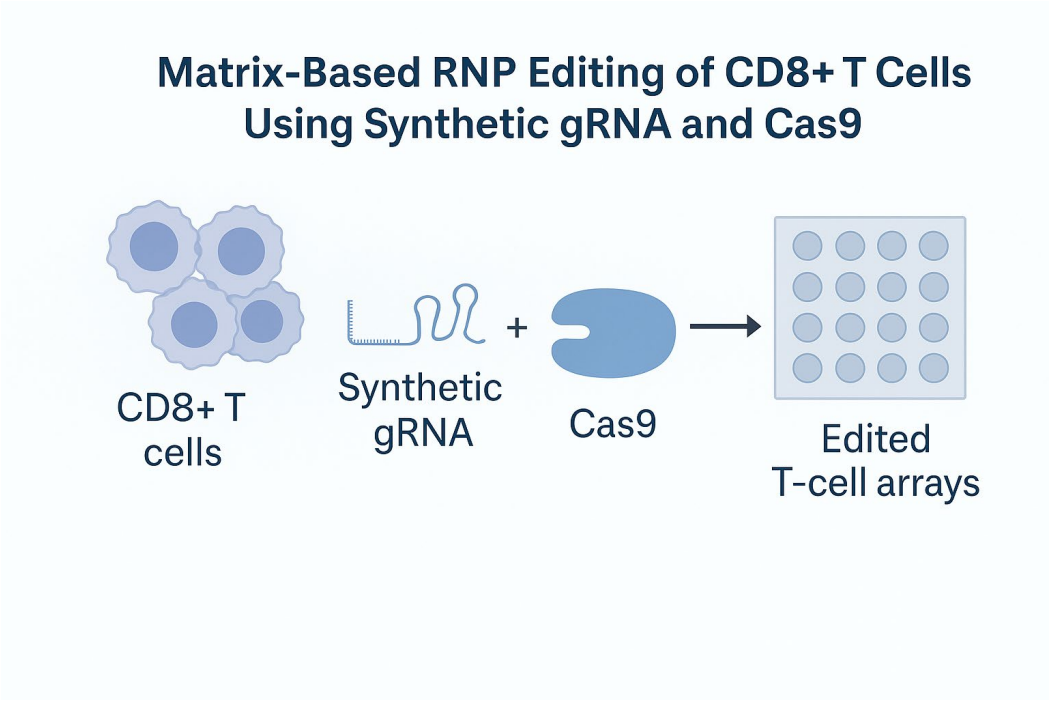


**Figure 2. Workflow of matrix-based RNP editing in CD8+ T cells using synthetic guide RNA (gRNA) and Cas9. CD8+ T cells are combined with synthetic gRNA and Cas9 to generate RNP complexes, which are then delivered using a matrix-based system to produce edited T-cell arrays.**

**Reprogramming strategies with CRISPR-Cas9 for CAR-T cells in GBM**

**Overcoming immune checkpoints**

The GBM microenvironment drives profound T-cell exhaustion through multiple inhibitory pathways. CRISPR-Cas9 enables the simultaneous disruption of several negative regulators, including:

1. PD-1/PD-L1, where knockout of *PDCD1* restores the effector function of CAR-T cells even in the presence of tumor PD-L1[35].
2. LAG-3 and TIM-3, inhibitory receptors co-expressed on exhausted T cells; their deletion enhances proliferation, cytotoxicity, and persistence[36].
3. TIGIT, a receptor that competes with CD226; its editing boosts T-cell activation and resistance to suppressive signals[37].

Preclinical studies indicate that multiplex editing of *PD-1*, *LAG-3,* and *TIM-3* synergistically strengthens resistance to tumor-induced exhaustion[38].

**Modulating cellular metabolism**

Activated T cells rely on a metabolic switch towards robust aerobic glycolysis to sustain effector functions. GBM competes aggressively for nutrients and shapes a metabolically hostile environment, prompting the development of several strategies:

1. A2AR knockout, targeting the adenosine receptor that promotes immunosuppression; its deletion improves CAR-T function in adenosine-rich microenvironments[39].
2. GLUT1 overexpression, which enhances glucose uptake and preserves cellular bioenergetics under hypoglycaemic conditions[40].
3. mTOR pathway modulation, by editing key regulators to fine-tune the balance between effector differentiation and memory formation[41].

Together, these interventions aim to bolster the metabolic resilience of CAR-T cells under the restrictive conditions imposed by the tumor.

**Enhancing infiltration and persistence**

The blood–brain barrier and the tumor extracellular matrix are major obstacles to CAR-T cell trafficking. CRISPR-Cas9 has enabled the introduction of genetic changes that improve infiltration:

1. Ectopic expression of CXCR1/CXCR2, chemokine receptors that guide migration along IL-8 gradients present in GBM[42].
2. Production of proteolytic enzymes, such as matrix metalloproteinases (MMPs), which degrade extracellular matrix components and facilitate tumor penetration[43].
3. Integrin modulation, by enhancing molecules such as LFA-1 or VLA-4, to improve adhesion to cerebral endothelium and extravasation into the tumor[44].

These strategies are intended to secure sustained CAR-T presence within the tumor, a key factor for durable anti-tumor effects.

**Reducing toxicities**

Neurotoxicity and systemic toxicity are major concerns in brain tumor treatment. CRISPR-based engineering has enabled the design of safety control systems, including:

1. Suicide genes, such as inducible caspase-9 (iCasp9), which small molecules can activate to eliminate CAR-T cells in the event of severe toxicity[45], rapidly;
2. higher-specificity CAR designs, using lower-affinity binding domains or Boolean logic (AND/NOT) to increase tumor selectivity and spare healthy tissue[46];
3. GM-CSF knockout, which reduces neuroinflammation and toxicity while maintaining anti-tumor efficacy[47].

These mechanisms aim to improve safety without compromising therapeutic performance.

### Generating universal products

Autologous CAR-T manufacturing is expensive, slow, and highly variable. CRISPR-Cas9 opens the door to allogeneic or "universal" CAR-T products through:

1. TCRαβ deletion, to prevent graft-versus-host disease (GVHD)[48];
2. B2M knockout, which reduces HLA class I expression and limits rejection mediated by host immune cells[49];
3. Insertion of the CAR transgene into the *TRAC* locus, ensuring more uniform, physiologic, and stable expression compared with random integration[50].

These platforms are designed to turn CAR-T therapies into standardized, off-the-shelf products that are more widely accessible.

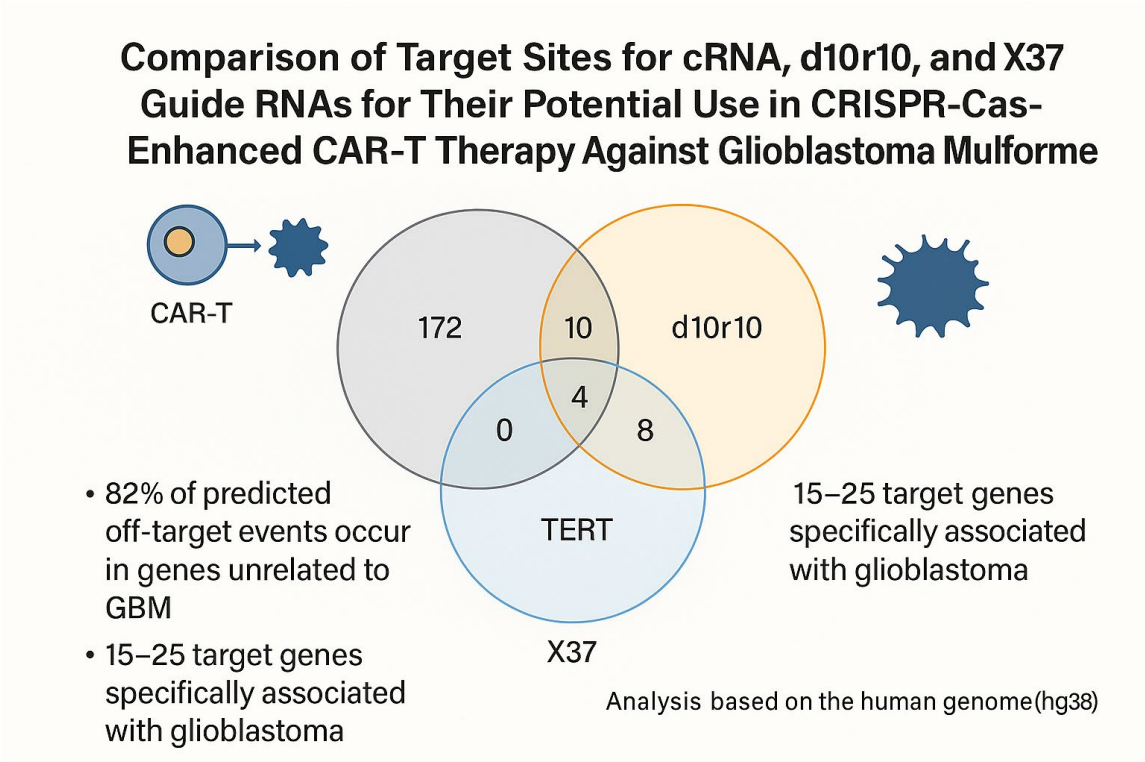


**Figure 3. Venn diagram comparing predicted genomic target sites for crRNA, d10r10, and X37 guide sequences for potential CRISPR-Cas9–enhanced CAR-T therapy against glioblastoma multiforme. Percentages of predicted off-target sites in non-GBM–related genes and the number of GBM-specific target genes (15–25) are shown. Analysis performed using the human genome assembly hg38.**

## Preclinical results and emerging clinical trials

### Preclinical evidence

Mouse models of human GBM have demonstrated the superiority of CRISPR-edited CAR-T cells. In one study, anti-EGFRvIII CAR-T cells with PD-1 knockout significantly prolonged survival, with treated mice surviving up to 90 days compared with 45 days for conventional CAR-T cells[35].

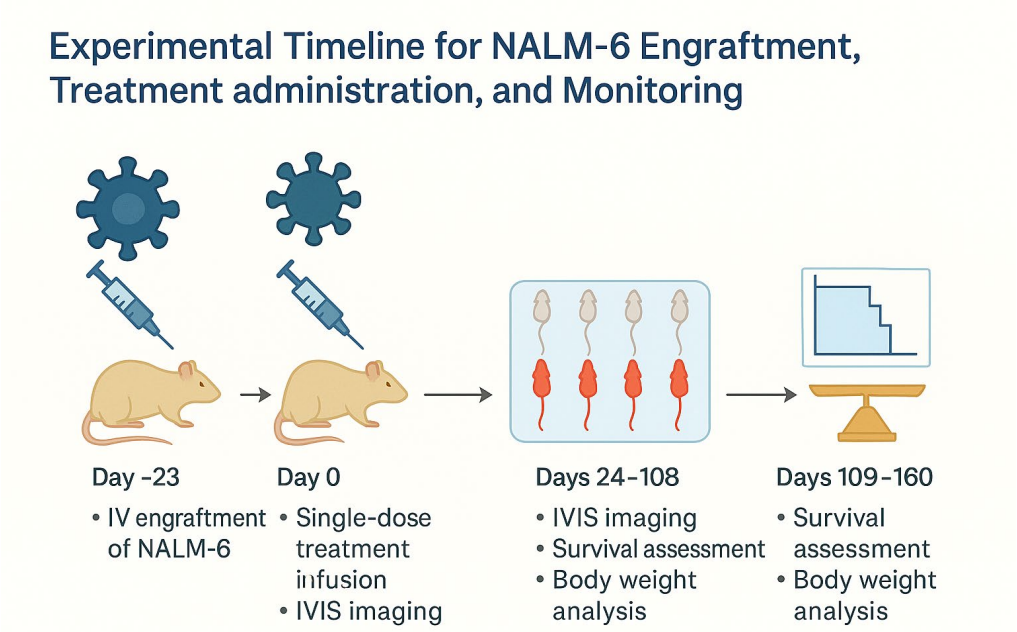


**Figure 4. Experimental timeline showing IV engraftment of NALM-6 cells (Day –23), single-dose treatment infusion and IVIS imaging (Day 0), longitudinal IVIS imaging, survival assessment, and body-weight monitoring (Days 24–108), followed by extended survival and weight analysis (Days 109–160).**

Bispecific CAR-T cells (EGFRvIII/IL-13Rα2) combined with A2AR knockout were able to eradicate tumors expressing either antigen[51]. Reduced toxicity has also been reported: CAR-T cells lacking GM-CSF showed less inflammatory brain infiltration while preserving anti-tumor efficacy[47].

**Ongoing clinical trials**

Although still in early stages, several clinical trials are now testing edited CAR-T products for GBM:

1. NCT04489420: anti-EGFRvIII CAR-T cells with CRISPR-mediated PD-1 knockout (Phase I).
2. NCT05366179: allogeneic anti-B7-H3 CAR-T cells with *TRAC* and *B2M* edits (Phase I/II).
3. NCT05660369: CAR-TEAM cells (combining CAR-T and bispecific engagers) for recurrent GBM[52].
4. NCT05063682: locoregional anti-IL-13Rα2 CAR-T cells incorporating a safety switch[53].

Initial data indicate acceptable safety profiles and some objective responses, although achieving durable efficacy remains a challenge[54].

**Biomarkers of response**

Transcriptomic and immunologic studies are beginning to identify predictors of response to edited CAR-T therapies, including:

1. Tumor immune signatures, such as IFN-γ expression and chemokine profiles associated with T-cell recruitment[55].
2. Tumor mutational burden, where a higher neoantigen load correlates with better responses[56].
3. Baseline immune contexture, especially the presence of pre-existing tumor-infiltrating lymphocytes[57].
4. Target antigen expression, in terms of both level and uniformity across tumor cells[58].

| Identifier | Target | CRISPR modification | Phase | Status | Preliminary results | Ref. |
|---|---|---|---|---|---|---|
| **NCT04489420** | EGFRvIII | PD-1 knockout | I | Recruiting | Acceptable safety (n=12) | [35] |
| **NCT05366179** | B7-H3 | TRAC/B2M knockout | I/II | Active | Responses in 3/15 patients | [34] |
| **NCT05660369** | EGFRvIII | Dual CAR-TEAM system | I | Active | Early evidence of tumor reduction | [52] |
| **NCT05063682** | IL-13Rα2 | iCasp9 safety switch | I | Completed | No ≥ grade 3 treatment-related toxicity | [53] |

**Table 2. Summary of clinical trials using edited CAR-T cells for GBM**

## Challenges and future considerations

### Technical limitations

The clinical application of CRISPR editing in CAR-T cells still faces several technical hurdles. One of the most relevant is the variable editing efficiency in primary human T cells, which can fluctuate widely depending on the donor source, delivery method, and specific protocol[59]. Although off-target effects have been reduced by optimizing guide design and using higher-fidelity Cas9 variants, the risk of unintended mutagenesis has not been completely eliminated[60].

Edited cells may also undergo epigenetic instability, with changes that alter their long-term function[61]. Manufacturing remains a multistep, complex, and costly process, which increases batch-to-batch variability and complicates industrial standardization[62].

### Biological barriers

Multiple biological barriers limit the clinical impact of these therapies. The selective pressure of the tumor microenvironment can promote the expansion of escape clones that lose expression of the targeted antigen[63]. In addition, the immunogenicity of exogenous components, such as bacterial Cas9 or the CAR itself, may trigger immune responses that curtail the persistence of infused cells[64].

The replicative senescence of T cells expanded ex vivo reduces their proliferative capacity and function in vivo[65]. Moreover, the complex network of non-genetic suppressive factors—including metabolites, cytokines, and hypoxia—within the GBM microenvironment can counteract the genetic modifications introduced by CRISPR[66].

### Regulatory and ethical considerations

The move towards clinical application raises substantial regulatory and ethical questions. Rigorous long-term safety evaluation is essential, including extended follow-up to monitor the theoretical risks of insertional mutagenesis and persistent genomic alterations[67].

Equitable access poses another major challenge, as the high costs of these therapies limit their availability. This has prompted interest in decentralized manufacturing models and automated production platforms to reduce costs and broaden access[68].

Finally, the informed consent process must ensure clear, transparent communication about expected benefits, inherent uncertainty, and potential risks associated with these experimental interventions[69].

Future perspectives and emerging innovations

### Technological advances

The next wave of CRISPR-edited CAR-T therapies will be driven by several promising technological innovations, including:

1. Base editing and prime editing, which allow single-nucleotide changes with high precision without inducing double-strand breaks[70].
2. CRISPR interference (CRISPRi) and activation (CRISPRa), enabling fine-tuned epigenetic regulation by repressing or activating genes without permanently altering the DNA sequence[71].
3. Non-viral delivery systems, such as nanoparticles or extracellular vesicles, are designed to improve transfection efficiency and support scalable manufacturing[72].
4. Automation and closed-system manufacturing aim to shorten production times and reduce both costs and product variability[73].

These tools are redefining what is technically possible in advanced cellular engineering.

### Combinatorial strategies

Combination approaches are emerging as a key route to overcome current limitations. Among the most promising strategies are:

1. Sequential therapies using different CAR-T products directed against multiple GBM antigens in a rationally planned sequence[74].
2. Integration with standard treatments, such as radiotherapy or chemotherapy, to debulk tumor mass and improve CAR-T activity[75].
3. Microenvironment modulation, using immune checkpoint inhibitors, antiangiogenic drugs, or metabolic modulators[76].
4. Neoantigen vaccines, administered before CAR-T infusion to prime endogenous immune responses[77].

Thoughtfully designed combinations could significantly enhance both efficacy and durability of responses.

### Precision medicine in practice

Precision medicine is reshaping how CAR-T therapies are conceptualized and deployed. Key areas of innovation include:

1. Individualized target selection, based on the genomic and transcriptomic profile of each patient's tumor[78].
2. Rational CAR design, tuning scFv affinity according to antigen density to balance efficacy and safety[79].
3. Engineering of specific T-cell subsets, such as stem cell–like memory T cells (TSCM) or γδ T cells, to improve long-term persistence[80].
4. Dynamic treatment monitoring, using liquid biopsy, circulating tumor DNA sequencing, or immune PET imaging to track therapeutic responses in real time[81].

These strategies point towards a future in which CRISPR-edited CAR-T therapies are more precise, safer, and more widely accessible.

## CONCLUSIONS

Reprogrammed CAR-T cells with CRISPR-Cas9 represent a novel strategy with the potential to transform glioblastoma treatment completely. Simultaneous targeting of multiple pathways can be used to design

immune cells that evade the immunosuppressive constraints of the brain tumor microenvironment, ultimately enhancing persistence and function while minimizing treatment-associated toxicities.

Preclinical evidence supports this concept, with greater efficacy demonstrated in GBM animal models. Preliminary phase I studies already demonstrate manageable safety profiles and encourage biological responses. However, significant obstacles persist, including editing efficiency, potential long-term adverse effects of genomic manipulation, tumor escape via antigen loss, and the burdens and availability of such treatment.

In the near term, progress will depend on optimizing manufacturing protocols, validating these approaches in well-designed clinical trials, and developing rational combination strategies. The integration of predictive biomarkers, improved safety switches, and precision-medicine frameworks will be crucial to maximizing clinical benefit.

As gene-editing technologies continue to improve in accuracy and specificity, and as our understanding of GBM immunobiology deepens, the synergy between CRISPR-Cas9 and CAR-T therapy is likely to continue to generate new opportunities against one of the most lethal human cancers. Successful translation of these innovative strategies into clinical practice could ultimately change the grim prognosis of glioblastoma and establish a new standard of care in neuro-oncology.

**Author Contributions**

Conceptualization, Saúl Emilio Guamba Cajas (S.E.G.C.), Arianna Rafaela Castillo Guevara (A.R.C.G.), and Diana Estefanía Paucar Fiallos (D.E.P.F.); methodology design, A.R.C.G.; clinical trial results and table preparation, D.E.P.F. and A.R.C.G.; literature support, manuscript review, and editing, Esteban Leonardo Guamba Cajas (E.L.G.C.).

All authors have read and approved the final version of the manuscript.

**Funding**

This research received no external funding.

The Article Processing Charge (APC) was self-funded by the corresponding author.

**Institutional Review Board Statement**

Not applicable.

This work is a scientific review and did not involve human participants, animals, or experimental interventions.

**Informed Consent Statement**

Not applicable.

This article is a literature-based review and did not involve human subjects or identifiable personal data.

**Data Availability Statement**

No new data were generated or analyzed in this study.

All supporting information is available within the references cited in the manuscript.

**Acknowledgments**

The authors thank the academic community of Universidad Yachay Tech for their support during the preparation of this manuscript and for providing access to scientific literature and institutional resources.

**Conflicts of Interest**

The authors declare no conflicts of interest.

The authors alone are responsible for the content and writing of this article.

**Data and AI Disclosure**

All figures and tables in this manuscript were created by the authors using publicly available scientific information and the references cited herein. Figures were designed using author-supervised AI-assisted illustration workflows (OpenAI), strictly for visualization, layout improvement, and English-language

polishing. No AI system was used for data generation, statistical analysis, scientific interpretation, or the creation of original scientific content. The authors independently verified all scientific statements, interpretations, and conclusions in accordance with *BioNatura Journal's Policy on AI-Assisted Content* (https://bionaturajournal.com/artificial-intelligence--ai-.html).

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**Received:** 26 Oct 2025 / **Accepted:** 18 Nov 2025 / **Published (online):** 15 Dec 2025 (Europe/Madrid)

**Citation**

Guamba Cajas S.E., Paucar Fiallos D.E., Castillo Guevara A.R., Guamba Cajas E.L.

*CRISPR-Cas9–Enhanced CAR-T Cell Engineering for Glioblastoma: Mechanisms, Preclinical Advances, and Emerging Clinical Applications.*BioNatura Journal. 2025; 2(4): 09. https://doi.org/10.70099/BJ/2025.02.04.09

**Additional Information**

**Correspondence should be addressed to:**

**Saúl Emilio Guamba Cajas**

Email: saul.guamba@yachaytech.edu.ec

ORCID: https://orcid.org/0009-0008-0518-1994

Tel.: +593 99 895 0919

**Peer Review Information**

BioNatura Journal thanks the anonymous reviewers for their valuable contribution to the peer-review process.

Regional peer-review coordination was conducted under the **BioNatura Institutional Publishing Consortium (BIPC)**, involving:

- Universidad Nacional Autónoma de Honduras (UNAH)
- Universidad de Panamá (UP)
- RELATIC (Panama)

Reviewer support via Web of Science Reviewer Locator:

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**Publisher Information**

Published by **Clinical Biotec S.L. (Madrid, Spain)** as the publisher of record under the *BioNatura Institutional Publishing Consortium (BIPC)*.

**Institutional co-publishers:**

- Universidad Nacional Autónoma de Honduras (UNAH)
- Universidad de Panamá (UP)
- RELATIC (Panama)

**Places of publication:**

Madrid (Spain); Tegucigalpa (Honduras); Panama City (Panama)

**Online ISSN:** 3020-7886