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Synthetic antigen-augmented SynNotch CAR T Cell therapy for Glioblastoma: a conceptual design review

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Abstract
Glioblastoma is the most aggressive primary brain tumor and remains highly resistant to surgery, radiotherapy, chemotherapy, and current immunotherapies because of antigen heterogeneity, immune suppression, and a dense extracellular matrix. Although EGFRvIII-targeted CAR T-cell therapies have demonstrated biological activity, clinical benefit has been limited by antigen escape, poor persistence, and restricted tumor infiltration. This conceptual design review proposes a multimodal post-resection strategy that integrates complementary immunoengineering technologies to address these barriers. An EGFRvIII-specific synthetic Notch (SynNotch) receptor is proposed to induce expression of an IL13Rα2-targeted CAR only after tumor recognition, improving specificity while reducing antigen escape. A CRISPR-mediated PD-1 knockout is incorporated to enhance T-cell persistence, while reactive oxygen species (ROS)-responsive hyaluronidase nanogels are designed to improve migration through the hyaluronic acid-rich extracellular matrix. A visible-light photocrosslinkable hyaluronic acid methacrylate hydrogel containing hypoxia-responsive IL-15 is further proposed to provide sustained, localized cytokine support within the resection cavity. Beyond this integrated therapeutic framework, the review discusses synthetic antigen engineering as a future strategy to further overcome antigen escape by introducing programmable tumor targets that may broaden CAR T-cell applicability in heterogeneous glioblastoma. Together, these approaches integrate advances in synthetic biology, biomaterials, and immunoengineering into a unified conceptual platform for improving CAR T-cell therapy against glioblastoma. Although experimental validation remains necessary, this framework identifies a rational direction for future translational investigation.

Keywords
Glioblastoma, CAR T cell therapy, Synthetic Notch (SynNotch), EGFRvIII, IL13Rα2, Tumor microenvironment, CRISPR-Cas9, Hydrogel drug delivery, Reactive oxygen species-responsive nanogels, Immunoengineering

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1. Introduction

Glioblastoma is the most aggressive and invasive form of glioma, a category of brain tumor that hijacks the cellular pathways of glial cells to accelerate cell replication and prolong survival. Glioblastoma is characterized by a highly immunosuppressive tumor microenvironment, highly invasive nature, complex genetic heterogeneity, and resistance to conventional treatments such as surgical resection, chemotherapy, and radiation, which frequently result in tumor recurrence (1,2).

Glioblastoma's resistance to surgical resection is mainly due to its characteristic tendrils that infiltrate nearby tissues, making it virtually impossible to extract the entirety of the tumor. Its resistance to chemotherapy and radiation is derived from the adaptable and complex DNA repair pathways within the tumor cells (1,2). Temozolomide (TMZ), a chemotherapy drug commonly used in glioblastoma patients, functions by transferring a methyl group to the DNA of malignant cells, leading to the incorporation of thymine instead of cytosine during DNA replication. This mismatch results in DNA damage that ultimately causes malignant cell death. However, cancer cells often develop resistance to this effect by upregulating DNA repair enzymes, such as O-6-methylguanine-DNA methyltransferase (MGMT), or by acquiring mutations in key proteins involved in the DNA mismatch repair system. These adaptations enable cancer cells to evade cell death and survive treatment with TMZ. Notably, such resistance mechanisms are more commonly observed in recurrent GBM, suggesting that they represent adaptive survival strategies (1,2). In conjunction with a highly acidic pH, metabolic stress, hypoxic conditions, and a lack of necessary nutrients for cell survival and persistence, these resistance pathways give rise to a tumor microenvironment that is hostile towards both endogenous immune cells and adoptively transferred immunotherapies. Therefore, while immunotherapy is a promising treatment approach, glioblastoma poses unique physical and immunologic constraints that, without supportive interventions, limit therapeutic efficacy.

Consequently, targeted immunotherapy has emerged as one of the most promising therapeutic strategies for overcoming the limitations of conventional glioblastoma treatments. These targeted therapies have the potential to be patient and cell-type-specific (3). A common immunotherapy treatment involves the use of Chimeric Antigen Receptor (CAR) T cells, which enhance the natural immune system reactions against cancer cells and are capable of escaping molecular mechanisms of resistance. CD8+ cytotoxic T cells and CD4+ helper T cells are crucial components of the adaptive immune system in targeting antigens. In targeting and eliminating cancer, CD8+ cytotoxic T cells rely on the presentation of Tumor-Associated Antigens (TAAs) via the tumor cells' Major Histocompatibility Complex (MHC). While this system works to eliminate baseline cancerous cells, cancers commonly adapt to reduce their expression of MHC, which results in immune evasion. The T cells used to generate CAR T cells can be obtained from either the patient (autologous) or from a donor (allogenic). They are engineered *ex vivo* to express a chimeric antigen receptor (CAR) on their surface, making them MHC independent and therefore

amplifying their efficacy in targeting malignant cells (4-7). Despite this, early clinical trials in Glioblastoma demonstrated that, despite their success in demonstrating on-target activity and eliciting a biological response, EGFRvIII-targeted CAR T cells failed to produce meaningful clinical improvement in patients because the harsh microenvironment limited physiologic function *in vivo*. This was made evident by tumor samples collected after the infusions, which indicated rapid antigen loss, increased expression of inhibitory ligands and cytokines, and widespread T cell exhaustion. Additionally, the dense hyaluronic acid-rich extracellular matrix characteristic of Glioblastoma, restricted CAR T cell infiltration and movement, resulting in insufficient distribution and limited penetration within and surrounding the tumor (8).

A fundamental limitation of current antigen-directed CAR T-cell therapies is that they remain dependent on endogenous tumor antigens, which are subject to selective pressure and antigen loss during treatment. An alternative conceptual strategy is to shift target recognition away from native tumor biology and toward therapeutically programmable targets. Rather than relying exclusively on naturally expressed tumor antigens, synthetic antigen engineering could enable tumor cells to be deliberately labeled with an artificial antigen that is absent from healthy tissues. Because the therapeutic target is introduced by the treatment itself rather than arising from tumor biology, this strategy has the potential to reduce selective pressure for antigen escape while providing a standardized target for subsequent CAR T-cell recognition.

Gene editing tools, such as Clustered Regularly Interspaced Short Palindromic Repeats CRISPR-Associated Protein 9 (CRISPR), are critical to enabling accurate genetic modifications of the cell genome. CRISPR-Cas9 is a gene-editing tool that has gained significant traction over the last decade due to its procedural simplicity and precision. This technique utilizes Cas9–guideRNA(gRNA) complexes that are designed to target DNA segments associated with the desired genetic modification (typically being a deletion, knock-out, or knock-in) (9). CHOP CHOP, a CRISPR-gRNA modeling software, is used to evaluate the appropriate guide gRNA for targeting the gene of interest, allowing for the deletion or insertion of any particular mutation or gene sequence. This software predicts potential off-target effects, enabling the design of a precise genome editing approach. The resulting CRISPR complex can be employed to genetically edit the T cells *ex vivo* into the appropriate CAR T cells. Utilizing CRISPR-Cas9 is expected to enable more accurate, timely, and efficient engineering of CAR T cells, which is crucial for overcoming the multifaceted challenges of treating refractory cancers such as glioblastoma (10). This proposal suggests employing the CRISPR-Cas9 genome editing technique to generate both a PD-1 knockout and a SynNotch receptor construct, along with other supplementary deletions, such as the deletion of the glucocorticoid receptor, which could reduce the severity of steroid-induced T cell suppression during glioblastoma treatment.

To address the complex therapeutic barriers of glioblastoma, this approach also targets immune

checkpoints. Immune checkpoints are transmembrane proteins, such as programmed cell death protein 1 (PD-1), cytotoxic T-lymphocyte-associated protein 4 (CTLA-4), or lymphocyte activation gene-3 (LAG-3), that stop the activation or promote the premature exhaustion of T cells and other immune cells (5,7,11). However, systemic immune checkpoint blockades have shown limited benefit in treating GBM as a monotherapy; therefore, integrating immune checkpoint resistance mechanisms into antigen-specific CAR T cells, such as a knockout, could be more effective at sustaining T cell activity within the tumor microenvironment.

Gene expression data from databases such as cBioPortal have identified the most significantly upregulated genes in glioblastoma, enabling the selection of Epidermal Growth Factor Receptor variant III (EGFRvIII) as the optimal target for CAR T cell therapy in treating glioblastoma. Due to the high heterogeneity of EGFRvIII expression in glioblastoma tumor cells, biological pressure imposed by immunotherapy agents, such as targeted CAR T cells, may result in antigen loss or downregulation in the tumor. Therefore, strategies that reduce dependence on endogenous tumor antigens are likely to be necessary for durable therapeutic efficacy. Pairing an EGFRvIII-recognition SynNotch receptor with an IL13Rα2-targeting CAR represents one approach to mitigating antigen escape, while future synthetic antigen engineering may further shift CAR T-cell recognition toward therapeutically programmable targets that are less susceptible to tumor-driven antigen loss.

This paper proposes a conceptual design review for a second-generation CAR T cell therapy engineered with CRISPR-Cas9 gene editing, hypothesizing increased efficacy in targeting malignant glial cells. Prior to the implementation of this proposed treatment approach, patients should undergo biopsy testing to confirm that their glioblastoma tumor expresses sufficient levels of EGFRvIII and IL13Rα2. If so, the EGFRvIII-specific CAR T cells would be administered into the tumor cavity post-surgical resection. These CAR T cells would have an EGFRvIII-specific SynNotch “priming” receptor component that, upon detection, triggers a downstream cascade resulting in the transcriptional activation of an IL13Rα2 “killing” CAR receptor component. Additionally, ROS-cleavable hyaluronidase nanogels could be tethered to the CAR T cell surface via metabolic glycoengineering and copper-free SPAAC click chemistry to dismantle the dense HA-rich GBM tumor scaffolding. This could be followed by the implantation of a visible-light photocrosslinkable hyaluronic acid methacrylate hydrogel that encapsulates hypoxia-responsive IL-15 ligands, an immunostimulant cytokine that is capable of prolonging the persistence of CAR T cells within the tumor microenvironment. These components were selected to potentially overcome some of the major barriers in GBM treatment, such as antigen escape, metabolic stress, and physical impediments, by improving precision, persistence, and CAR T cell trafficking within the tumor microenvironment.

2. Methods

To establish a comprehensive and accurate systemic literature review, PubMed, the National Institute of Health, and the Cancer Research Institute database were searched to find relevant papers by pairing the following key words: “CAR T cells”, “CAR T cells and glioblastoma”, “CRISPR Cas-9”, “Immune checkpoint inhibitors”, “ROS-cleavable nanogels”, “Combination therapies and glioblastoma”, “IL-15 mechanisms”, “Hydrogels” or “Hydrogel application to tumor treatment”. Relevant articles were assessed based on the number of authors who had previously cited the paper and their publication recency. This paper utilized cBioPortal and the Human Protein Atlas, which are public databases that provided access to a large variety and quantity of data surrounding glioblastoma tumor samples. More specifically, this paper drew data from The Cancer Genome Atlas (TCGA) Firehouse legacy dataset through cBioPortal to identify specific protein targets with the potential to increase the efficacy of CAR T cell treatment in glioblastoma. This dataset offered a spectrum of data to describe the most frequent DNA mutations in glioblastoma and their correlation with mRNA expression. CHOP CHOP (https://chopchop.cbu.uib.no), a CRISPR guideRNA modeling software, was used to illustrate the appropriate gRNAs for the completion of a CRISPR-initiated PD-1 (PDCD1) knock-out, and to predict off-target effects. Determining the most effective gRNA was an iterative process based on numerous factors, such as the number of predicted mismatches or the GC content, both of which are critical to ensuring guide stability and binding affinity.

2.1 EGFRvIII as a primary target for CAR T Cell therapy in Glioblastoma

Epidermal Growth Factor Receptor (EGFR) is a receptor tyrosine kinase, a type of transmembrane glycoprotein, that is amplified in approximately 40–60% of glioblastoma cases. Furthermore, EGFR amplification is observed in~ 40–60% of glioblastomas, whereas the tumor-specific mutant isoform EGFRvIII is present in ~ 20–30% of cases (12). EGFRvIII manifests from an in-frame deletion of exons 2 through 7, resulting in the formation of a novel glycine residue at the junction. The altered polypeptide forms a functional, yet truncated receptor that serves as a tumor-specific neoepitope (12). This neoepitope is absent in healthy Central Nervous System (CNS) tissues, whereas wild-type EGFR is expressed at low levels in numerous healthy CNS tissues, making EGFRvIII a highly specific target for immunotherapy treatment of glioblastoma (12,13).

According to the copy number segment graph for glioblastoma from cBioPortal, the genomic profile of glioblastoma exhibits multiple chromosome 7 amplifications (see Figure 1A). Amplification of the EGFR locus (7p11.2) is the most frequent transmutation present on chromosome 7. Consequently, EGFR is frequently amplified and overexpressed in glioblastoma, while the tumor-specific mutant isoform EGFRvIII arises from a characteristic genomic deletion. Together, these alterations constitute a distinctive molecular hallmark of glioblastoma and contribute to its aggressive growth, therapeutic resistance, and poor clinical prognosis.

Figure 1B illustrates mRNA expression and copy number in glioblastoma patient samples, and demonstrates a correlation between EGFR amplification and elevated mRNA expression. The less frequent isoform of EGFR in the tumor samples analyzed is diploid EGFR, whereas EGFR-amplified tumor samples display markedly increased expression levels. This increase in EGFR gene amplification drives an EGFR overexpression in glioblastoma cells. Since EGFRvIII is both highly expressed and restricted to malignant glial cells, it is an ideal target for CAR T cell therapy, allowing for effective recognition of glioblastoma cells while reducing the likelihood of off-target effects (13).

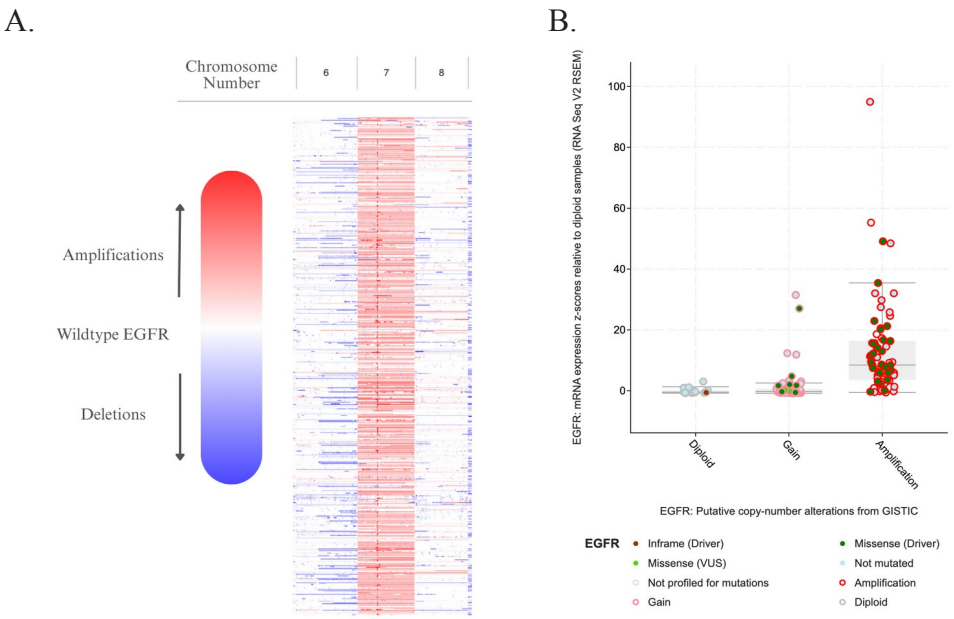


Figure 1. cBioPortal CN Segment and EGFR mRNA scatterplot. **A**. Copy Number segment showing glioblastoma chromosome 7 amplifications, in which the EGFR locus is found. **B**. mRNA scatterplot showing either diploid EGFR, gain of EGFR, or amplification of EGFR in patient tumor samples. Indicating that there is a significant increase in patients suffering from amplifications of EGFR at an mRNA level. The data from this figure was obtained from cBioPortal.

2.2 IL13Rα2 as a secondary target for CAR T Cell therapy in Glioblastoma

Interleukin-13 receptor alpha 2 (IL13Rα2) is an established tumor-associated antigen that is highly overexpressed in glioblastoma cells (see Figure 2A), yet has minimal expression in healthy brain tissue, making this antigen a valuable candidate for a safe and specific target for CAR-based immunotherapy treatment. Two IL-13 receptors naturally occur in the body: IL13Rα1 and IL13Rα2. IL13Rα1 is naturally expressed in healthy brain cells and is known to bind to IL-4Ra receptors; together, they facilitate healthy immune signaling and function through the JAK/STAT signaling pathway. Comparatively, IL13Rα2 is upregulated on tumor-associated cells, yet it does not effectively transmit immune signals. IL13Rα2 is

characteristically overexpressed in glioblastoma and thus is typically associated with more aggressive tumor behavior (14, 15). IL13Rα2 is structurally homologous to IL13Rα1. Hence, it can function like a decoy receptor where IL13Rα2 binds to IL-13 and prevents IL-13 from interacting with the IL13Rα1/IL-4Ra complex. Due to the fact that IL13Rα2 cannot effectively conduct immune signaling, this would reduce activation of the JAK/STAT signaling pathway (14, 15).

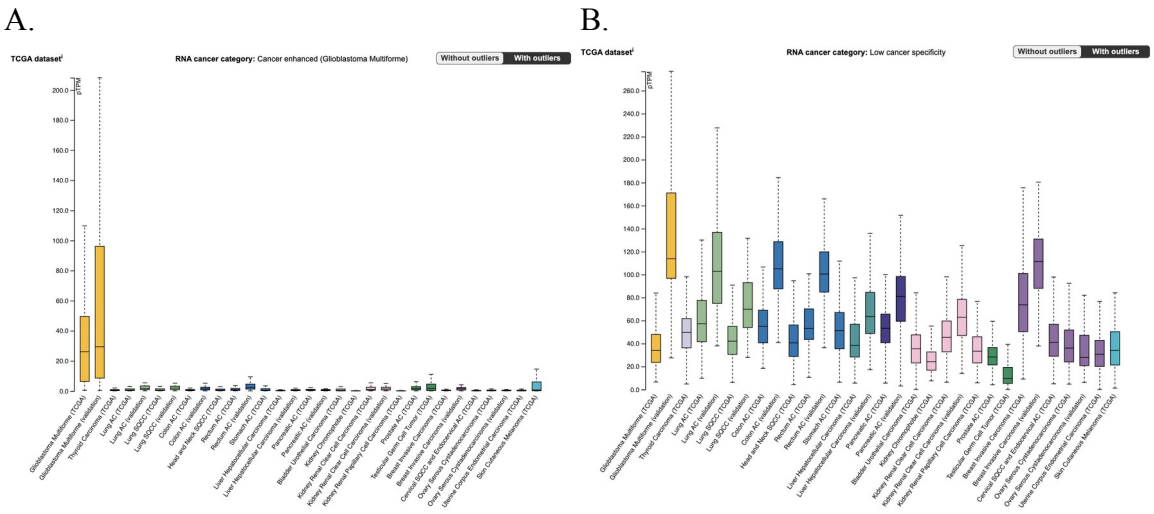


Figure 2. The Human Protein Atlas TCGA dataset IL13Rα2 and IL13Rα1 RNA expression overview. **A**. IL13Rα2 RNA expression is amplified in glioblastoma multiforme patient tumor samples relative to patient samples from multiple tumor types recorded in the TCGA dataset. **B**. IL13Rα1 RNA expression is elevated in GBM, supporting the autologous nature of the antigen.

Brown et al. (2022) demonstrated that IL13Rα2-targeted CAR T cells retain their ability to combat cancer cells even under exposure to steroids, a serious limitation for CAR T cell treatment for patients with brain tumors, who often receive frequent doses of dexamethasone to manage cerebral edema. This study demonstrated that IL13Rα2-CAR T cells engineered for glucocorticoid resistance (via knockout) maintain their cytotoxicity, persistence, and metabolic function *in vivo* (15).

These findings justify the employment of IL13Rα2 as a downstream “switch and kill" receptor in combination with EGFRvIII in a SynNoth dual-antigen strategy. In this proposal, CAR T cells would be engineered to, first, interact with EGFRvIII receptors on cancer cells through the SynNotch “priming” receptor, ensuring the CAR T cell is physically located within glioblastoma tissue, seeing as EGFRvIII is a more specific and unique GBM-associated antigen. Next, a downstream signaling cascade would result in the transcription of the IL-13Rα2-CAR, which will then interact with IL-13α2^{+} glioblastoma cells, navigating around EGFRvIII antigen escape, and conducting a more widespread attack on native cancer cells (16,17).

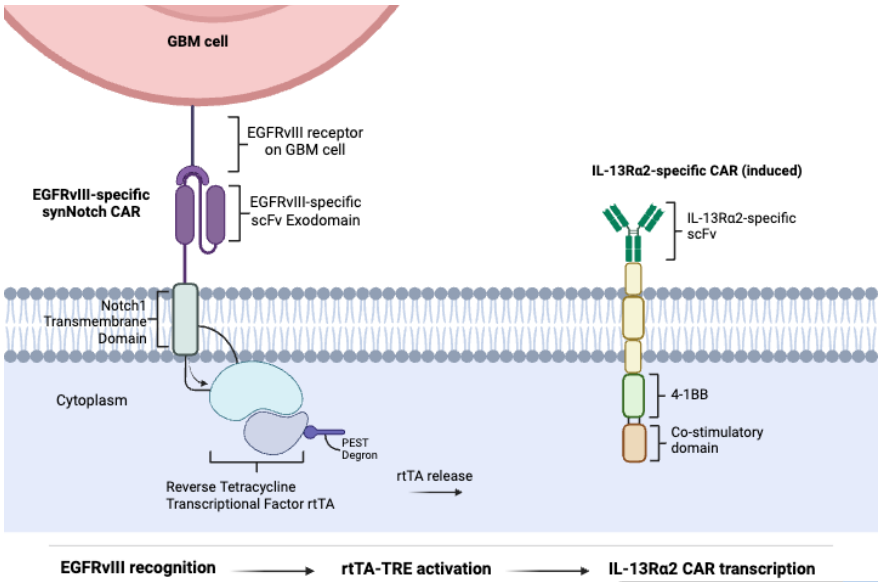


Figure 3. Structure of the EGFRvIII-specific synthetic Notch CAR receptor and IL13Rα2-specific CAR receptor. This figure models the CAR complex for a synthetic Notch (synNotch) receptor composed of three core components: an EGFRvIII-specific single-chain variable fragment (scFv) exodomain, fused to a Notch1 transmembrane domain, and a reverse tetracycline transcriptional factor (rtTA) endodomain with an additional PEST degron. The EGFRvIII-specific scFv exodomain recognizes the EGFRvIII receptor on the GMB cell, triggering a downwards signaling cascade, resulting in the transcription of an IL13Rα2-specific CAR. This figure was designed in BioRender.

The paper proposes a CAR T Cell engineered to express a synthetic Notch (synNotch) receptor composed of three core components: an EGFRvIII-specific single-chain variable fragment (scFv) exodomain, fused to a Notch1 transmembrane domain, and a reverse tetracycline transcriptional activator (rtTA) endodomain with an additional PEST degron (see Figure 3). The scFv is derived from an antibody structure with advanced binding affinity towards the neoepitope on EGFRvIII, making the scFv the antigen-specific portion of the CAR that actively binds with the target antigen. Upon binding, the Notch1 regulatory region (located between the EGFRvIII-specific scFv and the transmembrane domain) changes shape, enabling proteolytic cleavage via ADAM10/ADAM17 and γ-secretase, triggering the release of the intracellular rtTA transcription activator tail into the cytoplasm (18). The rtTA transcription activator then, in the presence of doxycycline, binds with the tetracycline-responsive element (TRE) upon entering the nucleus, which is strategically positioned upstream of a minimal CMV promoter (16,17). The rtTA will only activate transcription of the IL13Rα2-specific CAR in the presence of doxycycline. This downstream signaling cascade drives the transcription of an IL13Rα2-specific CAR. Therefore, by necessitating the binding with $EGFRvIII^{+}$ cells prior to activation of a "killer" IL13Rα2-specific CAR, there is a greater probability that the CAR T Cell is localized within the tumor tissue when targeting

IL13Rα2, a less specialized target antigen typical of Glioblastoma, but more widely expressed. Additionally, by engineering the endodomain with an rtTA-TRE element, the transcription of the IL13Rα2 CAR can be easily regulated or terminated by adjusting the concentration of administered doxycycline. This was accomplished in a study conducted by Sakemura et al. in 2016, where a Tet-On system was successfully employed to control CD19-targeted CAR receptor expression with doxycycline (19). As a final layer of control, the PEST degron is incorporated into the endodomain because it shortens the half-life of the rtTA. Once the CAR T Cell is no longer interacting with EGFRvIII, the PEST degron reduces the duration of CAR expression, minimizing off-target persistence towards the normal body cells (20). As part of this proposal, the genetic engineering method to design these CAR T cells is illustrated in the following plasmid map (see Figure 4).

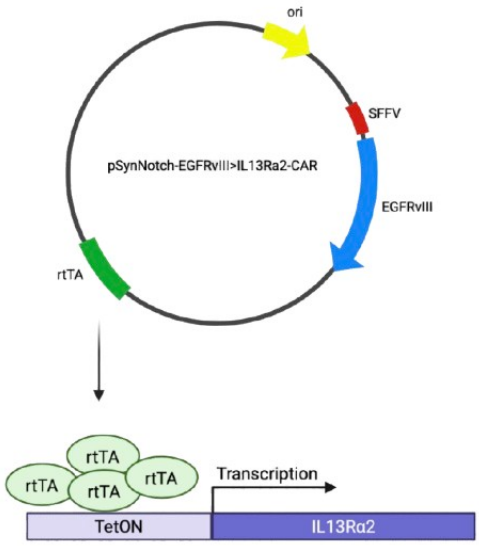


Figure 4. Plasmid Map Illustrating Proposed EGFRvIII-Activated rtTA-Mediated IL13Rα2 CAR Transcription. Ori is the origin of replication. The SFFV promoter drives the expression of the EGFRvIII synNotch receptor in CAR T Cells. Upon EGFRvIII binding and synNotch activation, rtTA is released and binds to the tetracycline-responsive promoter, resulting in the transcription of the IL13Rα2-targeting CAR.

Before receiving an EGFRvIII targeting CAR T cell treatment, it would be necessary for patients to undergo molecular profiling of their tumor, possibly by means of Next Generation Sequencing (NGS) or biopsy, to determine their eligibility for the treatment. If the patient does not exhibit EGFRvIII within their tumor, this treatment will be unsuitable for their tumor. When translating cancer therapy into the clinical setting, it is necessary to acknowledge that, due to the high specificity of this proposed therapy and the individualized nature of glioblastoma, this treatment is not universal.

In 2024, Choi et al. conducted a study that administered EGFRvIII targeting CAR v3-

TEAM-E T cells intraventricularly, or by means of an Ommaya reservoir, into three patients with recurrent glioblastoma. While the majority did not experience significant tumor shrinkage post treatment, the preponderance of patients experienced stabilization in their disease, and some of the patients experienced a slightly extended survival compared to retrospective controls. There was a noticeable decrease in EGFRvIII levels post-treatment, indicating that the CAR T cells imposed pressure on the tumor to adapt (21).

Similarly, in 2017, researchers at the University of Pennsylvania conducted a study that administered a single intravenous infusion of autologous EGFRvIII-targeted CAR T cells into ten patients who suffered from recurrent glioblastoma. While there was no evident tumor regression in the following MRI scans, they did notice a significant antigen escape, increased immunosuppressant cytokines, and increased checkpoint inhibitors within the tumor microenvironment, demonstrating that a physiological response to the therapy had taken place (8).

While both trials demonstrated that the CAR T cells elicited a biological response, and the treatment was well tolerated in patients, the infused cells failed to maintain persistence as time progressed, likely due to the marked antigen escape and to the immunosuppressive tumor microenvironment. This paper proposes a multipronged approach that addresses immune evasion, trafficking barriers, the tumor microenvironment, and the short-lived efficacy of CAR T cells in treating glioblastoma. Understandably, addressing these obstacles is not readily attainable through EGFRvIII monotherapy; therefore, it is necessary to administer this therapy in combination with other approaches, such as immune checkpoint inhibitors and immunostimulatory cytokines, to augment CAR T cell persistence and modulate the tumor microenvironment. Clinical efficacy remains speculative due to the lack of prior testing or clinical trials.

2.3 Targeting PD-1 to enhance CAR T Cell activity

Immune checkpoints, such as PD-1, CTLA-4, LAG-3, TIM-3, and TIGIT, play a critical role in inducing premature T cell exhaustion, functional impairment, and reduced persistence of CAR T cells in the tumor microenvironment (3,7). The process is initiated when the extracellular domain of immune checkpoint receptors presented on the T cell membrane binds to their corresponding ligand, such as the PD-L1 ligand that binds to the PD-1 receptor. This linkage triggers an intracellular signaling cascade where the tail of the PD-1 protein undergoes phosphorylation, triggering the recruitment of Src homology 2 domain-containing phosphatase-2, SHP-2, which dephosphorylates key TCR signaling molecules that are necessary for T cell activation. This cascade results in the suppression of T cell activity or premature exhaustion. This process inhibits T cell hyperactivity and prevents consequent damage to non-cancerous cells (22).

However, in treating cancer, these immune checkpoints present an obstacle in facilitating the most potent and effective T cell attack on cancerous cells. Therefore, immune checkpoint inhibitors are emerging as a leading treatment

approach. Immune checkpoint inhibitors block immune checkpoints from binding to their corresponding receptors on T cells, preventing any interaction with the T cell itself. The most common immune checkpoint inhibitors currently in circulation are Nivolumab, which targets PD-1/PD-L1/PD-L2 interactions, and Ipilimumab, which targets CTLA-4/CD-80/CD86 interactions (3,7).

Previous studies have demonstrated that employing systemic PD-1 checkpoint blockade antibodies puts patients at a higher risk of acquiring various immune-related toxicities, and their observed clinical impact in combating solid tumors has been generally inconsistent. Conventional immune checkpoint inhibitor therapies are administered intravenously; however, immune checkpoint inhibition can also be integrated into a treatment by engineering a knockout gene into the CAR T cells themselves. This eliminates the expression of the immune checkpoint receptor and reduces the likelihood of triggering systemic toxicities in patients (23).

Nevertheless, it is important to consider the critical role the PD-1 plays in preventing overactivation of T Cells and other immune cells in the body, and the risks of removing such a mechanism. Without PD-1 as a checkpoint for overactivity, CAR T Cells may remain active for longer than intended, which can lead to immune-mediated damage to healthy tissues, including increased inflammation or an alternate autoimmune response (24). The long term safety of a PD-1 knockouts in CAR T Cells has yet to be deemed safe by previous studies; thus, future studies should continue to analyze PD-1/PD-L1/PD-L2 methods of action, and continue to evaluate ways of maximizing antitumor activity while preventing any detrimental side effects to healthy tissue and overall patient wellbeing.

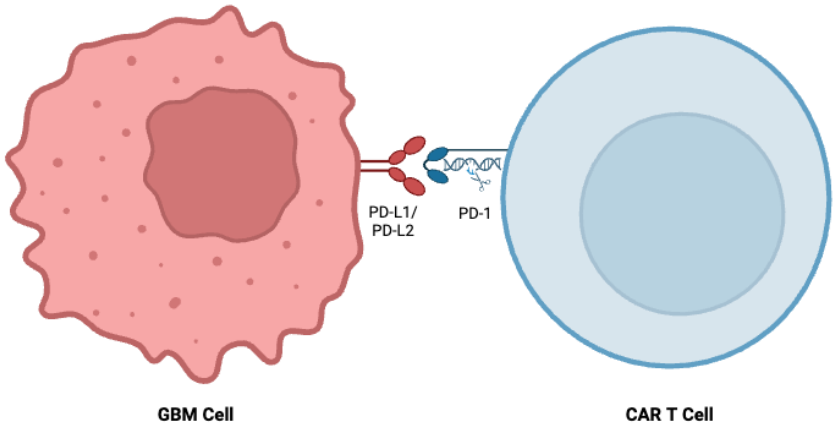
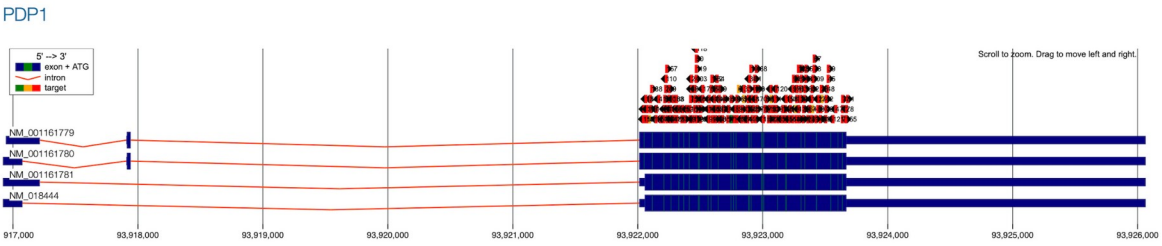


Figure 5. This figure illustrates the CRISPR-Cas9-mediated PD-1 knockout on the CAR T Cell. This figure was designed in BioRender.

The inconsistent clinical impact of conventional immune checkpoint monotherapies in solid tumors, such as glioblastoma, can be attributed to the lack of endogenous T cells within the tumor microenvironment. Immune checkpoint inhibitors require a sufficient population of endogenous T cells for them to carry out their function. However, the harsh tumor environment characteristic of glioblastoma and the nature of CNS immune privilege result in a severe lack of endogenous T cells and immune cells in general, making this therapy an ineffective option (3,22,25). However, if this therapy were to be administered in conjunction with a CAR T cell therapy, it would function as a protective factor that would prevent the engineered T cells from falling victim to the dense immune checkpoint population within the tumor microenvironment, therefore adding an aspect to the treatment that would both aid in increasing the persistence of T cells and aid in their survival within the tumor microenvironment (4,26).

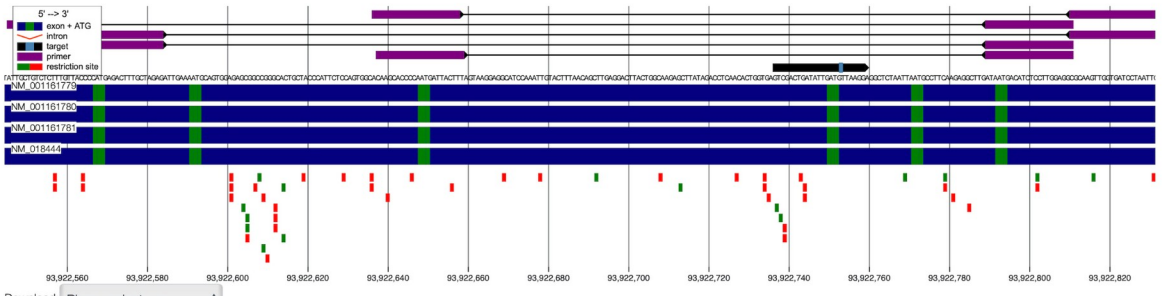
A.



Rank	Target sequence	Genomic location	Strand	GC content (%)	Self-complementarity	MM0	MM1	MM2	MM3	Efficiency
1	GTCGACTGATATTGATGTTAAGG	chr8:93922737	+	35	1	0	0	0	0	37.51
2	AATCGGTGGTAAGTTACCTCTGG	chr8:93923254	-	45	1	0	0	0	1	52.32
3	GTTCTGATCCTCAAATACCGAGG	chr8:93923468	-	45	1	0	0	0	2	74.67

B.

Target: **PDP1**
Rank: **1**
Target sequence: **GTCGACTGATATTGATGTTAAGG**



Off-targets		
Location	Number of mismatches	Sequence (including mismatches)
chr2_KI270776v1_alt:70896	0	ACCGCCCAGACGACTGGCCAGGG

Figure 6. PD-1 CRISPR KO gRNA design with CHOP-CHOP. **A**. This figure depicts the top three gRNAs designed to target the PD-1 gene. **B**. This figure depicts the top gRNA to target the PD-1 gene (gRNA 1). This gRNA (blue box) has the minimum of predicted mismatches, and therefore was chosen as the most reliable option to avoid off-target edits.

The design of the CRISPR KO guideRNA targeting the PD-1 gene was generated in CHOP-CHOP. The top three guideRNAs with the least likelihood of mismatches are illustrated in Figure 6A. Figure 6B depicts the most optimal gRNA designed to target the PD-1 gene (black box). This gRNA (blue box) has minimal predicted mismatches and, therefore, is the most reliable option to avoid off-target edits.

2.4 Hydrogel-based delivery of immunostimulants

Hydrogels, substances composed primarily of water and hydrophilic polymers, can be used to deliver a variety of immunomodulatory agents such as immune checkpoint inhibitors, nanoparticle constructs, cytokines, and chemokines into the tumor microenvironment. Systemic administration of therapeutic agents results in rapid dilution and an elevated risk of off-target toxicities. The hydrogel structure serves as a biocompatible drug reservoir that releases its contents in a gradual and controlled manner, reducing the risk of systemic toxicity and off-target effects. Hydrogel tunability in structural design, loading capacity, composition, and crosslink density allows for precise spatiotemporal regulation of drug release and increased adaptability to diverse treatment approaches. Furthermore, hydrogels can be implanted directly into the tumor bed, establishing a concentrated and prolonged source of therapeutic agents directly within the target tissue (27,28).

This paper proposes the deployment of a visible-light photocrosslinkable hyaluronic acid methacrylate (HA-MA) hydrogel that encapsulates IL-15 ligands post-surgical resection. Even after resection, the residual tumor cavity is hypoxic and continues to suppress T cell function. To address this, this paper proposes encapsulating the IL-15 ligands into oxygen-responsive nanocapsules embedded within the hydrogel itself. This could enable the release of IL-15 ligands into hypoxic areas, which are immunosuppressive to immune cells, and thus are in the greatest need of cytokine support (2).

Hyaluronic acid is a glycosaminoglycan that naturally occurs within the extracellular matrix, making it a biocompatible and biodegradable material suitable for mimicking its environment (29). More specifically, the glioblastoma tumor microenvironment has been shown to contain elevated levels of hyaluronic acid, making this material particularly suitable for this treatment approach (30). However, hyaluronic acid on its own is insufficient due to its vulnerability against rapid enzymatic degradation by means of hyaluronidases and its inability to naturally form stable covalent crosslinks, which are necessary to create the reticular framework of hydrogels (29). A solution to this problem could be achieved by chemically modifying hyaluronic acid with methacrylate groups, a process otherwise known as methacrylation. This enables the formation of crosslinks through photoinitiated polymerization of the methacrylate groups. This process is readily tunable depending on the circumstance and environment in which the therapeutic approach is being employed, making it especially appropriate for patient-specific tumors such as glioblastoma. The methacrylation process stems from a reaction between methacrylic anhydride and the hydroxyl groups in the hyaluronic acid

to form hyaluronic acid methacrylate HA-MA (31-34).

The hypoxia-responsive nanocapsules are dispersed throughout the HA-MA before polymerization. The nanocapsules are composed of polymers that include azobenzene (azo) or nitroimidazole (NI) linkers, which undergo enzymatic degradation in hypoxic environments, such as the tumor resection cavity. When the azo linkers interact with reductive enzymes, which have enhanced function in hypoxic conditions, they cleave at the -N=N- bond. Comparatively, under hypoxic conditions, the hydrophobic -NO_2 groups on NI linkers are enzymatically reduced to hydrophilic -NH_2 groups, enabling the capsule structure to soften and gradually expand. Incorporating both of these hypoxia-responsive linkers enables controlled release when hypoxia-induced reductases interact with the linkers (35-37).

IL-15, an immunostimulant cytokine, is administered to counteract the immunosuppressant tumor microenvironment, mitigate the onset of T cell exhaustion, and increase the population of endogenous T cells. IL-15 functions by binding with a heterotrimeric receptor complex involving IL-15Rα, IL-2/15Rβ, and the common γ chain. This interaction triggers several intracellular signaling pathways that exhibit overlapping roles, but each contribute distinct advantages: the JAK/STAT pathway, which induces the transcription of genes that are necessary to the survival and expansion of lymphocyte populations; the MAPK pathway, which is necessary for supporting effector cell maturation, the production of cytokines, and cell cycle progression; and the NF-κB pathway, which prevents premature cell death in activated immune cells, mediates inflammation, and promotes immune cell activation. Each of these pathways is important to modulate an immunosuppressant tumor microenvironment to drive the survival and persistence of CAR T cell therapy (38).

IL-15 is especially suitable for the immune-desert tumor microenvironment of glioblastoma because it is capable of sustaining CD8+ T cell activity independent of CD4+ regulatory T cells, and it supports the functions of other immune cells, such as Natural Killer cells. Additionally, IL-15 has a distinct impact on the functionality of antigen-presenting cells (APCs), which are necessary to maintain efficient immune cell crosstalk. IL-15 supports the maturation of APCs, especially dendritic cells; mitigates apoptosis in APCs; and heightens the expression of necessary co-stimulatory molecules and cytokines, such as CD80, CD86, and IFN-γ. It is important to consider that IL-15 has been shown to increase PD-1 expression on T cells. However, since this paper proposes a PD-1 knockout, this is not a significant limitation (38, 39).

Despite this, it is important to note the IL-15 has several reported risks of neurotoxicity, overactivation of NK cells, and increased patient risk of developing cytokine release syndrome (CRS). While IL-15 is beneficial because it has been shown to increase the longevity and efficacy of CAR T Cells, it can also overactivate certain aspects of the immune system, which can result in detrimental side effects. Excessive immune activation within the

central nervous system is especially detrimental because it increases the risk for immune-related neurotoxicity (40). Cytokine release syndrome is an inflammatory response in the body that can occur for a multitude of reasons, including the use of immunotherapies such as CAR T Cells (41). Thus, if IL-15 is employed to increase the proliferation and activation of CAR T Cells, it can indirectly result in a higher risk of CRS for patients. Additionally, IL-15 has been shown to activate natural killer (NK) cells, which are a type of immune cell that can be especially harmful when uncontrolled in the body. If too many NK cells are activated locally to the tumor, they have the potential to release harmful inflammatory molecules (40). It is important to consider that this proposed hydrogel administration would not deliver IL-15 throughout the entire body; the use of a hydrogel as the delivery method is what keeps the IL-15 local to the tumor resection area. The hydrogel's gradual release of IL-15 could prevent some of the harsh side effects described above. In future studies, it is paramount to determine the appropriate dosage and parameters of IL-15 administration, including the IL-15 loading per nanocapsule, the number of nanocapsules, and the speed in which the nanocapsules degrade, in order to maintain control over IL-15's impact on the brain and body.

The crosslink density of each layer of the hydrogel can be adjusted based on two main factors: polymer concentration (weight percent, wt%) and the degrees of methacrylation (DOM). The polymer concentration determines how much HA-MA is in the hydrogel solution, and the degree of methacrylation is the percentage of sites on an HA-MA backbone that have been attached to methacrylate groups; therefore, it is a measure of how many places on one HA-MA molecule are available to form crosslink bonds with other HA-MA polymers in the hydrogel matrix. These two measurements are important because the higher the polymer concentration and/or degree of methacrylation, the higher the crosslink density of the hydrogel, and conversely, the lower the polymer concentration and/or degree of methacrylation, the lower the crosslink density of the hydrogel (29,32).

The outer layer of the hydrogel, which this proposal defines as the bottom layer lining the tumor bed wall, is designed to have a low crosslink density, while the core, which this proposal defines as the luminally oriented, central region of the hydrogel, is conversely designed with a high crosslink density to enable a two-phase release profile that includes an initial rapid release followed by a gradual, sustained release. The crosslink density gradient between the two layers is governed by the spatial distribution of surgical light and local oxygen availability within the resection cavity during photocrosslinking. The outer layer will be applied along the tumor bed wall, and the remaining cavity will be filled with a high crosslink density interior. The implanted hydrogel will be exposed to the surgical light source directed into the resection cavity (preferably a green visible light source because it is less harmful to the patient than being exposed to UV rays). The hydrogel core receives earlier and more direct exposure to the surgical light source, promoting a higher degree of photocrosslinking and the formation of a dense, mechanically stable network. In contrast, the outer layer is exposed to lower light intensity

and greater oxygen availability, which limits photocrosslinking and produces a looser, more compliant hydrogel structure. This polymerization gradient reinforces the two-phase release profile by establishing a dense inner core and an outer layer that allows for fast release of immunomodulatory agents.

Nanocapsules near the outer shell, which is characterized by low crosslink density, will naturally provide a fast release of IL-15 ligands, while those within the dense core, which is characterized by high-crosslink density, will naturally provide gradual, sustained IL-15 delivery. To optimize IL-15 diffusion and responsiveness throughout the hydrogel and within the tumor microenvironment, it is paramount to regulate capsule size; the ratio of hypoxia-responsive linkers to polymer matrix in the capsule structure; IL-15 loading; as well as the polymer wt% and degree of methacrylation, as previously mentioned. Since glioblastoma recurrence most commonly arises at or near the resection margin, this approach will administer the necessary immunomodulatory agents at the site most in need of therapeutic intervention (42).

It is critical to consider the effects of oxygen inhibition, light attenuation, and light scattering on the crosslink density and general solidification of the hydrogel. Since oxygen inhibits free-radical crosslinking, there is a potential for oxygen exposure to have a detrimental impact on the degree of crosslinking. The hypoxic tumor microenvironment would mitigate oxygen inhibition during polymerization; however, it is paramount that this treatment employs techniques to further limit oxygen infiltration during the polymerization process or recognizes that this factor could influence the efficacy of the therapeutic approach (33). The proposed method establishes a crosslink density gradient throughout the hydrogel itself such that the layers touching or closest to the resection wall tissue have the lowest crosslink density, allowing for more rapid diffusion, while the layers approaching the top of the hydrogel have the greatest crosslink density, allowing for slower diffusion. Thus, light attenuation throughout the hydrogel is deliberately leveraged to establish the desired crosslink density gradient, enabling spatial control of therapeutic diffusion and release. However, to alleviate the risk of creating too low of a crosslink density at the resection border due to light attenuation and the risk of uneven crosslinking due light scattering, it would be beneficial to build up the hydrogel in layers starting at the layer directly touching the resection cavity and moving upwards towards the skull. Therefore, each layer will be exposed to visible light to guarantee crosslinking. Moreover, the crosslink density gradient could still be achieved by managing the time that each layer of the hydrogel is exposed to the visible light. Ideally, the layer closest to the reception cavity will have the least visible light exposure, and the final layer nearest the skull will have the most visible light exposure.

2.5 ROS responsive hyaluronidase-loaded nanogels for ECM modulation

To improve CAR T cells' ability to navigate glioblastoma's hyaluronic acid-rich extracellular matrix, this proposal suggests ROS-responsive, hyaluronidase-loaded nanogel

"backpacks" tethered to the surface of each CAR T cell. Cancer cells have been shown to exhibit approximately 100 μmol of ROS, which is elevated compared to normal, healthy cells. However, this measurement is patient and context-dependent, and is variable between cases, so it is not guaranteed and must be tested prior to administration of the treatment. ROS presence in cancer cells can be ~ 10 to 100 times greater than it is in normal, healthy cells (43). These nanogels are engineered to only release their enzyme payload in environments that exhibit high levels of ROS, such as the tumor microenvironment. Once released into the tumor tissue, the hyaluronidase enzymatically degrades the HA scaffolding, improving the CAR T Cell's ability to permeate and move throughout the tumor tissue. The nanogels are assembled using biocompatible host-guest chemistry between cyclodextrin and adamantane, which naturally self-assemble to form the nanogel's basic scaffolding. It is necessary to incorporate an ROS-cleavable thioketal linker into this nanogel design, to keep the nanogel intact until it encounters high levels of ROS. Healthy brain tissue exhibits normal H_2O_2 levels, so when the TK linker is exposed to elevated H_2O_2 levels, it will cleave, releasing the nanogel's hyaluronidase contents into the HA-dense extracellular matrix (44).

Appropriate nanogel loading can be assessed using flow cytometry. In this context, the nanogels are tagged with a fluorescent dye, and once linked to the CAR T Cells, are analyzed with a flow cytometer to evaluate the fluorescence intensity per CAR T Cell. This data is then compared to calibration beads with a known number of fluorophores per bead to establish a standard curve, which can be used to establish an optimal range for nanogel loading. Similarly, TK linker cleavage can be demonstrated using a statistical curve illustrating that elevated H_2O_2 levels result in an increase of TK linker cleavage, while normal H_2O_2 levels result in minimal cleavage. Additionally, in an experimental setting, T cell health and efficacy could be assessed using CD69/CD25 activation markers, viability staining, and tumor-cell killing assays to confirm that surface attachment of nanogels does not impair CAR-T cytotoxicity or function.

The attachment of nanogels to the T Cell surface can be achieved through the use of metabolic glycoengineering (MOE). In this process, T Cells are incubated with acetylated N-azidoacetylmannosamine (Ac_4ManNAz) for a defined period, which enables the initial uptake and metabolic incorporation of the acetylated monosaccharides into the cells. Structurally, Ac_4ManNAz is analogous to N-Acetylmannosamine (ManNAc), which is an endogenous molecule that contributes to the sialic acid biosynthesis pathway, which, in turn, supports the production and expression of glycolipids and glycoproteins on the cell membrane (44-46). Therefore, a T cell would react to and metabolize Ac_4ManNAz through the same pathway that it would for ManNAc. However, unlike ManNAc, the metabolism of Ac_4ManNAz would result in the expression of azide-functionalized sialic acids (SiaNAz) on the surface of the T cell's glycocalyx, which act as bioorthogonal "hooks". Then, nanogels themselves are modified with dibenzocyclooctyne (DBCO), a strained alkyne that naturally undergoes a copper-free strain-

promoted azide-alkyne cycloaddition (SPAAC) reaction upon interaction with the azide-functionalized sugars on the T cell surface. This reaction forms a covalent bond between the nanogels and the surface of the T-cell that minimizes the risk of off-target reactions (44-46). Based on prior studies conducted by Zhao et al, an individual T cell can retain approximately 10^3-10^4 nanogels on its surface. Assuming the diameter of a T cell to be approximately 7-10 μm, and the diameter of each nanogel to be approximately 100 nm, it is estimated that the nanogels would cover 5-40% of the T cell surface. It is paramount that the nanogel concentration per CAR T cell is engineered to administer a clinically significant hyaluronidase dosage, while simultaneously avoiding any interference with T cell function, brain tissue damage, or edema (44).

Combining a hyaluronic acid based hydrogel with hyaluronidase containing nanogels can lead to degradation of the hydrogel itself. To prevent either therapy from reducing the other's effectiveness, a staggered timing of administration of the nanogels and the hydrogel would be ideal. However, since the administration of both systems is contemporaneous during an operative procedure and temporal staggering is not ideal, spatial cohorting of the two mediums should be employed. The nanogels are injected into the surrounding tissue post surgical resection to aid the CAR T-cells in penetrating and navigating the hyaluronic acid dense glioblastoma tumor tissue. Following implantation, the hydrogel itself would remain localized to the resection cavity, and would not penetrate the surrounding tissue; whereas the hyaluronidase nanogels are expected to remain within the brain tissue and spread outwards towards infiltrating tumor cells. Thus, there would be spatial separation between the hydrogel and the hyaluronidase in this proposed treatment strategy. Consequently, hyaluronidase-mediated degradation would be expected to preferentially target the hyaluronic acid-rich extracellular matrix of the glioblastoma microenvironment rather than the implanted HA-MA hydrogel itself. Nevertheless, future studies should investigate engineering the hydrogel polymers to resist hyaluronidase-mediated degradation, thereby further minimizing the potential for therapeutic interference.

2.6 Perspectives

One of the primary obstacles in treating tumors with CAR receptors designed to target tumor-associated antigens is antigen escape (5). In other words, the tumor cells recognize the repeated attacks on a particular antigen and downplay their expression of the antigen as a defensive measure. In this instance, CAR T Cells engineered to target a specific tumor-associated antigen would no longer be able to target the tumor cells. This defensive mechanism is similar to how tumor cells evade detection of autologous T-cells by reducing their expression of MHC class 1 molecules (25).

The therapeutic treatment approach outlined in this paper attempts to reduce antigen escape through a dual-antigen recognition system. However, future studies would benefit from exploring the applications of artificial antigens in combating antigen escape. Specifically, employing artificial antigens as the primary targets of CAR T Cells. This strategy would

require a two-layered approach towards CAR T Cells administration. The first round of CAR T Cells would act as the "taggers", and the second round would act as the "attackers". The first round of CAR T Cells would deliver the artificial antigen (such as CD19 fragments, peptide tags, or other synthetic receptors compatible with this treatment type) to the tumor cells so that they display them on their surface, making them identifiable for the second round of CAR T Cells. The second round of CAR T Cells, those of which would have been engineered with CAR receptors that target this artificial antigen, will be able to identify and attack the flagged tumor cells. Since this artificial antigen is being introduced to the body by a therapeutic system rather than relying on an antigen derived from endogenous tumor biology, the synthetic antigen may be less susceptible to antigen escape because it is expected that the tumor cells would be unable to remove it. Even if the tumor cells try to persist by eliminating EGFRvIII from their surfaces, it would not impact the success of the treatment because they would have already been tagged with the artificial antigen. Additionally, due to its artificial nature, this antigen is not found on any other body cells that may be within or surrounding the tumor, eliminating the risk of off-target effects.

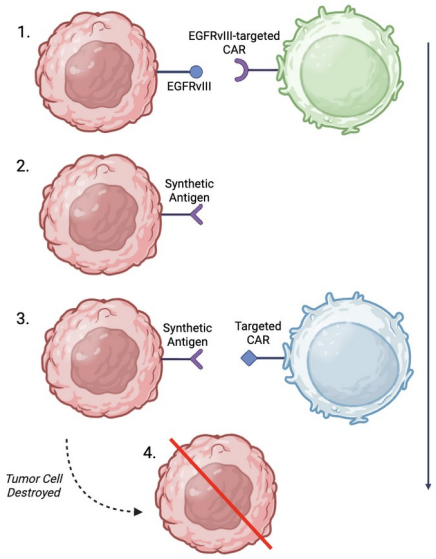


Figure 7. The steps of the proposed two-phased CAR T-cell strategy for targeting glioblastoma tumor cells. The first round of CAR T-cells are displayed in green, while the second round of CAR T-cells are displayed in blue. The first round of CAR T-cells targets the EGFRvIII^{+} tumor cells via an EGFRvIII-targeted CAR T receptor, and the synthetic antigen is administered to the tumor cell. Then, the second round of CAR T-cell, designed to target the artificial antigen, identifies and eliminates the marked tumor cell. This figure was designed in BioRender.

This strategy could be especially valuable in treating glioblastoma due to its severe heterogeneity and the likelihood of antigen escape and variability due to high levels of immunosuppression within the tumor microenvironment (25,11). Gliomas are often

variable in their antigen profiles, both from patient to patient and within different parts of the tumor itself (25,11). This is one of the primary obstacles in treating glioblastoma. By establishing a universal antigen that can be easily targeted by engineered T Cells, the risk posed by the heterogenic nature of this tumor type, and the likely loss of target antigens, would be alleviated.

Ideally, based on the prior evidence in this study, the first round of CAR T Cells should be engineered to target EGFRvIII, due to its consistent presence in glioblastoma patient samples, and its success in past studies (8). Additionally, these CAR T Cells should also be engineered to administer the artificial antigen into the tumor cell upon binding with the EGFRvIII ligand. This could be accomplished in a number of ways, including, but not limited to, SynNotch gene circuits, delivery via an engineered viral vector, or through membrane protein transfer.

2.6.1 *SynNotch gene circuits application*

A SynNotch receptor, as outlined in the previous section, *IL13Rα2 as a Secondary Target for CAR T Cell Therapy in Glioblastoma* and in Figure 3, is often a dual–antigen system. Upon binding between the primary tumor-associated antigen receptor on the CAR T cell, and the target antigen expressed on the tumor, a downwards signaling cascade is triggered within the CAR T cell that releases a transcription factor into the nucleus, activating a specific gene that ultimately results in the establishment of a beneficial protein (47). In this proposal, a system was proposed in which upon binding of EGFRvIII, a IL13Ra2 receptor was produced. Alternatively, the SynNotch system could be designed so that upon binding with EGFRvIII, a synthetic antigen is delivered to the tumor cell.

2.6.2 *Viral vector application*

A viral vector is a replication incompetent virus engineered to deliver DNA into cells. In other words, instead of acting like an infectious virus, it uses its delivery mechanisms to administer a predetermined portion of DNA into a cell to produce a beneficial result or protein (48). In this particular system, upon binding with EGFRvlll, a viral vector that contains the necessary DNA (programing for the production of the synthetic antigen) is administered to the tumor cell, delivers this DNA package into the cell, and results in the permanent display of the synthetic antigen on the tumor cell surface.

2.6.3 *Membrane protein transfer application*

Membrane protein transfer physically attaches the synthetic antigen to the tumor cell surface, instead of encoding for its production via DNA (48). There are many different methods of conducting membrane protein transfer, such as through engineered membrane vesicles, or transposon-based delivery systems, which are able to carry larger payloads and are less expensive to manufacture than viral vectors (48).

Compared to the dual-antigen SynNotch approach proposed in this paper, which is dependent on glioblastoma-specific tumor associated antigens, this strategy would target and identify tumor cells via an established synthetic target, thereby reducing the treatment's dependence on endogenous antigen expression.

3. Results & Discussion

The administration of PD-1 knockout, EGFRvIII-targeting CAR T Cells supported by a hydrogel that delivers IL-15 to the tumor bed post resection, offers a multifaceted therapeutic approach for overcoming the adverse tumor microenvironment and improving the efficacy and persistence of CAR T Cell treatment. Current treatments for glioblastoma, such as surgical resection, chemotherapy, radiotherapy, and the administration of corticosteroids, fail to address the numerous obstacles characteristic of this form of cancer, such as severe tumor heterogeneity, immune suppression, and tumor recurrence post-resection. Using EGFRvIII as the initial target in a dual-antigen SynNotch approach improves therapeutic precision by enabling MHC independence and exclusively targeting malignant cells while preventing off-target effects, while IL13αR2 functions as the secondary receptor to ensure widespread tumor elimination and to reduce the effects of antigen escape on treatment efficacy. Although this dual-antigen strategy is designed to mitigate antigen escape, future iterations of the platform could further enhance therapeutic durability through the localized delivery of viral vectors encoding programmable synthetic antigens from the implanted hydrogel. By inducing residual tumor cells to express a uniform synthetic target, CAR T-cell recognition could become less dependent on heterogeneous endogenous antigen expression, thereby providing an additional strategy for overcoming antigen escape and broadening the applicability of the SynNotch platform. The PD-1 knockout fortifies the CAR T Cells against inhibitory signals that induce premature exhaustion and inactivation, as PD-1 is a major immune checkpoint that suppresses antitumor T-cell activity. ROS-cleavable nanogels further support sustained CAR T Cell activity in the harsh tumor microenvironment by leveraging the elevated oxidative stress characteristic of glioblastoma to enable controlled degradation of the HA-dense tumor scaffolding. The incorporation of an intracavitary hydrogel IL-15 delivery system improves upon prior CAR T Cell treatment strategies for solid tumors by enabling prolonged, localized cytokine support and minimizing CAR T Cell exposure to immunosuppressive constraints and systemic toxicities. IL-15 acts as an immunostimulant that further modulates the tumor microenvironment to support CAR T Cell activity and overall immune activation. As a final step, this treatment is designed to be administered post tumor resection so that it has the potential to bypass the blood-brain barrier and to improve treatment localization.

While each component of this proposed treatment strategy has undergone individual experimentation, not all of them have been applied in combination with other therapies. Moreover, these treatments have not all been combined into one combination strategy before, which is the ultimate proposal of this paper. Consequently, the complexity of this proposed platform may present challenges for clinical translation and large-scale manufacturing, particularly in terms of cost, production scalability, and the storage and handling of biological materials and reagents. This proposal requires intricate and complex genetic engineering on multiple fronts, including an EGFRvIII SynNotch circuit with inducible IL13Ra2 CAR expression, a PD-1 knockout,

potentially a glucocorticoid receptor knockout; as well as the construction of biomaterials such as ROS-responsible hyaluronidase nanogels, metabolic glycoengineering, and a hypoxia-responsible IL-15 releasing HA-MA hydrogel. Coordinating the creation, storage control, and reproducibility of each of these components, as well as transportation in alignment with treatment timelines, would be considerably complex and would present barriers of accessibility, especially in more rural or socioeconomically challenged communities.

Furthermore, translating this treatment method into the clinical setting would be difficult to manage due to the numerous components and sensitive timing windows. CRISPR-Cas9 is the recommended strategy for conducting the numerous genetic alterations to the analogous T cells at the start because of CRISPR's accessibility, and its rapid timeframe. Viral vectors represent another potential approach for implementing these complex genetic modifications. However, the size and complexity of the engineered genetic construct may approach or exceed vector packaging limits, potentially reducing delivery efficiency and increasing the risk of off-target effects or unintended genomic alterations. From a translational perspective, each component of the proposed engineering strategy would require independent validation for safety, stability, and manufacturing consistency to ensure reproducibility, scalability, and suitability for clinical application. While this proposal presents a conceptual framework rather than a product that would be immediately translated into the lab, it is paramount that future studies prioritize simplification of this design, as well as streamlining the engineering process to make it more efficient and accessible to research centers around the world. However, it is critical that the overall methodology maintains its ability to combat the primary obstacles this proposal targets, including antigen escape, tumor microenvironment toxicity and heterogeneity, immunosuppression, and the blood brain barrier.

When developing the therapeutic components of the treatment proposed in this paper, and translating them into a clinical setting, it is of utmost importance to consider how steroids, such as Dexamethasone, and other palliative drugs, may influence or interfere with the treatment, and the disparities between the cellular mechanisms and manifestation of glioblastoma across gender and sex. Corticosteroids, especially dexamethasone, are the cornerstone of glioblastoma symptom management in patients; however, their immunosuppressive effects interfere with the biological mechanisms necessary to conduct an effective CAR T-cell treatment approach.

Dexamethasone is a widely used corticosteroid that helps to reduce edema and manage patient symptoms during immunotherapy. However, in recent studies, it has been shown to have a detrimental impact on T cells, more specifically their proliferation, functionality, and maturation, which are key components of numerous immunotherapy techniques and the immune response as a whole (49). This is a major limitation of current immunotherapy treatments because patient well-being and symptom management must coexist with therapeutic efficacy for a treatment plan to be

successful; thus, if these factors inadvertently undermine each other, the therapy is unlikely to be viable in a clinical setting. Expanding research of this nature is essential to understanding the full spectrum of obstacles presented by cancer treatment, and therefore, is critical to guaranteeing the efficacy of immunotherapy treatments such as the one proposed in this paper. A potential solution is the CRISPR-mediated deletion of the glucocorticoid receptor in engineered CAR T cells, which would alleviate steroid-induced immunosuppression while preserving patient well-being during cancer treatment.

Historically, biomedical research has been male-centric, and still, there remains disproportionately little insight into female biology, especially into how the female body responds to and develops certain conditions differently from the male body. The role that sex and gender play in the development, diagnosis, and outcome of glioblastoma must be recognized in glioblastoma research for treatments to be the most effective and potentially life-saving in the future. The higher incidence in females compared to males (27.85 vs. 21.62 per 100,000), as reported in the 2023 CBTRUS Statistical Report based on the average annual age-adjusted incidence rate (AAIR), further underscores the importance of developing safe and inclusive treatment strategies for female patients and highlights the need for sex-specific research (50). While female biology is more complex than male biology, and therefore presents additional obstacles and parameters to research studies, this does not justify neglect. Several studies have identified distinct trends that differentiate female and male pathogenesis of glioblastoma, highlighting a critical aspect of glioblastoma research that cannot be ignored by means of underrepresentation (51).

4. Conclusion

Glioblastoma remains the most difficult CNS tumor to treat due to its heterogeneous tumor microenvironment, highly invasive nature, anatomical location, and its high likelihood of immune evasion, antigen escape, and recurrence. Routine clinical treatments, such as surgical resection, chemotherapy, and radiation therapy, are inadequate due to their lack of specificity and personalization in patients, their vulnerability to genetic adaptation, and their failure to address the various elements that contribute to the refractory nature of glioblastoma, most notably the hostile tumor microenvironment. Additionally, these modalities often work independently rather than cohesively, which limits their long-term success in combating the tumor microenvironment and immune evasion. Such a heterogeneous and treatment-resistant malignancy requires a comprehensive treatment approach that incorporates multiple therapeutic agents that act in synergy with each other to target and eliminate malignant cells. This conceptual design review underscores the necessity of a multifaceted therapeutic approach for glioblastoma. While CAR T-cell therapies have advanced into clinical trials, their limited efficacy demonstrates the need for further optimization. By employing a combination therapy that integrates antigen-specific, CRISPR-mediated targeting of EGFRvIII, a dual-antigen targeting system facilitated by a SynNotch–IL13αR2 receptor, immune

checkpoint resistance through the use of a PD-1 knockout, ROS-responsive hyaluronidase-loaded nanogels for extracellular matrix modulation, prolonged IL-15-driven immunostimulatory support, and localized intracavitary delivery through a photocrosslinkable HA-MA hydrogel, this approach addresses the primary obstacles that have limited the efficacy of prior CAR T-Cell therapies in treating glioblastoma.

Beyond this proposed therapeutic platform, future iterations could incorporate localized hydrogel-mediated delivery of viral vectors encoding programmable synthetic antigens into residual tumor cells within the resection cavity. By engineering a uniform, controllable antigen directly onto surviving glioblastoma cells, this strategy could reduce dependence on heterogeneous endogenous antigen expression, complement the SynNotch-regulated CAR T-cell platform, and provide an additional mechanism for overcoming antigen escape.

This study is grounded in an extensive review of the literature addressing the major challenges associated with GBM, CAR T-cell therapy, and T-cell exhaustion. Subsequently, an intracavitary delivery of EGFRvIII-targeting, dual-antigen SynNotch–IL13Rα2 engineered, PD-1 CRISPR knockout CAR T cells supported by ROS-responsive hyaluronidase-loaded nanogels and an IL-15-enriched, visible-light photocrosslinkable hyaluronic acid methacrylate hydrogel is proposed. On the basis of these identified challenges, the present work seeks to hypothesize a therapeutic strategy designed to comprehensively address the obstacles inherent to GBM treatment while also identifying synthetic antigen engineering as a promising future extension to further improve therapeutic specificity, persistence, and resistance to antigen escape in personalized glioblastoma treatment.

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