

Peer-Review

Coppo, Julia M. 2026. "Synthetic Antigen-Augmented SynNotch CAR T Cell Therapy for Glioblastoma: A Conceptual Design Review." *Journal of High School Science* 10 (3): 26–55. <https://doi.org/10.64336/001c.164664>.

A well thought out, in-depth, combination of existing treatments. Congratulations. However, even this combined treatment is unlikely to prove efficacious. More elements need to be incorporated into this treatment (points 3, 4 and the suggestion). I list my concerns and comments below that will need to be addressed in the manuscript.

1. the individual components of your therapy (IL-15, PD-KO and CART targeting EGFRvIII) are well known and have been described in the literature; some with in vivo studies. You have combined these in an obvious, evident way thus making your paper not an original contribution; but rather, a plug-and-play combination of existing therapies in the public domain. Therefore, your paper falls into the category of a 'review manuscript'.

2. As a review manuscript, the paper is not acceptable for publication in the Journal since it only presents content already in the public domain and does not satisfy the Journal's expectations for a review paper as seen here: <https://jhss.scholasticahq.com/for-authors>, types of manuscripts, review papers.

3. The challenge is that - while a combination of the approaches is logically sound, many have demonstrated no clinical benefit in-vivo and in phase I clinical studies (see references below). For example, a PD-1 + EGFRvIII targeting CART showed no clinical efficacy in phase I studies, secondary tumors appeared (presumably due to poor CART infiltration) in patients treated with EGFRvIII targeting CART and patients relapsed eventually even though the primary tumor regressed. Antigen escape and exhaustion still occurred, even with IL15 (the latter, presumably due to the hypoxic and metabolically deficient TME). There have been advances in designing CART which are active even in hypoxic or metabolically deficient environments (see representative references below). Discuss in the manuscript.

4. HA is expressed by the ECM of the tumor. This slows down infiltration of CART throughout the brain tissue and may be responsible for the development of secondary cancers. Hyaluronidase can be encapsulated into the polymer matrix (PLGA or collagen based) which can increase the infiltration area of CART into the brain tissue adjoining the resection cavity. Discuss in the manuscript.

Suggestion: also incorporate SynNotch receptors into CART cells (when SynNotch receptor detects EGFRvIII, it binds to this target. If EGFRvIII is not detected, SynNotch allows transcription of a second CAR (such as IL13R α 2-CAR). This mitigates the phenomenon of antigen escape. Furthermore, incorporate HRE (hypoxia responsive elements) driving IL15 and maintaining CART efficacy in hypoxic tumor environments, Steroid resistant elements into CART. In addition, incorporate hyaluronidase into biodegradable polymer matrix.

5. Even if points 3 and 4 (and the resulting suggestion) are incorporated into the manuscript (and you will need to incorporate these), this is still evident, and obvious extrapolation from the literature. You will need to describe or present some non-obvious or non-evident attributes of your combined therapy to have this paper confirm to the 'review paper' guidelines for this Journal.

6. Here's what you can propose: inject a second population of CART with the exact elements incorporated into it as the first. However, this will work a 'prime-and-kill' trajectory. i.e. it will force engaged (tumor) cells to express a synthetic antigen (perhaps even EGFRvIII) thereby enabling the same CART cells to kill the tumor cells EVEN if immune escape occurs. Please research the literature. This represents a non-obvious, non-evident mechanism that is publishable in the Journal's rubric. Present this proposal with a vector map and a gene circuit diagram. Take the help of chatgpt if you must. I will be very happy to review your manuscript revised along these lines.

Additional references that need incorporation into the manuscript:

Regression of Glioblastoma after Chimeric Antigen Receptor T-Cell Therapy, Brown et al., N Eng J

Med. 375(26).

<https://doi.org/10.1093/neuonc/noac024>

<https://clinicaltrials.gov/study/NCT03726515>

<https://doi.org/10.1038/s43018-023-00709-6>

<https://doi.org/10.1126/scitranslmed.aaa0984>

<https://doi.org/10.1172/jci.insight.177141>

<https://doi.org/10.3390/ijms24065936>

<https://doi.org/10.1016/j.jbc.2021.100549>

<https://doi.org/10.26599/NR.2025.94907359>

<https://doi.org/10.1038/s41423-025-01290-x>

<https://doi.org/10.1146/annurev-chembioeng-092120-092914>

<https://doi.org/10.3389/fimmu.2023.1186383>

<https://doi.org/10.1016/j.xcrm.2021.100227>

<https://doi.org/10.1172/jci.insight.177141>

<https://doi.org/10.1111/imr.12773>

<https://doi.org/10.1016/j.cell.2016.09.011>

<https://doi.org/10.1038/s43018-025-00968-5>

I cannot review this iteration unless the author includes a word file describing my previous comments verbatim - each followed by a response as to how and where in the revised manuscript, that comment is now addressed.

This was communicated in the earlier review.

The challenge is that, while a combination of the approaches is logically sound, many have demonstrated no clinical benefit in vivo and in phase I clinical studies (see references below). For example, a PD-1 + EGFRvIII targeting CART showed no clinical efficacy in phase I studies; secondary tumors appeared (presumably due to poor CART infiltration) in patients treated with EGFRvIII targeting CART, and patients eventually relapsed, even though the primary tumor regressed. Antigen escape and exhaustion still occurred, even with IL15 (the latter, presumably due to the hypoxic and metabolically deficient TME). There have been advances in designing CART, which are active even in hypoxic or metabolically deficient environments (see representative references below). Discuss in the manuscript.

I appreciate the reviewer highlighting the importance of acknowledging the previously established limitations of EGFRvIII-targeted CAR-T cell therapy, as demonstrated in recent studies. In response, this revised manuscript discusses relevant Phase I and Phase II studies and their results. The proposed reasoning behind such limitations, including antigen escape, high heterogeneity in the tumor microenvironment, and hypoxic conditions, is also addressed in both the abstract and introduction paragraphs and referenced through relevant sections of the manuscript to provide rationale and context. Additional therapeutic components, focused on addressing persistence within a hypoxic and metabolically deficient tumor microenvironment, were combined alongside PD-1 knockout and IL-15 release, providing further support towards the clinical efficacy of the CAR T cells.

HA is expressed by the ECM of the tumor. This slows down infiltration of CART throughout the brain tissue and may be responsible for the development of secondary cancers. Hyaluronidase can be encapsulated into the polymer matrix (PLGA or collagen-based), which can increase the infiltration area of CART into the brain tissue adjoining the resection cavity. Discuss in the manuscript.

I am appreciative of the reviewer's suggestion to address the role of an HA dense extracellular matrix in preventing CAR T cell infiltration into tumor tissue. To address this, this revised

manuscript proposes the implementation of reactive oxygen species (ROS)-responsive hyaluronidase-loaded nanogels. These nanogels are attached to the CAR-T cells through metabolic glycoengineering (MOE), which is a bioorthogonal method compatible with T cell metabolism. This strategy capitalizes on the elevated ROS levels within the tumor tissue to trigger the release of hyaluronidase, which would degrade the HA-dense extracellular matrix localized to the CAR-T cell, improving infiltration and persistence. This manuscript includes a detailed explanation of the nanogel design, release mechanism, methods of evaluation and regulation, and attachment chemistry within the section titled: *ROS-Responsive Hyaluronidase-Loaded Nanogels for ECM Modulation*.

Suggestion: also incorporate SynNotch receptors into CART cells (when the SynNotch receptor detects EGFRvIII, it binds to this target. If EGFRvIII is not detected, SynNotch allows transcription of a second CAR (such as IL13R α 2-CAR). This mitigates the phenomenon of antigen escape. Furthermore, incorporate HRE (hypoxia responsive elements) driving IL15 and maintaining CART efficacy in hypoxic tumor environments, Steroid resistant elements into CART. In addition, incorporate hyaluronidase into a biodegradable polymer matrix.

I value the reviewer's suggestion to incorporate a SynNotch receptor to address antigen escape, a significant barrier in EGFRvIII-targeted CAR T cell therapy towards glioblastoma. In response, the revised manuscript includes a dual-antigen SynNotch system that, upon interaction with tumor-associated EGFRvIII, enables the transcription of a secondary IL13R α 2-targeted CAR receptor on the surface of the CAR T cell. EGFRvIII is more tumor-specific than IL13R α 2; thus, this combination of target antigens establishes a prime-and-kill system, in which the CAR T cells cannot target IL13R α 2, a more widespread antigen, without binding to EGFRvIII. This provides an extra layer of protection for healthy body cells. This revised manuscript also provides a detailed and unique design, including a TetOn system and PEST degron applications that enhance patient safety when employing a dual-antigen SynNotch receptor. The section titled *IL13R α 2 as a Secondary Target for CAR T Cell Therapy in Glioblastoma* expands on the rationale behind a dual-antigen system; supports the employment of IL13R α 2 as the secondary antigen with genomic data presented in The Human Protein Atlas, and recalls the results of several papers that support the safety and efficacy of targeting IL13R α 2 in CAR T cell therapy. Additionally, this section includes an original plasmid map that illustrates the proposed EGFRvIII-activated rtTA-mediated IL13R α 2 CAR transcription, and an original diagram outlining the intrinsic and extrinsic mechanisms.

Furthermore, I appreciate the reviewer's suggestion to incorporate more hypoxia-responsive elements (HRE) to drive IL-15 and maintain CAR T cell efficacy in hypoxic tumor microenvironments. Thus, in recognition of the hypoxic nature of the residual tumor cavity post resection, this revised manuscript proposes the encapsulation of the IL-15 ligands into oxygen-responsive nanocapsules embedded within the hydrogel itself. These nanocapsules incorporate hypoxia-responsive linkers that degrade under low-oxygen conditions, allowing for localized cytokine release in low-oxygen environments, which are characteristic of the tumor microenvironment, immunosuppressive towards engineered immune cells, and in the greatest need of cytokine support. This description of the system is described in the *Hydrogel-Based Delivery of Immunostimulants* section of this manuscript.

Additionally, I value the reviewer's suggestion to expand upon steroid resistance mechanisms. In response, this revised manuscript proposes CRISPR-mediated deletion of the glucocorticoid receptor in engineered CAR T cells. Dexamethasone is a widely used corticosteroid that helps to reduce edema and manage patient symptoms during immunotherapy, but it presents a legitimate barrier to employing this treatment strategy in a clinical setting where patient well-being is equally as important as treatment efficacy. This strategy would prevent steroid-induced immunosuppression

while preserving patient well-being during cancer treatment. In addition, this paper discusses published research conclusions describing the success of IL13R α 2-targeted CAR T Cells, which demonstrated resistance to corticosteroids. This proposal and rationale are discussed in the *Results & Conclusion* section of this paper.

Even if points 3 and 4 (and the resulting suggestion) are incorporated into the manuscript (and you will need to incorporate these), this is still an evident and obvious extrapolation from the literature. You will need to describe or present some non-obvious or non-evident attributes of your combined therapy to have this paper conform to the 'review paper' guidelines for this Journal. Here's what you can propose: inject a second population of CART with the exact elements incorporated into it as the first. However, this will work a 'prime-and-kill' trajectory.

i.e., it will force engaged (tumor) cells to express a synthetic antigen (perhaps even EGFRvIII), thereby enabling the same CART cells to kill the tumor cells EVEN if immune escape occurs. Please research the literature. This represents a non-obvious, non-evident mechanism that is publishable in the Journal's rubric. Present this proposal with a vector map and a gene circuit diagram. Take the help of ChatGPT if you must. I will be very happy to review your revised manuscript along these lines.

I appreciate the reviewer's suggestion to incorporate a non-obvious mechanism that strengthens this manuscript's proposed therapeutic strategy. In response, this revised manuscript describes the employment of a prime-and-kill dual-antigen system to improve the efficacy of CAR T cell targeting within the tumor. EGFRvIII is the initial target in a dual-antigen SynNotch approach, while IL13R α 2 functions as the secondary receptor to ensure widespread tumor elimination and to reduce the effects of antigen escape on treatment efficacy. An original plasmid map that illustrates the proposed EGFRvIII-activated rtTA-mediated IL13R α 2 CAR transcription within the section titled: *IL13R α 2 as a Secondary Target for CAR T Cell Therapy in Glioblastoma*.

Thank you for trying to address my comments. The manuscript now appears to be heading in the right trajectory. However, you have misunderstood my 'non-obvious' suggestion (see chatgpt pdf file attached for step-by-step instructions. In addition, please address additional inconsistencies and deficiencies that i have listed below. Please also make a concerted effort to include more recent and pertinent references in the manuscript (you will need to do this anyway to properly address my concerns below).

1. You have misunderstood my previous suggestion regarding the introduction of a second CAR-T population designed to induce expression of a synthetic antigen on tumor cells. (see attached pdf file for a chatgpt step-by-step explanation). In my comment, the intent was to propose a mechanism in which an initial CAR-T population would engage tumor cells and subsequently force or deliver expression of a non-native, synthetic surface antigen, thereby creating a stable and uniform target that could be recognized by a second CAR-T population. This approach is conceptually distinct from the SynNotch dual-antigen system currently described in the manuscript, which simply activates a secondary CAR directed at an existing tumor antigen (IL13R α 2). While the dual-antigen SynNotch strategy is reasonable, it does not address the specific concept suggested in my comment, namely the induction of a synthetic antigen to mitigate antigen escape and tumor heterogeneity. The manuscript would benefit from clarifying this distinction or from more directly engaging with the originally proposed mechanism. (see attached pdf file from chatgpt which takes you step-by-step). Please incorporate this suggestion in your manuscript in a subsection titled (for example), "perspectives". (also see last paragraph of review.)

2. Since you have zero computational modeling, remove 'in silico' and replace with 'conceptual design review'.

3. Incorporating all my comments means that you now have unrealistic system complexity (which is fine), but then you need a brief discussion on manufacturing feasibility, regulatory barriers, vector size limits, CAR construct packaging, etc. Please include this discussion in the manuscript.

System complexity = SynNotch circuit

, IL13R α 2 CAR, PD-1 knockout, glucocorticoid receptor knockout, ROS nanogels, metabolic glycoengineering

hypoxia nanocapsules, IL15 release....

4. Correct this claim “EGFRvIII detected in 50–60% of GBM cases.” to “EGFR amplification ~40–60%

EGFRvIII mutation ~20–30%”

5. Discuss IL-15 toxicity in terms of CRS, neurotoxicity and NK overactivation. Discuss dosing control of the hydrogel.

6. You only state “reduces systemic toxicities” for PD-1 KO. please also discuss risk of autoimmunity and uncontrolled T cell activation.

7. you assume that tumor ROS $\approx$ 100 μ M. However, This is extremely context-dependent. ROS levels vary widely by tumor region. Therefore trigger reliability is unclear. Discuss in the manuscript.

8. Remove all “clinical efficacy” claims, or explicitly state that these remain speculative because there is no modeling, no animal data or prior studies. Restate the claim as being hypothesis-generating. In addition, you claim “zero predicted mismatches”. This is unrealistic. Off-target prediction tools never guarantee zero mismatches across genome. Please correct. Furthermore, remove all claims of “unique design” (except in the perspectives section).

9. Database analysis unclear: you mention TCGA and cBioPortal, but provide no statistical analysis. Figures appear to be screenshots rather than original analysis.

10. Because the hydrogel is intended to be photocrosslinked intraoperatively, the issue of intracranial light penetration may be less restrictive than in non-surgical contexts, as the resection cavity is directly exposed to illumination. However, the manuscript should still clarify how uniform crosslinking throughout the hydrogel volume will be achieved given potential light attenuation, scattering within the hydrogel matrix, and oxygen inhibition during free-radical polymerization.

11. A potential design conflict arises from the simultaneous use of hyaluronidase-loaded nanogels and a hyaluronic-acid-based hydrogel scaffold. Because hyaluronidase enzymatically degrades hyaluronic acid, it is unclear how the HA-MA hydrogel would maintain structural integrity and controlled IL-15 release in the presence of enzyme activity intended to digest the tumor ECM. The manuscript would benefit from clarifying whether the hydrogel is engineered to resist hyaluronidase degradation, whether spatial separation between these systems is expected, or whether an alternative hydrogel polymer might be more appropriate.

Explanation of non-obvious proposal and its special relevance for glioblastoma (you can rewrite for the manuscript).

The proposal changes the logic of the system. Instead of relying only on naturally occurring tumor antigens, the first CAR-T population would induce expression of a synthetic antigen on tumor cells after initial engagement. Once tumor cells are “tagged” with this artificial marker, a second CAR-T population targeting the synthetic antigen can recognize and eliminate them. Because the target antigen is artificially introduced rather than dependent on endogenous tumor biology, the therapy becomes less vulnerable to antigen heterogeneity and antigen loss, which are major causes of treatment failure in glioblastoma.

In other words, the proposal converts a system that chases variable tumor markers into one that creates its own uniform target, which could substantially reduce the impact of antigen escape; which is the most significant reason why existing glioblastoma CAR therapies fail.

You have misunderstood my previous suggestion regarding the introduction of a second CAR-T population designed to induce expression of a synthetic antigen on tumor cells. (see attached pdf file for a chatgpt step-by-step explanation). In my comment, the intent was to propose a mechanism in which an initial CAR-T population would engage tumor cells and subsequently force or deliver expression of a non-native, synthetic surface antigen, thereby creating a stable and uniform target that could be recognized by a second CAR-T population. This approach is conceptually distinct

from the SynNotch dual-antigen system currently described in the manuscript, which simply activates a secondary CAR directed at an existing tumor antigen (IL13R α 2). While the dual-antigen SynNotch strategy is reasonable, it does not address the specific concept suggested in my comment, namely the induction of a synthetic antigen to mitigate antigen escape and tumor heterogeneity. The manuscript would benefit from clarifying this distinction or from more directly engaging with the originally proposed mechanism. (see attached pdf file from chatgpt which takes you step-by-step). Please incorporate this suggestion in your manuscript in a subsection titled (for example), “perspectives”. (also see last paragraph of review.)

I appreciate the reviewer’s suggestion to propose a more unique perspective for combatting antigen escape in treating glioblastoma, and I apologize for my previous misunderstanding. To fulfill this request, I added a section titled *Perspectives* that outlines the methodology described in this comment and the ChatGPT summary provided to me. Additionally, I described the necessity of such a strategy and how it differs from the previously described dual-antigen SynNotch circuit, and included a brief discussion of each of the suggested methods of administering the artificial antigen to the tumor cell. Finally, I designed a new figure in BioRender and added to this section that illustrates the steps of the methodology (first round of CAR T cells bind with EGFRvIII on tumor cell, transmit the artificial antigen to its surface, then the second round of CAR T Cells bind to the artificial antigen and eliminate the cancer cell)

Incorporating all my comments means that you now have unrealistic system complexity (which is fine), but then you need a brief discussion on manufacturing feasibility, regulatory barriers, vector size limits, CAR construct packaging, etc. Please include this discussion in the manuscript.

System complexity = SynNotch circuit

, IL13R α 2 CAR, PD-1 knockout, glucocorticoid receptor knockout, ROS nanogels, metabolic glycoengineering

hypoxia nanocapsules, IL15 release....

I thank the reviewer for highlighting the importance of recognizing this limitation of the proposal. To address this, I added two paragraphs into the *Results and Discussion* section of this paper describing the limitations of this proposal within the context of manufacturing, replication, and its clinical application, and describe what I believe should be the primary goals of future studies when aiming to alleviate this barrier to translating this theoretical approach into clinical care

Discuss IL-15 toxicity in terms of CRS, neurotoxicity and NK overactivation. Discuss dosing control of the hydrogel.

I appreciate this suggestion from the reviewer, and these obstacles were described more thoroughly in the section titled *Hydrogel-Based Delivery of Immunostimulants*. This section now provides information on the risk of IL-15 in causing cytokine release syndrome, what cytokine release syndrome is, and the risk of Natural Killer cell overactivation, as well as the consequences. Future studies are advised to prioritize managing dosage and evaluating treatment administration parameters based on the needs of the patient and with the ultimate goal of minimizing immune-related toxicities while still providing enough of the cytokine to support CAR T Cell function.

You only state “reduces systemic toxicities” for PD-1 KO. please also discuss risk of autoimmunity and uncontrolled T cell activation.

I appreciate the reviewer’s suggestion, for this was a significant gap in my description of PD-1 in this proposal. To address this, I added a paragraph to the section titled *Targeting PD-1 to Enhance CAR T Cell Activity* describing the risks posed by a lack of PD-1 towards CAR T Cell overactivation and potential autoimmune responses that may result as a consequence. Additionally, I

make further suggestions for future studies on what they should prioritize when aiming to minimize the immune-related toxicities of PD-1 knockouts while maintaining anti-tumor activity.

You assume that tumor ROS $\approx 100 \mu\text{M}$. However, This is extremely context-dependent. ROS levels vary widely by tumor region. Therefore trigger reliability is unclear. Discuss in the manuscript.

I appreciate the reviewer's suggestion, as this is a significant limitation. When this statement is made in the proposal, a brief description was added that outlines this limitation and the necessity of evaluating the reliability of this treatment from patient to patient based on their ROS levels.

Remove all "clinical efficacy" claims, or explicitly state that these remain speculative because there is no modeling, no animal data or prior studies. Restate the claim as being hypothesis-generating. In addition, you claim "zero predicted mismatches". This is unrealistic. Off-target prediction tools never guarantee zero mismatches across genome. Please correct. Furthermore, remove all claims of "unique design" (except in the perspectives section).

I appreciate the reviewer's suggestion. These claims were removed or altered to suggest that they were hypothetical in nature, or that they were not unique, but instead, based on prior research and experimentation conducted by others. Additionally, the zero predicted mismatches statement was removed.

Database analysis unclear: you mention TCGA and cBioPortal, but provide no statistical analysis. Figures appear to be screenshots rather than original analysis.

I value the reviewer's suggestion and will clarify as follows. These database images are not any form of data analysis; instead, they are there to be viewed as evidence to support a specific claim. I conducted no independent data analysis, just worked with what the databases provided, and described what I was seeing and how it aligned with my claims. Additionally, these figures are screenshots from the databases because I was unable to access the png's.

Because the hydrogel is intended to be photocrosslinked intraoperatively, the issue of intracranial light penetration may be less restrictive than in non-surgical contexts, as the resection cavity is directly exposed to illumination. However, the manuscript should still clarify how uniform crosslinking throughout the hydrogel volume will be achieved given potential light attenuation, scattering within the hydrogel matrix, and oxygen inhibition during free-radical polymerization.

I am grateful for the reviewer's suggestion to clarify how this method of hydrogel administration aims to overcome the challenges of light attenuation, light scattering, and oxygen inhibition. Oxygen inhibition was already addressed in the manuscript, but was restated alongside descriptions of light attenuation and scattering, and their impact on the effectiveness of the hydrogel. I suggested creating the hydrogel in layers starting from the resection cavity wall; as well as measuring the timing of light exposure on each layer to guarantee that crosslinking occurs in every part of the hydrogel, while also maintaining the proposed crosslink density gradient throughout.

A potential design conflict arises from the simultaneous use of hyaluronidase-loaded nanogels and a hyaluronic-acid-based hydrogel scaffold. Because hyaluronidase enzymatically degrades hyaluronic acid, it is unclear how the HA-MA hydrogel would maintain structural integrity and controlled IL-15 release in the presence of enzyme activity intended to digest the tumor ECM. The manuscript would benefit from clarifying whether the hydrogel is engineered to resist hyaluronidase degradation, whether spatial separation between these systems is expected, or whether an alternative hydrogel polymer might be more appropriate.

I appreciate this suggestion from the reviewer, as it is a critical limitation to this proposed strategy if not addressed. I suggested spatial separation between the two systems to prevent either from interfering with the effectiveness of the other. The nanogels would be injected into the tissue surrounding the resection wall and would spread, for their job is to strictly degrade the hyaluronic acid-dense extracellular matrix of remaining tumor tissue, increasing the likelihood of CAR T Cell success and penetration. The hydrogel would be inserted directly into the resection cavity and would not spread into the tissue, and thus would not interact with the hyaluronidase-containing nanogels. However, I also recommended that future studies explore other polymers to be used in creating hydrogels for glioblastoma treatment or engineering the HA-MA to be resistant to hyaluronidase.

Thank you for addressing my comments. Accepted.