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Combined Immunotherapy of Dual-Targeted CAR NK Cells and Modified Oncolytic Virus Against Glioblastoma

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Abstract. Glioblastoma (GBM) is a highly aggressive and treatment-resistant brain tumor with limited therapeutic options. This study explores a novel combined immunotherapy approach using dual-targeted CAR NK cells and a modified oncolytic virus (OV) to enhance anti-tumor efficacy. The CAR NK cells are engineered to target IL13Rα2 and CD19, while also incorporating IL6, IL21, and together with constitutively active STAT3 signalling to boost persistence and activity. The OV, derived from herpes simplex virus (HSV-1), is designed to express CD19 and the chemokine CCL5, facilitating NK cell recruitment and tumor targeting. Combined therapy in immunodeficient and immunocompetent mouse models shows significant tumor regression, prolonged survival, and increased immune cell infiltration compared to monotherapies. These results highlight the potential of this dual-mechanism strategy to overcome GBM's immunosuppressive microenvironment and heterogeneity. However, challenges such as off-target effects on healthy B cells and testicular tissue warrant further investigation. This study provides a promising foundation for advancing combined CAR NK and OV therapies against GBM.

Keywords: Glioblastoma, CAR NK, Combined therapies, Oncolytic virus

1. Introduction

Glioblastoma (GBM) is the most common primary malignant brain tumor of the central nervous system characterized by aggressive invasion, rapid growth and poor prognosis [1]. At the molecular level, GBM exhibits some predominant genetic alterations: amplification and/or high mutation of the epidermal growth factor receptor (EGFR), telomerase reverse transcriptase (TERT) promoter mutations, and unique chromosomal abnormalities like amplification, deletion, and aneuploidy [2]. The main current therapies for Glioblastoma Multiforme (GBM) include surgical resection, chemotherapy, molecularly targeted inhibitors, and immunotherapy [3]. Surgical resection often lacks in precision owing to brain shift [4], leading to local tumor recurrence in almost all patients [5]. Chemotherapy has limited efficacy because of the hematological toxicity and the restrictive

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properties of the blood-brain barrier [6]. Small- molecular inhibitors have emerged as an adjunctive therapeutic strategy for the treatment of glioblastoma, but the therapeutic benefit remains modest.

There are two emerging immunotherapies for GBM: Chimeric Antigen Receptor (CAR)-engineered cells and oncolytic viruses (OVs). The development of novel CAR has significantly transformed cancer treatment, particularly through CAR-T and NK cell therapies. Engineered lymphocytes provide unique approaches which they modify T cells or natural killer (NK) cells to express a CAR that can recognize specific antigen on GBM cells and initiate immune response against GBM tumor. However, CAR T cell therapy in GBM may face challenges including graft versus-host disease (GVHD) [7], indirect killing activity against tumor cells. In contrast, CAR NK cells decrease the ricks of GVHD largely compared with CAR T cells via specific inhibition of donor NK cells [8] and they are capable of eliminating tumor cell directly. OVs are a category of therapeutic viruses that have been genetically engineered to infect and selectively replicate within tumor cells, leading to selective pro-inflammatory viral oncolysis, converting the TME into a virus-inflamed, immune infiltrated state, allowing for accurate targeting against GBM cells, thereby achieving localized treatment [9]. Through the attenuation or removal of viral virulence factors, OVs are rendered incapable of replicating in healthy tissues.

However, both CAR NK cell therapy and OVs have their individual problems. NK cells showed restricted persistence [10], potential off-target cases and blocked tumor infiltration [11] against GBM cells. Delivery of OVs is an existing obstacle for this promising therapy. A major limitation of widely used intratumoral delivery is that it requires intricate neurosurgery and highly selective patient eligibility, making repeated administration very challenging [12]. In order to solve these drawbacks, we design a combined therapy of CAR NK and OVs, which prolongs persistence of NK cells and facilitate infiltration of NK cells via secretion of chemokine and specific antigen of CAR NK cells. Modified HSV-1 is delivered into NSG-IL15 mouse model by intranasal administration and injected HSV-1 is modified to secrete antigen CD19 and chemokine CCL5 in HSV-infected GBM 30 cells. NK cells are engineered to produce CAR1 and CAR2 which target IL13Rα2 and CD19 (Fig.1), respectively. Also, genes encode for IL6, IL21 and its transcription factor STAT3 are inserted into DNA sequence of CAR1 and CAR2, as IL21 plays an important role in maintain NK cells' activity [13]. Wang et al. has already demonstrated impact of CCL5 which it enhanced migration of NK cells towards GBM 30 tumor cites. Thus, the combined therapy would amplify therapeutic efficacy of CAR NK cells in terms of NK cells infiltration and duration of immune response.

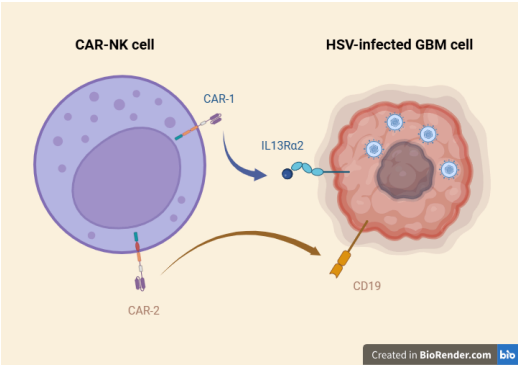

Figure 1. Molecular mechanism of proposed therapy

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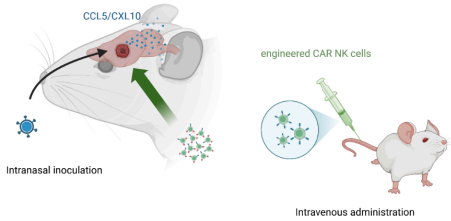

Figure 2. In vivo experiment schematic diagram

2. Method

2.1. Construct HSV-1

Firstly, a homologous recombinant vector containing CD19 and CCL5 expression cassettes will be constructed, and the recombinant vector and BAC-HSV-1 will be co-transformed into Escherichia coli. The CD19 and CCL5 genes will be inserted and the ICP34.5 and ICP6 genes will be deleted by the Red/ET recombinant system. After screening positive clones, the recombinant virus will be purified by Plaque Assay.

2.2. Flow Cytometry (FACS)

For the in vitro phase, the modified HSV-1 will be used to infect GBM30 cells. After infection, cells will be collected by FACS. The percentage of CD19-positive cells and the mean fluorescence intensity (MFI) will be quantitatively analysed via anti-CD19-APC antibody staining. For the in vivo phase, a single-cell suspension will be prepared from harvested tumor tissue, and the expression of CD19 in tumor cells will be analysed by FACS.

2.3. Enzyme-Linked Immunosorbent Assay (ELISA)

Cell supernatants from the in vitro infection will be collected synchronously, and the secretion dynamics of CCL5 will be detected using a CCL5 ELISA kit. For the in vivo phase, cerebrospinal fluid will be collected, and the concentration of CCL5 will be detected by ELISA.

2.4. In vivo animal model

Transgenic NSG-Tg(Hu-IL15) mice expressing human IL15 will be used as animal model. Engineered HSV-1 intranasal inoculation (Figure.2) will be performed on GBM30-transplanted NSG mice. Tumor tissue will be harvested for subsequent analysis. Control groups, including an uninfected group and an empty vector virus group, will be set for both the in vitro and in vivo experiments.

2.5. Real-time label-free cellular analysis

The xCELLigence real-time cell analysis instrument will be used for impedance experiments to determine tumor cell killing according to the manufacturer’s protocol. Briefly, tumor cells will be

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plated at 25,000 cells per well, cocultured with Ovs. Fresh media will be added to remove any residual viral particles. Then, CAR-NK cells will be added and cultured with them. The tumor cells index will be detected by the xCELLigence real-time cell analysis instrument to analyze the tumor-killing efficiency of CAR-NK cells.

2.6. Mice

The immunodeficient humanized mice NSG-hIL15 and NSG-SGM3 purchased from Jackson Laboratory will be used for the study. They have characteristics of less immune interference and being close to the clinical situation. The BLT model based on NSG-SGM3 will be constructed through the combined transplantation of human fatal liver, thymic tissue, and bone marrow stroma, which will be used to explore the enhancing effect of combination therapy on the endogenous immune system.Mice will be sacrificed when they become moribund with neurologic impairments or obvious weight loss (up to 20%).

2.7. Construction of GBM model

Tumor cells from human GBM-30 patients are genetically recombined by delivering the luciferase gene Luc2 via adenovirus to form GBM-30 cells expressing luciferase, which facilitates the measurement of tumor volume.An equal amount of luciferase-expressing GBM-30 will be injected intracranially into the mice NSG-hIL15 and the BLT model to construct GBM model.

2.8. In vivo imaging

A PBS solution containing D-luciferin will be delivered into the cranial cavity via intracranial injection. After 10 minutes, the IVIS Lumin III system will be used to capture the bioluminescent signal through the Living Image software. This technology will be used in conjunction with luciferase to measure tumor volume.

2.9. Aerosol atomization drug delivery

Venturi effect will be utilized to enable the drug be rapidly absorbed in the form of high-concentration small particles, effectively reducing irritation and significantly enhancing the absorption effect. This technology will be used in the OV drug delivery process for mice in order to increase the probability of OV successfully reaching the lesion.

2.10. Luminex

The liquid-phase chip flow fluorescence detection system provided by Luminex Corporation specifically binds fluorescently stained polystyrene microspheres to multiple target molecules to detect molecular-level indicators, featuring high efficiency with small sample volumes. It will be used to detect the levels of cytokines such as perforin, granzyme, TNF-α, TNF-β, INF-γ, IL-2, IL-15, IL-12, CCL3, CCL4, GM-CSF, and TGF-β.

2.11. Multicolor immunohistochemistry

Prepare pathological tissue section. Then use microwave and antigen retrieval solution with a pH of 6 - 9 to restore antigen activity. Incubate the primary antibodies with anti - CD56 antibody, anti -

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CD4 antibody, and anti - CD8 antibody. Then use HRP secondary antibody combined with TSA fluorescent dye for staining, and use microwave to remove non - covalent antibodies. After completing the labeling, use HALO software to analyze the infiltration degree of the above three types of cells.

3. Anticipated results

Table 1. Treatment of the experimental group

Mice model		Treatment			
		CD19	CCL5	IL13Rα2R	CD19R
NSG-hIL15-GBM model	Group A	-	-	-	-
	Group B	-	-	+	+
	Group C	+	+	-	-
	Group D	+	+	+	+
BLT-GBM model	Group E	-	-	-	-
	Group F	+	+	+	+

PCR and sequencing analyses are expected to demonstrate specific amplification bands consistent with the designed constructs, confirming the accurate insertion of the CD19 and CCL5 expression cassettes into the intended loci of the HSV-1 genome, alongside successful deletion of the ICP34.5 and ICP6 genes without nonspecific recombination in other genomic regions. Plaque assays will reveal that the recombinant virus forms morphologically uniform and well-defined plaques on Vero cells, indicating preserved infectivity and successful purification. For in vitro validation in GBM cell lines, flow cytometric analysis (FACS) is expected to detect a substantially increased proportion of CD19-positive cells with markedly elevated fluorescence intensity in the recombinant virus-infected group compared to the empty vector and uninfected controls, showing clear upward trends in both the percentage of positive cells and signal intensity over time. Concurrently, ELISA of cell culture supernatants is anticipated to demonstrate a significant rise in CCL5 secretion levels in the infected group, with concentrations escalating over time and exhibiting a dose-dependent enhancement, while controlling groups maintain minimal background levels. In mouse models, FACS analysis of tumor-derived single-cell suspensions is projected to reveal a distinct population of CD19-positive cells that co-express the tumor cell marker GFAP but lack the hematopoietic marker CD45, confirming successful viral infection and heterologous CD19 induction specifically in tumor cells. ELISA of cerebrospinal fluid samples should show a pronounced increase in CCL5 concentration in the treatment group, affirming virus-driven CCL5 secretion within the intracranial tumor environment, whereas plasma CCL5 levels remain comparable across all groups, indicating localized production and action of the chemokine.

To prepare CAR-NK cells harboring the IL21 circuit, CAR-NK cells will be engineered using constructs cloned into a second-generation self-inactivating lentiviral backbone (pHR). Based on the fluorescent protein marker added during lentiviral construction, when sorting transduction-positive cells using the FACS Aria Fusion, and obtain CAR-NK cells with high purity. In in vitro experiments, it is anticipated that when culture supernatants from CAR-NK cells engineered with an IL21 circuit are analyzed by ELISA, the concentration of IL21 will be 60% higher than that produced by CAR-NK cells lacking this circuit. Furthermore, in co-culture experiments with GBM30 cells, real-time monitoring of the cell index via the xCELLigence real-time cell analysis

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instrument is expected to show that the CAR-NK cells with the IL21 circuit will exhibit significantly enhanced cytotoxic activity and efficacy against GBM30 cells compared with control CAR-NK cells.

The relative tumor volume measured in Group A (Table 1) is expected to be the largest at the final stage. Minimal evidence of NK cell infiltration or elevated cytokine secretion will be observed, and almost all the mice died after 12 days. In Groups B and C, due to the intervention of CAR - NK and OV, tumor growth will be inhibited. The relative tumor volume will be smaller than that in Group A, and the survival time of the mice will be longer. NK cell infiltration will be limited in Group B and nearly absent in Group CThere will be insufficient NK cell infiltration in Group B and almost no NK cell infiltration in Group C. The cytokine levels in the two groups are expected to be significantly higher than those in Group A.

The combination therapy administered in Group D is expected to be achieve robust and durable anti-tumor, and sufficient to eliminate tumor cells in immunodeficient humanized mice. The NK cell infiltration and cytokine level will also be significantly increased, and the majority of mice are expected to survive. Overall, the efficacy of combination therapy on GBM - 30 is supposed to surpass that of monotherapies. For group E and F, the immune cell infiltration and cytokine levels in group F are expected to be significantly higher than those in group E, and the relative tumor volume of group F will be smaller than that of group E, meanwhile the survival rate of group F will be higher. It can be concluded that the combination therapy has a certain enhancing effect on the endogenous immune system to eliminate GBM - 30.

4. Discussion

GBM is known to be one of the most challenging tumor subtypes because of its complex intratumor heterogeneity. Consequently, major breakthrough of immunotherapy have highlighted CAR NK cells and OVs as two promising approaches against GBM. Here, we propose a combined therapy of CAR NK cells and Ovs. The engineered NK cells are designed to express dual CARs targeting IL13Rα2 and CD19, together with IL21 to promote persistence and long-term anti-tumor activity. In addition, CAR NK cells are further modified with IL6 and STAT3 signaling to upregulate expression of IL21. The OVs are engineered to express CD19 and CCL5 upon infection of GBM30 cells. thereby providing additional CAR targets and enhancing NK cells recruitment to tumor site [14].

Our experimental approach firstly is designed to test whether CD19 and CCL5 can be secreted by HSV-infected GBM30 cells. The anticipated results of second aim are expected to show that expression of CD19 and CCL5 largely increases in TME. Also, expected results indicate that expression pathway of IL21 is stimulated under intervention of IL6 and transcription factor STAT3. The combined therapy is expected to exert a profound effect on enhancing anti-tumor activity.

However, there are still several limitations that might occur if we want to further apply this combined therapy on clinical trials. First, healthy B lymphocytes and testicles may be attacked by engineered CAR NK cells, because target of CAR NK cells, CD19 and IL13Rα2 are physiologically expressed in healthy B cells and testis, respectively [15,16]. Especially for healthy testis cells, they are the only tissue that produce huge amount of IL13Rα2 among all human tissues, which incorrect recognition of engineered CAR NK cells leads to expression of IL21 and sustained killing activity of NK cells and inflammatory cytokine release. As for healthy B cells, one of the most significant markers, CD19 can be mistakenly targeted by CAR NK cells, and loss of B cell cause patients to a state of immunodeficient, which increases risks of other diseases. Second, the specific mechanism of how IL6 and STAT3 stimulate expression of IL21 is not fully discovered, scientists have already uncovered that expression of IL21 is induced by IL6 [17].

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In order to improve efficacy of combined therapy, preventing intrinsic antiviral immune response against HSV-1 is a future direction for salutary headway of curative effect of HSV-1. HSV-1 can easily trigger antiviral immune response and it would constrain efficacy of viral replication and propagation within GBM 30 tumors [18-20]. Recent studies have demonstrated that genetic modification of HSV-1 to express E-cadherin suppresses NK cell-mediated antiviral activity and enhances intratumorally viral dissemination. And they found that overexpression of E-cadherin not only reduced HSV-1 susceptibility to NK cell killing, but also enhanced its intratumorally spread [21]. Developing targeted strategies to inhibit antiviral immune response will be crucial in accomplishing sustainable antitumor activity of remaining NK cells and maximizing the therapeutic potential of CAR NK cells and OVs combination therapy.

5. Conclusion

To sum up, we propose a combined therapy of CAR NK cells and OVs against GBM 30 tumor which is designed to address fleeting antitumor activity of CAR NK cells and to promote migration of intrinsic NK cells towards tumor cite via secretion of chemokine CCL5 by HSV-1-infected GBM 30 cells. Further validation and refinement including feasibility of incorporating inhibitory mechanism to modulate NK cell responses and evaluation of the potential off-target susceptibility of B lymphocytes and healthy testicle cells to engineered CAR NK cells, will be requisite to maximize its clinical potential and ensure patient safety.

Author contributions

Xiaoheng Zhang, Shuhao Wu, Yueheng Qin contributed equally to this work and should be considered co-second authors.

References

[1] Ostrom, Quinn T et al. “CBTRUS Statistical Report: Primary Brain and Other Central Nervous System Tumors Diagnosed in the United States in 2013-2017.” Neuro-oncology vol. 22, 12 Suppl 2 (2020): iv1-iv96. doi: 10.1093/neuonc/noaa200

[2] Singh, Shilpi et al. “Glioblastoma at the crossroads: current understanding and future therapeutic horizons.” Signal transduction and targeted therapy vol. 10, 1 213. 9 Jul. 2025, doi: 10.1038/s41392-025-02299-4

[3] Wu, Wei et al. “Glioblastoma multiforme (GBM): An overview of current therapies and mechanisms of resistance.” Pharmacological research vol. 171 (2021): 105780. doi: 10.1016/j.phrs.2021.105780

[4] Oszvald, Agi et al. “Glioblastoma therapy in the elderly and the importance of the extent of resection regardless of age.” Journal of neurosurgery vol. 116, 2 (2012): 357-64. doi: 10.3171/2011.8.JNS102114

[5] Ringel, Florian et al. “Clinical benefit from resection of recurrent glioblastomas: results of a multicenter study including 503 patients with recurrent glioblastomas undergoing surgical resection.” Neuro-oncology vol. 18, 1 (2016): 96-104. doi: 10.1093/neuonc/nov145

[6] Sabahi, Mohammadmahdi et al. “Proposing a tandem AND-gate CAR T cell targeting glioblastoma multiforme.” Medical hypotheses vol. 137 (2020): 109559. doi: 10.1016/j.mehy.2020.109559

[7] Sabahi, Mohammadmahdi et al. “CAR-engineered NK cells versus CAR T cells in treatment of glioblastoma; strength and flaws.” Journal of neuro-oncology vol. 171, 3 (2025): 495-530. doi: 10.1007/s11060-024-04876-z

[8] Olson, Janelle A et al. “NK cells mediate reduction of GVHD by inhibiting activated, alloreactive T cells while retaining GVT effects.” Blood vol. 115, 21 (2010): 4293-301. doi: 10.1182/blood-2009-05-222190

[9] Muik, Alexander et al. “Re-engineering vesicular stomatitis virus to abrogate neurotoxicity, circumvent humoral immunity, and enhance oncolytic potency.” Cancer research vol. 74, 13 (2014): 3567-78. doi: 10.1158/0008-5472.CAN-13-3306

[10] Burga, Rachel A et al. “Improving efficacy of cancer immunotherapy by genetic modification of natural killer cells.” Cytotherapy vol. 18, 11 (2016): 1410-1421. doi: 10.1016/j.jcyt.2016.05.018

DOI: 10.54254/2753-8818/2025.AU27984

[11] Carlsten, Mattias, and Richard W Childs. “Genetic Manipulation of NK Cells for Cancer Immunotherapy: Techniques and Clinical Implications.” Frontiers in immunology vol. 6 266. 10 Jun. 2015, doi: 10.3389/fimmu.2015.00266
[12] Zeng, Jiayi et al. “Oncolytic Viro-Immunotherapy: An Emerging Option in the Treatment of Gliomas.” Frontiers in immunology vol. 12 721830. 5 Oct. 2021, doi: 10.3389/fimmu.2021.721830
[13] Shanley, Mayra et al. “Interleukin-21 engineering enhances NK cell activity against glioblastoma via CEBPD.” Cancer cell vol. 42, 8 (2024): 1450-1466.e11. doi: 10.1016/j.ccell.2024.07.007
[14] Wang, Jiao et al. “Multispecific targeting of glioblastoma with tumor microenvironment-responsive multifunctional engineered NK cells.” Proceedings of the National Academy of Sciences of the United States of America vol. 118, 45 (2021): e2107507118. doi: 10.1073/pnas.2107507118
[15] Masir, N et al. “Loss of CD19 expression in B-cell neoplasms.” Histopathology vol. 48, 3 (2006): 239-46. doi: 10.1111/j.1365-2559.2005.02317.x
[16] Yin, Yibo et al. “Checkpoint Blockade Reverses Anergy in IL-13Rα2 Humanized scFv-Based CAR T Cells to Treat Murine and Canine Gliomas.” Molecular therapy oncolytics vol. 11 20-38. 28 Aug. 2018, doi: 10.1016/j.omto.2018.08.002
[17] Nurieva, Roza et al. “Essential autocrine regulation by IL-21 in the generation of inflammatory T cells.” Nature vol. 448, 7152 (2007): 480-3. doi: 10.1038/nature05969
[18] Ikeda, K et al. “Oncolytic virus therapy of multiple tumors in the brain requires suppression of innate and elicited antiviral responses.” Nature medicine vol. 5, 8 (1999): 881-7. doi: 10.1038/11320
[19] Alvarez-Breckenridge, Christopher A et al. “NK cells impede glioblastoma virotherapy through NKp30 and NKp46 natural cytotoxicity receptors.” Nature medicine vol. 18, 12 (2012): 1827-34. doi: 10.1038/nm.3013
[20] Han, Jianfeng et al. “TGFβ Treatment Enhances Glioblastoma Virotherapy by Inhibiting the Innate Immune Response.” Cancer research vol. 75, 24 (2015): 5273-82. doi: 10.1158/0008-5472.CAN-15-0894
[21] Xu, Bo et al. “An oncolytic herpesvirus expressing E-cadherin improves survival in mouse models of glioblastoma.” Nature biotechnology, 10.1038/nbt.4302. 26 Nov. 2018, doi: 10.1038/nbt.4302