Prospects and applications of NK therapy in the treatment of gliomas (Review)

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Abstract. Brain tumours are in the spotlight of oncology research due to their intractability and resistance to conventional treatments. High-risk craniotomies must be performed on patients during tumour resection surgeries due to the specificity of the brain structure, and the complexity of the brain structure also leads to the fact that brain tumours usually cannot be removed completely. Besides, the inability of foreign small molecules to cross the blood-brain barrier has led to the inability of conventional drug therapy to reach the tumour location in the brain. Furthermore, the damage to healthy brain tissue caused by conventional radiotherapy cannot be ignored. Therefore, brain tumours represented by gliomas are in urgent need for a novel therapeutic approach. Glioma is the most common brain tumour, accounting for 81% of malignant tumours in the central nervous system, and is characterized by high morbidity, recurrence, mortality and low cure rate. In recent years, natural killer (NK) cell immunotherapy for gliomas has gradually emerged and numerous studies have shown surprising therapeutic effects. NK cells have been demonstrated to traverse the blood-brain barrier and numerous studies have confirmed their ability to kill glioma cells both *in vivo* and *in vitro*. This article begins by introducing conventional therapies for glioma, followed by an overview of the potential of NK cell-based immunotherapy in glioma treatment and the regulatory mechanisms of NK cells within the glioma immune microenvironment. It then summarizes preclinical studies on CAR-NK cells and clinical advancements in NK cell therapy for glioma. Finally, the paper discusses recent progress in immunotherapy for gliomas and explores novel therapeutic strategies combining NK cell immunotherapy with other treatment modalities.

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1. Introduction

In recent years, cerebral neoplasms have garnered heightened research interest in medical science, particularly from those specializing in oncological research, owing to cerebral neoplasms' recalcitrant nature and resistance to conventional treatment modalities. Such neoplasms manifest within the encephalic tissue and are categorized into primary cerebral tumours and secondary encephalic metastases, constituting 1.4% of the totality of malignant growths (1). Divergent from the typical symptomatology associated with oncological ailments, individuals afflicted with primary cerebral malignancies frequently exhibit an absence of symptoms until the malignancy presents with conspicuous clinical indications, attributable to the cerebral organ's intricate architecture. Treatment efficacy, as a result, is diminished when traditional oncological treatments are employed. Statistical analysis divulges that in the US alone, an excess of 15,000 mortalities annually are attributed to malignant primary cerebral neoplasms. Of note, the incidence rate of this serious condition increases drastically with advanced age, with the average quinquennial survival rate hovering at ~36%, bereft of viable curative strategies (2). Gliomas, constituting a formidable 81% of malignancies within the central nervous framework (3), predominantly originate from astrocytic, oligodendrocytic or ventricular meningeal progenitors, subsequently developed into astrocytic tumors, ependymomas or oligodendroglial neoplasms.

Gliomas are classified into quartets of escalating severity based on their anticipated survival outcomes: Grade I, II III and IV glioma. Grades I and II represent the less aggressive cohort, known as low-grade gliomas, whilst Grades III and IV epitomize the more highly fatal high-grade gliomas. Prognostically, low-grade gliomas confer a more auspicious outlook, with the average decennial survival rate standing at 47%. By contrast, the decennial survival likelihood for corticoblastic astrocytoma reaches a remarkable 96%, a stark juxtaposition to diffuse infiltrating glioma's more somber fate, which has a median survival duration of 5.6 years for World Health Organization Grade II astrocytomas. For patients with Grade III gliomas, the median overall continuance is reduced to approximately three years. Glioblastomas (GBM), predominant amongst Grade IV gliomas, are encumbered with a median persistence of a scant 4.8 months, and a quinary survival rate languishing at 7.2% (4).

This review explores the role of natural killer (NK) cells in the brain tumour microenvironment, discusses the advantages and prospects of NK cells compared to traditional methods of treatment and investigates NK cell treatment strategies for overcoming the blood-brain barrier (BBB) in the tumour microenvironment by combining treatment with other cell treatments, looking at the future directions in highly effective brain tumour therapies.

2. Common treatments for gliomas

Gliomas present significant clinical challenges due to their high rates of morbidity, recurrence and mortality. Current standard treatments include surgical resection, radiation therapy and chemotherapy, each with notable limitations. Surgical resection remains the cornerstone of glioma treatment. However, complete tumor removal is often unachievable due to the infiltrative growth pattern of gliomas, leading to high recurrence rates. Furthermore, craniotomy procedures bear a risk of causing permanent damage to surrounding healthy brain tissue, potentially resulting in significant functional impairments (5).

Radiation therapy delivers high-dose localized irradiation using advanced modalities such as γ-knife, X-knife and proton knife systems. Despite these technological advances, treatment efficacy remains limited. Minniti *et al* (6) demonstrated that GBM (accounting for 50% of glioma cases) typically recur at or near the primary site following radiotherapy. While medulloblastomas show notable radiosensitivity, other glioma subtypes respond poorly to radiation treatment. The combination of temozolomide with radiotherapy has emerged as an improved therapeutic approach. Compared to radiotherapy alone, this regimen demonstrates superior outcomes, increasing 2-year survival rates from 10.9 to 27.2% and 5-year survival rates from 1.9 to 9.8% (2). However, this benefit comes with significant toxicity, including grade 3-4 hematological adverse events in 7% of patients (7). Additionally, whole-brain irradiation carries the risk of radiation necrosis, which can profoundly impact neurological function.

Chemotherapeutic approaches have demonstrated even greater limitations in glioma treatment compared to radiotherapy, primarily due to the restrictive properties of the BBB. The BBB consists of endothelial cells in the capillary wall, astrocyte ends encasing the capillaries and pericytes embedded in the basement membrane of the capillaries. The tight junction of endothelial cells restricts the entry of most hydrophilic small molecules into the brain region. Besides, there are a series of ATP-dependent efflux transporter proteins on the luminal side of the BBB and the polarised expression of efflux transporter proteins significantly blocks the delivery of numerous therapeutic drugs, such as the typical BBB efflux transporter proteins, breast cancer resistant protein and P-glycoprotein (8,9). Therefore, only a small number of drugs are approved for the treatment of gliomas, and limited clinical data are available (10). While the BBB serves the crucial physiological function of protecting the central nervous system (CNS) from neurotoxic substances, this protective mechanism simultaneously prevents most conventional chemotherapeutic drugs from reaching therapeutic concentrations in brain tumors (11). This fundamental limitation has created an urgent need for alternative treatment strategies. Recent advances in cancer immunotherapy have shown promise for both hematological and solid malignancies (12,13). After surgical removal of the primary tumour lesion, cytotoxic T-lymphocytes (CTL) or NK cells may be infused in the local area. In addition, it has been shown that NK cells may be attracted to the tumour site (14).

3. A cell therapy based on an immunotherapy strategy

NK cells are the main innate lymphocyte subpopulation that eliminates tumours, not only recognising and killing tumour cells that escape the T-cell response, but also promoting the anti-tumour response of other immune cells, such as T cells (15). NK cell-based cancer therapies have been rapidly evolving in recent years and numerous studies have demonstrated that NK cells have an important place in the treatment of a wide range of cancers, which is now a major area of innovation in immunotherapy (16-18).

In 2021, Shaim *et al* (19) assessed the abundance by analysing excised glioma specimens from 21 patients with primary GBM and 2 patients with low-grade gliomas and found that the number of NK cells entering the microenvironment of GBM was much greater in high-grade gliomas. By analysing >1,746 NK cell samples from tumour patients and >530 NK cell samples from peripheral blood mononuclear cells of healthy donors, it was found that unedited NK cells from healthy individuals were able to exert toxicity on patient-derived GBM stem-like cells (GSCs) without affecting normal brain cells (astrocytes). NK cells were first demonstrated to be able to target and kill GSCs (GBM) *in vitro*. In addition to this, in the treatment of gliomas in the brain, the BBB is another major limitation to treatment, preventing the systemic delivery of most targeted drugs to the brain tumour site, which makes the treatment of brain tumours difficult. By contrast, NK cells can cross the BBB and migrate into brain tumour tissue (1). This renders NK cell-based brain tumour therapies to be of great potential and value.

For the past few years, NK cells have been thought to play an immunosurveillance role in the brain and exert toxic effects on abnormal cells (20,21). First, activated T cells enter the CNS via lymphatic drainage through the cerebellar vessels, choroid plexus epithelium and peripheral regions of the barrier, and

subsequently, antigen and antigen-presenting cells in the brain interact with activated CD4+ T cells traversing the barrier, and this interaction generates an inflammatory response that permits immune cells to enter the CNS parenchyma. NK cells, represented by the immature CD16-CD56bright subtype, are able to cross the BBB and choroid plexus into the CNS and settle in substantia parenchyma induced by the chemokines C-X3-C motif chemokine ligand CX3CL1, C-C motif chemokine ligand (CCL)2 and C-X-C motif chemokine ligand (CXCL)10 (22).

4. The potential of NK cells in glioma therapy

The ability of NK cells to cross the BBB to exert toxic effects has brought them much attention in the treatment of gliomas. GBM, a representative of highly malignant primary brain cancers, has a median survival time of 14 months, a low overall survival (OS) rate, limited therapeutic options and is highly susceptible to recurrence after treatment (23). One of the reasons why GBM is difficult to treat is due to its unique tumour microenvironment. In the microenvironment of GBM, glioma cells upregulate the secretion of immunosuppressive factors like programmed cell death 1 ligand and indoleamine 2,3-dioxygenase (IDO), which limit the expression of antigens (24,25). In addition, gliomas promote the secretion of interleukin 10 (IL-10) and transforming growth factor β (TGF-β) by glioma-associated macrophages, which reduces the activity of immune cells (26,27). More often, In the microenvironment, gliomas can mediate immunosuppressive effects by eliminating cytotoxic T lymphocytes around the tumour via regulatory T (Treg) cells (28,29).

Current approaches used for the treatment of GBM include virotherapy, which uses released lysogenic viruses to destroy glioma cells (30), and dendritic cell (DC) vaccine therapy, which kills glioma cells by exploiting the efficacy of DC antigen presentation and the ability to induce activation of CTLs (31). Blocking immune checkpoints, i.e., preventing glioma cells from evading immune surveillance by inhibiting programmed cell death 1 (PD-1) (32,33), cytotoxic T-lymphocyte antigen 4 (34,35) and IDO (36,37). Furthermore, one immunotherapy strategy involves activating type 1 T helper (Th1) cells via cytokines, thereby enhancing CTL-dependent antitumor immunity. In addition, NK cells can be activated by IFN-α and IFN-β to eliminate tumour cells via antibody-dependent cytotoxicity (ADCC) (38-40). With chimeric antigen receptor (CAR)-T therapy, IL-13Rα2, epidermal growth factor receptor vIII and CD70 and other related antigens on gliomas can be recognised by CTLs, and the recognition of gliomas by CTLs can be enhanced by using these antigens as targets for transgenic CAR-T cells (41-48). In addition, MMP-9 and TGF-β1 secreted by tumour-associated macrophages activated with colony-stimulating factor (CSF)-1 and CCL2 promote glioma cell invasion, so the spreading of glioma cells can also be slowed down by inhibiting the secretion of MMP-9 and TGF-β1 by tumour-associated macrophages (49-51). However, despite the advances in efficacy of these strategies compared to traditional surgical radiotherapy, the treatment of GBM still has its own limitations and drawbacks. That is why a portion of researchers have focused their attention on novel NK cell therapies and have made surprising discoveries.

Previously, tumour-killing tests in immunodeficient mice suggested NK cells may help destroy human tumour cells *in vivo* (52). Castriconi *et al* (53) isolated tumor cells from surgical specimens obtained from nine glioblastoma (GBM) patients exhibiting classic clinical and radiographic features. Following *in vitro* expansion, these GBM cells were characterized for neural stem cell marker expression, differentiation potential and tumorigenic capacity in immunodeficient mice. Notably, their study also revealed the susceptibility of these stem-like GBM cells to lysis by both resting and lymphokine-activated allogeneic and autologous NK cells (53). In addition, the types of receptor and ligand interactions recognised by NK-associated cancer cells were measured, as well as the amount of. All GBM analysed in this study showed susceptibility to NK-mediated cytotoxicity, demonstrating that recipient mice can be cured by intra-tumoural injection of IL-21 inducing a cytotoxic response involving NK cells in a mouse model of GBM (54), and discovered a new strategy that can be used to eliminate residual cancer cells by activating NK cells. Although NK cells were not detected in GBM tumour homogenates from primary tumours, the ability of NK cells to reach tumours located in the CNS can be judged by the overshoot of activated NK cells in NOD/SCID mice carrying human GBM. Since GBM cells do not have human leukocyte antigen class I molecules, NK cells do not show activated receptor downregulation and dysfunction as they do in other tumours (55,56). In addition, lymphokine-activated NK cells are able to achieve the highest goal of conventional therapies, i.e., killing cancer cells with stem cell-like properties (57,58). Avril *et al* (59) similarly found that GSCs can be killed by lectin-activated NK cells. More recently, cancer stem-like cells (CSC) have been found to be present in GBM specimens, and it is hypothesised that CSC are responsible for this recurrence (60-62). GSCs are self-expanding *in vitro* and can differentiate into CNS cells. The study by Avril *et al* (59) confirmed the sensitivity of GSCs to antibody-mediated cytotoxicity as well as to cytotoxicity exerted by IL-2-activated NK cells and tumour-specific T cells through experiments using the therapeutic antibody cetuximab. More importantly, GSCs were more sensitive to NK- and T-cell-mediated lysis relative to GBM cells cultured with the corresponding serum obtained from the same initial tumour specimen. In addition, for the first time, researchers demonstrated that GSCs are sensitive to ADCC mediated by NK cells that use the anti-EGFR antibody cetuximab. These results demonstrate the sensitivity of GSCs to NK cytotoxicity and show the great potential of NK cells in the treatment of gliomas.

In addition, Poli *et al* (63) identified a method of combining the antibody mAb9.2.27 with overt NK cells to reduce GBM cell proliferation and improve cell survival. Previously, studies have shown that increased levels of neuron-glial antigen 2/chondroitin sulfate proteoglycan 4 (NG2/CSPG4) proteoglycans on GBM cells and angiogenic material are associated with more severe tumour expansion (64,65). A strategy targeting NG2/CSPG4 in combination with mAb9.2.27 and NK cells was found, using cytokines released by NK cells to reverse the anti-inflammatory axis and, in combination with mAb9.2.27, to eliminate tumours in a GBM animal model. Expression of major histocompatibility complex (MHC) class II molecules and ED1 (CD68) was upregulated in microglia from animals

co-treated with NK + mAb9.2.27 compared to controls, enabling them to present GBM antigens (66). NK + mAb9.2.27 combination therapy effectively inhibited tumour growth and was associated with apoptosis and prolonged survival compared to controls. In addition, combination therapy reduced ED2 scavenger receptor-positive microglia, suggesting that a decrease in perivascular microglia may promote the development of GBM (67). NK cell-mediated mAb9.2.27 immunotherapy significantly reduced the proportion of CCR2-expressing macrophages, a pro-invasive subpopulation known to drive glioblastoma (GBM) progression. (68). Furthermore, *in vitro* tests demonstrated that mAb9.2.27 effectively reduced the tumour-promoting effects of tumour-associated macrophages (TAM) and microglia. To summarise, these results suggest that NK + mAb9.2.27 treatment may be a viable therapeutic strategy for the treatment of NG2/CSPG4-expressing GBMs using NG2/CSPG4 as a therapeutic target.

5. Mechanisms of NK cytotoxicity regulation in the glioma microenvironment

Despite the efficacy of NK cell-mediated cytotoxicity for glioma treatment, glioma cells have acquired their own unique immune microenvironment over a long period of evolution. The antitumor cytotoxicity of NK cells is governed by a dynamic equilibrium between activating and inhibitory signals. Activating receptors facilitate tumor cell recognition through antigen detection, triggering cytotoxic responses, while inhibitory receptors maintain immune tolerance by suppressing NK cell activity (69). In the tumour microenvironment, gliomas inhibit NK cell activity by inducing upregulation of inhibitory receptors on NK cells and downregulation of activating receptors, as well as by releasing tumour-associated factors. Activating receptors typically bind to linker proteins containing immunoreceptor tyrosine-activating motifs, such as FcεRIγ, CD3ζ and DAP12, in mutant cells. Inhibitory receptors recognise inhibitory motifs (ITIMs) with immunoreceptor tyrosines on normal cells (70). ITIM is a conserved amino acid sequence located in the cytoplasmic domains of certain inhibitory receptors in immune cells. It transduces inhibitory signals to suppress immune activation, such as blocking B cell, T cell and NK cell responses. Binding of the activating receptor leads to a phosphorylation response in NK cells and the inhibitory receptor leads to a dephosphorylation response in NK cells. At the molecular level, these counteracting receptor signals are integrated through phosphorylation-dephosphorylation dynamics, which bidirectionally regulate NK cell cytotoxic function by modulating its activation-inhibition balance. The main receptors that activate NK cells include natural cytotoxicity receptor (NCR), CD16 and natural killer group 2D (NKG2D). The NCR family includes three members of type I membrane-penetrating receptors known as NKp46, NKp44 and NKp30, which are encoded by the respective NCR genes, and play important roles in the activation of NK cells (71). CD16, encoded by the Fc gamma receptor IIIa gene, is a receptor for the Fc segment of immunoglobulin G (IgG), which assists NK cells in recognising IgG-bound cells, thus exerting ADCC (72). NKG2D, encoded by the killer cell lectin-like receptor K1 gene, is required to bind to the junction protein DNAX-activating protein of 10 kDa on the surface of NK cells to stabilise the receptor complex, and its ligand is expressed on a variety of tumour cells (73). When NK cells are stimulated and activated by the corresponding ligands of NKG2D, ADCC, degranulation and cytokine production of NK cells are promoted (74).

The main inhibitory receptors include PD-1, T-cell Ig and mucin-domain containing-3 (TIM3), ITIM structural domain protein (TIGIT) and CD96. PD-1 is considered to be a marker of T-cell depletion, and it can also be expressed in NK cells (75). Hepatocellular carcinoma cells can induce high expression of PD-1 in tumour-infiltrating NK cells by secreting the exosome circular RNA ubiquitin-like with PHD and ring finger domains 1, thereby suppressing their anti-tumour immune function (76). TIM3 is also a marker of NK cell depletion. Compared to TIM3-PD-1-NK cells, TIM3+PD-1+ NK cells within a variety of tumours are defective in their ability to secrete IFN-γ and granzyme B and have reduced cytotoxicity (77). TIGIT is a typical inhibitory receptor and T-cell Igs are as well (78), with a ligand of CD155, which has been found to be highly expressed in a variety of tumours. CD155 is expressed at low levels in normal tissues but is highly expressed in numerous tumour cells, as well as primary tumour tissues. It is associated with tumour cell proliferation and migration (Fig. 1). In addition, CD155 expression is upregulated in tumour-associated antigen-presenting cells, which also regulate the immune response of NK cells (79).

In addition to the regulation of receptor signalling, the regulatory cytokines IL-2, IL-15 and type I IFN activate NK cells by enhancing signalling downstream of the activated receptor, while IL-21 stimulates NK cell differentiation and IFNγ secretion. By contrast, IL-10 and TGF-β exert inhibitory effects on NK cells (80,81). In addition, chemokines, including CXCR3, CXCR4, CCR2-CCR5 and CX3CR1, induce NK cells to be recruited into tissues (82). Cytokines and growth factors such as IFNγ, TNF, granulocyte-macrophage-CSF and CCL5, can enhance cytotoxic effects, and they can also coordinate anti-tumour immune responses by recruiting immune cells, such as DCs, to achieve a joint anti-tumour effect of innate and adaptive immune responses (83).

In the glioma microenvironment, TAM in microglia or circulating monocytes gained an anti-inflammatory and pro-tumour M2-like phenotype under the influence of GBM-secreted factors. These TAMs secrete immunosuppressive cytokines such as TGF-β and IL-10, which inhibit effector immune cells and recruit regulatory T cells (Tregs) (84,85). Furthermore, widespread PDL1 expression in GBM further dampens antitumor immunity (86,87). The extracellular nucleosidase CD39 acts on Treg and TAM cells and CD73 on glioma cells, and adenosine produced by extracellular ATP produces a wide range of immunosuppressive effects, such as via the A2A adenosine receptor, which inhibits NK cells. Cholesterol is converted to 25-hydroxycholesterol with the help of cholesterol 25-hydroxylase and inhibits NK-cell metabolism through inhibition of critical transcription factor sterol regulatory element binding protein (88). In addition, oxygen and nutrient deprivation reduces NK cell activity, and prostaglandin E2 restricts DC recruitment by NK cells, thereby inhibiting the joint DC-NK cell antitumour effect, whereas lactic acid impedes NK cell activity by decreasing perforin and granzyme B expression (89,90). In IDH-mutant gliomas,

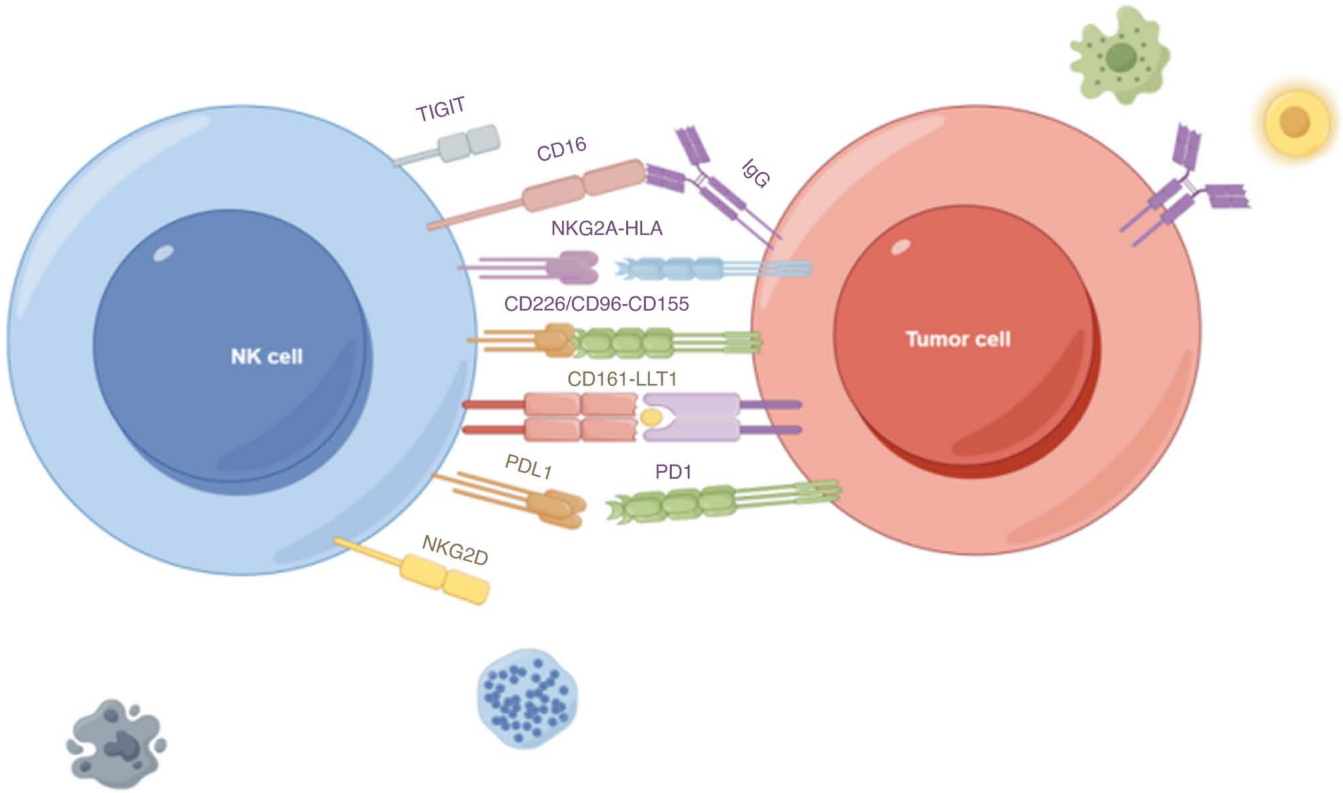

Figure 1. Receptor interactions between NK cells and tumour cells. Natural cytotoxic receptors on NK cells, such as NCR, CD16 and NKG2D, stimulate NK activation by binding to the corresponding ligands on tumours. CD16, encoded by the FCGR3A gene, interacts with IgG to help NK cells recognise IgG-bound cells, thus exerting antibody-dependent cell-mediated cytotoxicity. NKG2D, encoded by the KLRK1 gene, binds to the NK cell surface connexin DAP10 to stabilise the receptor complex. The suppressive receptors NKG2A, PDL1 and CD155 bind to the corresponding ligands to initiate NK cell inhibition. TIGIT, T-cell immunoreceptor with Ig and ITIM domains; CD16, cluster of differentiation 16; NKG2A-HLA, natural killer group 2A-human leukocyte antigen; CD226/CD96-CD155, cluster of differentiation 226/cluster of differentiation 96-cluster of differentiation 155; NKG2D, natural killer group 2D; CD161-LLT1, cluster of differentiation 161-lectin-like transcript 1; PDL1, programmed death ligand 1; PD1, programmed cell death protein 1; IgG, immunoglobulin G; NK, natural killer.

R-enantiomer of 2-hydroxyglutarate-mediated epigenetic reprogramming suppresses CXCL9 and CXCL10 expression, impairing the chemotaxis of tumor-infiltrating lymphocytes, including NK cells (91,92).

6. CAR-NK in tumour therapy

In order to increase the ability of NK cells to kill tumours without being inhibited by the tumour microenvironment, a new type of NK cell, CAR-NK, was created. CAR-NK is a genetically engineered NK cell that incorporates a chimeric antibody, CAR, which recognises tumour cells and at the same time activates the NK cell to kill the tumour cell. The CAR includes extracellular recognition domains used to recognise tumour-specific antigens, as well as a transmembrane structural domain and an intracellular signalling structural domain, enabling NK cells to precisely and efficiently kill tumour cells *in vivo*. The CAR approach uses NK cells derived from patients with transduction of the CAR gene to generate CAR, and after expanding CAR-NK cells *in vitro*, these cells are injected back into the tumour patient. So far, CAR-NK has shown good efficacy in the treatment of various tumours.

In 2018, Tang *et al* (93) found that CD33-CAR-NK-92 cells showed stronger cytotoxicity than CAR-T cells against CD33-positive leukaemia cells. On the other hand, CD33-CAR-NK cells are more effective than NK-92 cells in treating HL-60 and are safe in patients with relapsed and refractory acute myeloid leukemia (93). CD19-CAR-NK-92 cells significantly suppressed tumor cell proliferation in a B-cell lymphoma (BCL) model using NOD-SCID IL-2Rγ-deficient mice, demonstrating the feasibility of generating CAR-engineered NK-92 cells with potent tumor-targeting capabilities (94). Furthermore, CD4-CAR-NK cells are also selectively toxic to a wide range of CD4+ human T-cell leukaemia and lymphoma cells (95).

Of note, with the rapid progress of research in non-solid tumours, CAR-NK has also shown great potential in solid tumours. Liu *et al* (96) showed that EGFR-specific CAR-NK-92 cells and NK cells *in vitro* inoculated with mammary carcinoma cell lines MDA-MB-231 and MDA-MB-468 showed strong cytotoxicity, with epidermal growth factor receptor-specific CAR-NK-92 having better therapeutic efficacy compared to parental NK cells. Furthermore, EGFR-CAR-NK-92 cells or oncolytic herpes simplex virus 1 were effective in stopping breast tumour progression (96). Furthermore, Ng *et al* (97) found that mesothelin-positive ovarian cancer cells could be eliminated by mesothelin-specific CAR-NK cells, whereas they did not respond to mesothelin-negative cells. Notably, redirected NK cells were highly potent against ovarian cancer cells in either subcutaneous or peritoneal tumour models,

demonstrating that in addition to folate receptor α, mesothelin may be a viable target for targeted therapy in ovarian cancer. In the same year, this was also confirmed in a study by Ueda *et al* (97,98).

In a preclinical study, CAR-KHYG-1 NK cells were used to target c-Met, folate receptor α and AXL receptors, all of which are highly expressed in GBM cells. It has been shown that anti-EGFR/EGFR vIII and anti-human EGFR2 (HER2) CAR-NK cells are highly efficient against GBM and are effective in controlling tumor growth in animal models (1). In 2023, by experimenting with diffuse intrinsic pontine glioma (DIPG) cells and primitive pontine neural progenitor cells derived from five patients, Zuo *et al* (99) conducted the first comprehensive evaluation of GD2-CAR NK-92 cells for the treatment of DIPG, and the anti-tumour capacity and safety of this therapeutic strategy were confirmed. The results indicated that this novel treatment had significant anti-tumor effects and showed no significant side effects. Furthermore, tumor killing tests showed that GD2-CAR NK-92 cells were effective in killing high GD2-expressing DIPG cells with less activity against low GD2-expressing DIPG cells. In animal experiments, GD2-CAR NK-92 cells successfully inhibited tumour growth in GD2-overexpressing DIPG xenograft mice, which survived longer compared to controls. Nevertheless, the efficacy of this strategy in low GD2-overexpressing DIPG tumours was not significant. In another report, ErbB2/HER2-specific NK cells were targeted against GBM. Following intratumoral administration of $2x10^6$ ErbB2-specific NK-92/5.28 cells administered weekly for 11 consecutive weeks, significant suppression of tumor growth was observed (100). Of note, while CAR-NK therapy has shown promising results in preclinical studies, there are currently no large-scale clinical trials reporting its effectiveness in treating brain tumors.

7. Clinical advances in NK cell therapy for gliomas

Clinical trials investigating NK cell therapy for gliomas remain limited, with scarce reported outcomes. As early as 2004, the clinical trial 'Autologous NK Cell Therapy for Recurrent Malignant Gliomas in Humans' demonstrated that the administration of autologous NK cells to patients with recurrent malignant gliomas has proven to be safe and partially effective: By intravenous administration of autologous NK cells and low-dose IFN-β, 3/9 patients had a partial response and 2/9 patients had a mild response to treatment. No serious neurotoxicity was observed in any patient (101).

In 2020, a Phase I clinical trial (NCT02271711) on 'Expanded NK Cell Infusion in Treating Younger Patients With Recurrent/Refractory Brain Tumors' (NCT02271711) was completed at the M.D. Anderson Cancer Center. Patients in the trial received intravenous injections of autologous expanded NK cells over 3 min once a week for several weeks. In the absence of disease progression or unacceptable toxicity, treatment was repeated every 4 weeks for up to 3 courses. Patients were followed up within 30 days after completion of the study treatment. The results showed no dose-limiting toxicity after 112 intracerebroventricular infusions of NK cells; 8 patients progressed and 1 stabilized (102).

In the same year, a Phase II trial, the 'STIR Trial: Haploidentical Transplant and Donor NK cells for Solid Tumors' was completed. Results showed that patients tolerated NK infusion well and had no cytokine release syndrome (CRS). The median follow-up was 1.3 years, with 1- and 2-year OS of 64 and 40%, respectively, for the entire cohort. The disease control rate at 6 months was 72% (103).

In 2021, a phase I/IIa clinical trial on 'Autologous Adoptive Immune-cell Therapy Elicited a Durable Response With Enhanced Immune Reaction Signatures in Patients with Recurrent GBM' was completed (KCTOO03815). Autologous permissive immune cell therapy elicited durable responses and enhanced immune response characteristics in patients with recurrent GBM and no serious adverse events were observed in the trial. The median OS was 22.5 months and median progression-free survival was 10 months. A total of five patients survived >2 years and demonstrated durable responses with enhanced transcriptomic profiles of immune responses and no clinical decline (104).

In 2023, to assess the safety and feasibility of cerebrospinal fluid infusion of activated NK cells in recurrent gliomas to patients with drug-resistant brain tumours, a clinical phase I trial was performed with a mean follow-up of 8.08 months for recurrent brain tumours, GBM among them (105). Patients underwent several injections of activated NK cells. The first dose was administered two weeks after surgery and the other injections were frozen cells activated after thawing. Depending on the size of the tumor, $2x10^6$ to $100x10^6$ NK cells were injection straight into the tumour site. NK cells were embedded after surgical removal of the tumour or direct injection of NK cells was performed via vein/subcutaneous injection. At two weeks following the completion of chemotherapy for patients with inoperable diffuse tumours, $49.3\text{-}60x10^6$ NK cells were injected into the cerebrospinal fluid. Locally administered NK cells for malignant brain tumours were ultimately shown to be safe and feasible, with tolerance rising with increasing dose.

A phase II clinical trial of 'Injection of Active Allogeneic NK Cells in Patients With Gliomas' at the Royan Institute in Tehran, Iran is currently recruiting (NCT06687681). The research team has conducted a Phase I clinical trial in glioma patients, the results of which have not been disclosed. Additionally, a Phase I clinical study on 'Engineered NK Cells Containing Deleted TGF-βR2 and NR3C1 for the Treatment of Recurrent GBM' (NCT04991870) is currently recruiting at the M.D. Anderson Cancer Center (Respondent Party), Texas, US (Table I).

8. Anti-tumor therapy with combination of multiple immunotherapies

In addition to directly modifying NK cells through genetic engineering, there is another approach to attenuate the inhibitory effects of the glioma microenvironment on NK cells through the adjuvant effects of NKT cells or drugs. NKT cells have been shown to exert anti-tumor effects *in vivo*. Yamada *et al* (106) found that invariant NKT cells differentiated from induced pluripotent stem cells (iPSC) were able to effectively activate autologous NK cells and, when activated by ligand-pulsed DCs, were able to produce large amounts of IFN-γ, which resulted in more effective elimination of tumors. Furthermore, in 2024, Peng *et al* (107) invented a glioma immunotherapy that enhances the tumor-killing

Table I. Clinical trials of NK cell therapy for glioma.

NCTID	Trial description	Year	Phase	Therapeutic approach	Outcome	Notes	(Refs.)
-	Autologous NK cell therapy for recurrent malignant gliomas in humans	2004	Phase I	Intravenous infusion of autologous NK cells + low-dose interferon β	Partial response in 3/9 patients; mild response in 2/9 patients; no serious neurotoxicity observed	-	(100)
NCT02271711	Expanded NK cell infusion in treating younger patients with recurrent/refractory brain tumors	2020	Phase I	Intravenous injection of expanded autologous NK cells, once weekly for several weeks	8 patients progressed; 1 patient stabilized; no dose-limiting toxicity	-	(101)
-	STIR Trial: Haploidentical transplant and donor NK cells for solid tumors	2020	Phase II	Infusion of haploidentical donor NK cells	Median follow-up: 1.3 years; 1-year OS: 64%; 2-year OS: 40%; 6-month disease control rate: 72%	No cytokine release syndrome; well-tolerated by patients	(102)
KCT0003815	Autologous adoptive immune-cell therapy elicited a durable response with enhanced immune reaction signatures in patients with recurrent GBM	2021	Phase I/IIa	Autologous permissive immune cell therapy	Median OS: 22.5 months; median progression-free survival: 10 months; 5 patients survived >2 years	No serious adverse events; enhanced immune response signatures observed	(103)
-	Intra-lesion injection of activated NK cells in recurrent malignant brain tumors	2023	Phase I	Local administration of activated NK cells, some via cerebrospinal fluid	Safe and feasible; tolerance increased with higher doses	-	(104)
NCT06687681	Injection of active allogeneic NK cells in patients with gliomas	Ongoing	Phase II	Infusion of active allogeneic NK cells	-	Recruiting	-
NCT04991870	Engineered NK cells containing deleted TGF-βR2 and NR3C1 for the treatment of recurrent GBM	Ongoing	Phase I	Engineered NK cells with deleted TGF-βR2 and NR3C1	-	Recruiting	-
NCT01588769	A phase I study to investigate the tolerability and efficacy of ALECSAT administered to GBM multiforme patients (ALECSAT-GBM)	Completed	Phase I	ALECSAT therapy	-	Results not disclosed	-
NCT051080	NK cell therapy for recurrent GBM multiforme patients	Unknown	Phase I	NK cell therapy	-	-	-
NCT04254419	Intra-tumoral injection of NK cells in high-grade gliomas (NK HGG)	Not yet started	Phase I	Intra-tumoral injection of NK cells	-	-	-

Table I. Continued.

NCTID	Trial description	Year	Phase	Therapeutic approach	Outcome	Notes	(Refs.)
NCT01525459	Gene expression, immunological status and metabolome in glioma patients	Ongoing	Observational	Analysis of gene expression, immunological status and metabolome in glioma patients	-	-	-
NCT04489420	NK cell (CYNK-001) IV infusion or IT administration in adults with recurrent GBM	Terminated	Phase I	Intravenous or IT administration of CYNK-001 NK cells	-	-	-

NK, natural killer; OS, overall survival; GBM, glioblastoma; IT, intrathecal; NR3C1, nuclear receptor subfamily 3 group C member 1.

activity of T cells and NK cells. The study reports a nanocomposite consisting of a phospho-dendrimer macromolecule (AK128) combined with an antibody to PD-1 (aPD1). Since α4 and β1 integrins highly expressed on M1-type macrophage membranes (M1m) can bind to vascular endothelial adhesion molecule-1 on the surface of endothelial cells, the nanocomplexes could effectively pass the BBB after being encapsulated by M1m. Furthermore, the nanocomplexes modified by M1m had a lower monocyte clearance and longer blood circulation time. The results showed that after successfully crossing the BBB, AK128 in the complex promotes rapid proliferation and activation of NK cells, whereas the aPD-1 effectively reduced the inhibitory impact of the glioma microenvironment on both NK cells and T cells (107).

9. Advantages and prospects of NK cell therapy in glioma treatment

CAR-T as a cell therapy has been shown to play a role in alleviating hematologic tumors and clinical studies are currently underway for gliomas (47,108). However, this treatment has a non-negligible drawback: The heterogeneity of natural immune host defenses. Since T cells mainly stimulate cytokines, including IL-1/2/6/8/10/15 and TNF-α, they are prone to neurotoxicity and CRS even if the raw material is derived from themselves (109). Therefore, a homologous and safe therapeutic modality, such as NK cell therapy, is urgently needed.

NK cells are not prone to eliciting an overactive autoimmune response due to a lack of MHC. The feasibility and safety of autologous NK cells for over-the-counter cell therapy of recurrent gliomas in humans was also confirmed as early as 2004 (101). In addition, the ability of NK cells to cross the BBB gives them a unique advantage in the treatment of gliomas. However, NK cells are dependent on external ILs for proliferation and activation, and they are unable to produce IL-2 on their own as T cells do, making sustained delivery of IL to brain tumor sites challenging in clinical therapy. This makes it difficult for NK cells to have durability in the fight against gliomas and makes them more prone to depletion. Therefore, genetic engineering is needed to solve this problem.

Despite the paucity of research data on CAR-NK for glioma treatment, the advantages of CAR-NK make it highly promising for the treatment of glioma. First, modification of NK cells through gene editing techniques such as CRISPR/Cas9 or through the introduction of specific receptors (e.g. CARs) can improve their recognition and cytotoxicity against specific tumour markers, allowing NK cells to rapidly recognise antigens on tumour cells, maintain a higher proliferative capacity and *in vivo* persistence. It can also recognise and kill tumour cells through natural receptors for NK cells that are independent of CAR engineering, and are less likely to escape disease by downregulating CAR antigens. It enables improved tumour infiltration and is able to overcome the drug-resistant tumour microenvironment (110). In 2024, Shanley *et al* (111) increased the killing effect and persistence of NK cells against gliomas by engineering NK cells to express IL-21 via overexpression of CCAAT/enhancer-binding proteins and CCAAT/enhancer-binding protein delta in particular. The results showed that IL-21 NK cells promoted the expression of Ki67, CD25, perforin, TNF-α and granzyme B, achieved

sufficient tumor infiltration and were able to effectively alleviate or eliminate gliomas *in vivo*, as well as maintained a potent killing ability after multiple consecutive attacks on gliomas. In addition, the engineered NK cells enhanced metabolic adaptation by generating ATP through the oxidative phosphorylation pathway to counteract the metabolic inhibition of NK cells in the glioma microenvironment (111).

Secondly, autologous immune cells from cancer patients usually have low activity and cytotoxicity effects as well as limited cell expansion capacity, which can be well solved by allogeneic transfusion. CAR-NK is able to use an unlimited source of allogeneic NKs without the fear of graft vs. host disease (GVDH) compared to CAR-T cells. Meanwhile, the allogeneic environment counteracts the limitation of NK cells by self MHC-I molecules, allowing NK cells to achieve a more potent killing effect on tumors. Clinical trials of allogeneic CAR-NK were first completed in 2024 (NCT03056339) (112). There were no findings of any cytokine release syndrome, neurotoxicity or GVHD during the treatment of 37 patients with CD19+ B-cell malignancies. The objective response rate at day 100 was 48.6% and the 1-year OS rate was 68%. Thus, the safety of CAR-NK allogeneic transfusion was confirmed. It also demonstrates that CAR-NK can be generated 'off-the-shelf' from NK cell lines or iPSC-NK with a relatively short production time (113). Several preclinical studies have demonstrated that iPSC-derived CAR-NK has better anti-tumor capabilities compared to autologous CAR-NK cells (114-116).

Of note, NK cells have the ability to acquire adaptive immunity and become memory-like NK cells in a tumor environment or in the presence of pro-inflammatory cytokine stimulation (117). This means that the memory-like NK cells left behind after NK therapy eliminate the tumour and create a long-term immune environment against the specific tumour, enabling them to respond rapidly to escaped cancer cells as well as those that enter dormancy that cannot be killed by chemotherapy. The risk of tumour recurrence is markedly reduced and the prognosis for patient survival is improved. Several studies have shown memory-like NK cells that can be induced by cytokines, such as IL-12, IL-15 and IL-18 (118).

Finally, in addition to the advantages of CAR-NK itself, a large number of studies have shown that CAR-NK is able to interact with other immune-immune cells to achieve better therapeutic outcomes. For instance, Parihar *et al* (119) found that CAR-NK cells may be able to be used in combination with T-cell-based therapies for solid tumours. Furthermore, it has been shown that NK cells can coordinate anti-tumour immune responses by recruiting DCs, thereby facilitating the coupling of innate and adaptive immune responses (120).

In clinical practice, the treatment of NK cells delivered to the circulating medullary fluid by intraventricular catheter and subcutaneous routes after surgical removal of gliomas has yielded good results (105). Besides, several clinical studies on NK-cell therapy for glioma have demonstrated the safety of NK-cell therapy. Although clinical studies using NK cells for glioma treatment are currently limited, these advantages that NK autogenously possesses, as well as the safety shown in the clinic and the potential demonstrated in preclinical studies of CAR-NK, portend that the use of modified CAR-NK in combination with other therapies after surgical resection may be a major breakthrough in the treatment of malignant gliomas.

However, although current clinical results show improvement in clinical symptoms in patients treated with cell therapy via NK cells, the very limited number of successfully enrolled clinical studies means that there are still unknown challenges in the actual treatment. A number of conditions may affect the treatment of patients with GBM with NK cells: First, the timing and site of NK-cell infusion have not been clarified. In current research and clinical trials on GBM, the timing of infusion varies, with the most efficacious infusion site and optimal infusion regimen yet to be discovered. Second, cell counts and infusion intervals can also affect patient outcomes. Finally, tumor residue or residual tumor mass after surgery or tumor regrowth after radiation/chemotherapy may reduce the effectiveness of NK therapy. How to combine NK cells with radiotherapy, antibody drugs and immune pathways represented by T cells/NKT cells/DC cells after surgical resection, as well as exploring optimal combination therapies, is a critical step in the future of NK immunotherapy for gliomas.

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ZX, YL and CS structured the article, compiled the review, drafted the manuscript, and reviewed and edited the manuscript. QQ, XW and NW contributed to the conception of the article. All authors provided contributions to the article and have read and approved the final version submitted. Data authentication is not applicable.

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Competing interests

The authors declare that they have no competing interests.

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