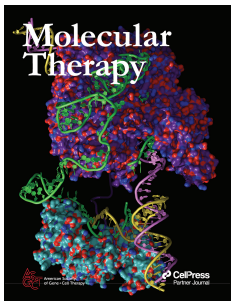
Journal Pre-proof



Killing the Killers: Natural Killer Cell Therapy Targeting Glioma Stem Cells in High-Grade Glioma

Poorva Poorva, Jensen Mast, Bihui Cao, Mitesh V. Shah, Karen E. Pollok, Jia Shen

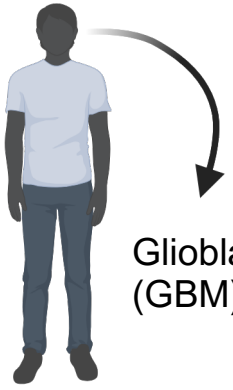
PII: S1525-0016(25)00168-6

DOI: https://doi.org/10.1016/j.ymthe.2025.02.043

Reference: YMTHE 6806

To appear in: *Molecular Therapy*

Please cite this article as: Poorva P, Mast J, Cao B, Shah MV, Pollok KE, Shen J, Killing the Killers: Natural Killer Cell Therapy Targeting Glioma Stem Cells in High-Grade Glioma, *Molecular Therapy* (2025), doi: https://doi.org/10.1016/j.ymthe.2025.02.043.



Glioblastoma
(GBM)

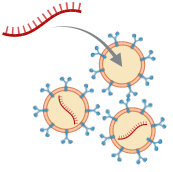
Diffuse intrinsic pontine
glioma (DIPG)

NK cell-based therapies

NK cell

CAR-NK cell

NK engager



NK exosome

Killing the Killers: Natural Killer Cell Therapy Targeting Glioma Stem Cells in High-Grade Glioma

Poorva Poorva[1,7], Jensen Mast[2,7], Bihui Cao[1], Mitesh V. Shah[3], Karen E. Pollok[4,5], Jia Shen[1, 5, 6, *]

[1] Medical Sciences Program, Indiana University School of Medicine, Bloomington, IN 47405, USA

[2] Biochemistry Graduate Program, Indiana University, Bloomington, IN 47405, USA

[3] Department of Neurological Surgery, Indiana University School of Medicine, Indianapolis, IN 46202, USA

[4] Department of Pediatrics, Indiana University School of Medicine, Indianapolis, IN 46202, USA

[5] Indiana University Melvin and Bren Simon Comprehensive Cancer Center, Indianapolis, IN 46202, USA

[6] Department of Medical and Molecular Genetics, Indiana University School of Medicine, Indianapolis, IN 46202, USA

[7] These authors contributed equally

[*] Corresponding author. Jia Shen, Ph.D. 202 Biology Building, 1001 E. 3rd St., Bloomington, IN 47405, USA. Phone: (812) 855-4724. E-mail address: shen17@iu.edu

Short Title: Natural Killer Cell Therapy Targeting Glioma Stem Cells in High-Grade Glioma

Abstract

High-grade gliomas (HGGs), including glioblastoma (GBM) in adults and diffuse intrinsic pontine glioma (DIPG) in children, are among the most aggressive and deadly brain tumors. A key factor in their resilience is the presence of glioma stem cells (GSCs), which drive tumor initiation, progression, and resistance to treatment. Targeting and eradicating GSCs holds potential for curing both GBM and DIPG. Natural Killer (NK) cells, as part of the innate immune system, naturally recognize and destroy malignant cells. Recent advances in NK cell-based therapies, such as chimeric antigen receptor (CAR)-NK cells, NK cell engagers, and NK cell-derived exosomes, offer promising approaches for treating GBM and DIPG, particularly by addressing the persistence of GSCs. This review highlights these advancements, explores challenges such as the brain-blood barrier and the immunosuppressive tumor microenvironment, and proposes future directions for improving and clinically advancing these NK cell-based therapies for HGGs.

Introduction

High-grade gliomas (HGGs) are a group of highly aggressive and often fatal brain tumors, affecting both adults and children[1,2]. Classified as grade 3 or 4 based on their level of malignancy and growth rate[3], these tumors include some of the most dangerous forms of gliomas, such as glioblastoma (GBM) in adults and diffuse intrinsic pontine glioma (DIPG) in children. GBM causes over 15,000 deaths annually in the United States[4]. Standard treatment includes surgical resection followed by radiation and temozolomide (TMZ) chemotherapy[5-7]. However, despite these interventions, the prognosis remains bleak, with a median survival of less than 15 months[8,9]. DIPG, primarily affecting children between ages 5 and 10, is equally devastating. This inoperable brainstem tumor, located in the pons, presents with severe neurological symptoms and shares the resistance of GBM to radiation and chemotherapy. The median survival time for DIPG is less than one year[10,11]. A major contributor to the persistence and recurrence of both GBM and DIPG is the presence of glioma stem cells (GSCs)[12,13]. GSCs are highly efficient at initiating and sustaining tumor growth and showing resistance to standard therapies[14-16]. Targeting and eliminating GSCs could offer a potential cure for both GBM and DIPG.

Natural Killer (NK) cells, key players in the innate immune system, have gained attention for their ability to target and eliminate cancer cells without prior sensitization[9,17]. Previous research suggests that NK cells preferentially attack stem-like cancer cells over differentiated ones[18]. Present in the microenvironment of both GBM and DIPG[19-22], NK cells exert cytotoxic effects independent of cancer cell proliferation, making them effective against both proliferating and dormant GSCs. Recent advances in NK cell-based therapies, such as chimeric antigen receptor (CAR)-NK cells, NK cell engagers, and NK-derived exosomes, show great potential in eradicating cancer cells across various types, offering a promising strategy to improve outcomes in HGGs like GBM and DIPG.

This review provides an in-depth analysis of GBM and DIPG, focusing on the challenges their aggressive nature and treatment resistance present, particularly due to the role of GSCs. It discusses the function of NK cells within the tumor microenvironment and highlights recent advances in NK cell-based cancer therapies. Additionally, it identifies the obstacles in advancing NK cell therapies and explores future directions for combating HGGs in both adult and pediatric patients.

GBM and GSCs

GBM is the most prevalent and lethal form of malignant brain tumor in adults, accounting for nearly half of all primary brain tumors[16,23]. Its aggressive behavior poses significant treatment challenges. GBM typically arises in the cerebral hemispheres, most often affecting the frontal and temporal lobes, though it can occur in other regions, including the parietal and occipital lobes[4,23,24]. The location of tumor often dictates clinical symptoms such as headaches, seizures, cognitive impairment, and motor deficits. It can develop as a primary tumor, arising without a preceding lower-grade glioma (LGG), or as a secondary tumor, evolving from pre-existing LGG. A key molecular marker is the mutation status of the isocitrate dehydrogenase (IDH) gene. IDH-mutant GBMs, which often arise from LGG, are less aggressive, while the more common IDH-wild type GBMs are associated with poorer prognosis[25-27].

The Cancer Genome Atlas (TCGA) classifies GBMs into three primary molecular subtypes: proneural (PN), classical (CL), and mesenchymal (MES). These subtypes, distinguished by specific genetic alterations, differ in clinical behavior and drug response, contributing to the failure of multimodal therapies like radiotherapy, chemotherapy, and targeted treatments[6,7,27]. For instance, EGFR amplification and mutations are common in the CL subtype, while the MES subtype is linked to NF1 mutations and increased immune cell infiltration[28-30]. However, recent single-cell RNA sequencing (scRNA-seq) studies have revealed that these subtypes are not strictly compartmentalized within individual tumors. Multiple subtypes may coexist within different regions of the same tumor, and the molecular characteristics can shift over time and in response to treatment. For the GBM cells, scRNA-seq has identified four cellular states: (1) neural-progenitor-like (NPC-like), (2) oligodendrocyte-progenitor-like (OPC-like), (3) astrocyte-like (AC-like), and (4) mesenchymal-like (MES-like)[13,28,31,32].

These cellular states align with the TCGA subtypes, with the CL and MES subtypes corresponding to the AC-like and MES-like states, while the PN subtype aligns with the OPC-like and NPC-like states. This phenotypic plasticity, where tumor cells can switch between states in response to genetic mutations or environmental changes, poses a great challenge for treatment. Targeting a single subtype is often insufficient, as GBM tumors are composed of cells in multiple cellular states that can adapt and evolve throughout therapy[13,16].

Central to the persistence and recurrence of GBM are GSCs, which exhibit stem cell-like properties such as self-renewal and the ability to differentiate into various tumor cell types[9,33,34]. They utilize mechanisms like enhanced DNA repair, drug efflux, and quiescence to evade treatments, positioning them as critical drivers of tumor progression and relapse[27,33,35]. GSCs thrive in hypoxic environments, which enhance their survival and promote tumor growth. Key molecular markers that identify GSCs include CD133[36-38], a transmembrane glycoprotein linked to increased tumorigenic potential and treatment resistance; SOX2[39-41], a transcription factor vital for maintaining cell stemness and plasticity; Nestin[42-44], an intermediate filament protein associated with neural progenitor-like states and tumor invasiveness; CD44[45-47], a cell surface glycoprotein that serves as a receptor for hyaluronic acid, facilitating cell-cell interactions, adhesion, and migration within the extracellular matrix; ALDH (Aldehyde Dehydrogenase)[48,49], a family of enzymes essential for detoxifying aldehydes by converting them into carboxylic acids; and OLIG2[50-53], a transcription factor important for oligodendrocyte development. However, the search for universal GSC markers remains controversial, primarily due to significant interpatient and intratumoral variability. Recent advances in scRNA-seq have revealed four primary GSC cellular states in GBM: NPC-like, OPC-like, AC-like, and MES-like, each associated with distinct stemness markers[13,28,31,32]. Specifically, CD133 is linked to OPC-like cells, CD24 to NPC-like cells, and CD44 to MES-like cells. This phenotypic plasticity, along with genetic alterations in EGFR, PDGFRA, CDK4, and NF1, influences GSC behavior, allowing them to further evade therapeutic interventions and contribute to tumor regeneration.

DIPG and GSCs

DIPG is among the deadliest pediatric brain tumors, representing about 10-15% of brain tumors in children. Following diagnosis, the median survival time is around 11 months, and fewer than 10% of patients survive beyond two years[54-57]. DIPG is located in the pons, a crucial part of the brainstem that regulates essential functions such as breathing, heart rate, sleep, and motor control. As a result, DIPG significantly disrupts these processes, leading to severe neurological symptoms. The invasive DIPG cells distort and destroy nerve fibers, further contributing to neurological decline[58]. Due to its infiltrative nature and brainstem location, DIPG is extremely difficult to treat surgically[59-61]. Current therapies like chemotherapy have been largely ineffective because of the inherent resistance of the tumor, while radiotherapy provides only temporary relief, extending survival by just a few months without stopping tumor progression[56,62]. This dismal prognosis highlights the urgent need for new therapeutic approaches.

One of the key genetic alterations driving the behavior of DIPG is the H3K27M mutation, found in about 70-80% of cases. These tumors, now classified as Diffuse Midline Glioma (DMG)[63,64], H3K27-altered, are characterized by a mutation in the histone H3 gene. This mutation disrupts normal chromatin regulation by inhibiting polycomb repressive complex 2 (PRC2), causing a global loss of H3K27 trimethylation and leading to widespread gene expression dysregulation[59,60]. Recent scRNA-seq studies have identified three distinct cell states in H3K27M DIPG: OPC-like, oligodendrocyte (OC)-like, and AC-like[13,65,66]. Notably, OPC-like cells, which represent undifferentiated progenitors, account for up to ~80% of the tumor population, indicating a differentiation block and highlighting the aggressive nature of the disease. Additionally, 20-30% of DIPG cases lack both the H3K27M and IDH mutations. These tumors are classified as DIPG, H3 wildtype, and IDH wildtype[63], but their underlying genetic drivers remain less understood, underscoring the need for further research into these subgroups.

A growing body of research has identified GSCs as key drivers of aggressiveness and treatment resistance in H3K27M DIPG. The H3K27M mutation disrupts the normal differentiation of OPCs by inhibiting PRC2 function, leading to an accumulation of undifferentiated cells[66,67]. A distinct stem-like profile has been observed in H3K27M-altered DIPG cells, which show elevated expression of stem cell markers such as Bmi1, Nestin, CD15, and SOX2[67-69]. These markers, associated with self-renewal and stem cell maintenance, suggest that these tumors retain significant stem-like characteristics. DMG cell lines like HSJD-DIPG-007 and HSJD-DIPG-012 demonstrate a strong capacity for self-renewal and can form neurospheres from a single cell, further supporting their stem-like behavior[67]. SOX2, a key transcription factor that regulates stem cell pluripotency, is notably overexpressed in these cells[15,39-41]. This stem-like profile, combined with the impact of mutation on chromatin and epigenetic regulation, underscores the aggressive nature of GSCs and highlights the challenges in developing effective therapies.

NK Cells in Tumor Microenvironment

NK cells are a vital component of the innate immune system, uniquely capable of identifying and eliminating virally infected or tumor cells, making them a critical first line of defense[17,70-73]. They originate from lymphoid progenitors in the bone marrow and circulate in the bloodstream. NK cells are characterized by the expression of CD56 and the absence of CD3, which distinguishes them from T cells. They are categorized into two subsets: $CD56^{bright}$ $CD16^{dim/-}$ cells and $CD56^{dim}$ $CD16^{+}$ cells. $CD56^{bright}$ $CD16^{dim/-}$ cells are primarily located in lymphoid tissues and are known for producing cytokines such as IFN-γ, which play a pivotal role in modulating both adaptive and innate immune responses. These cells are not inherently cytotoxic but can exhibit cytotoxicity following activation[74-76]. In contrast, $CD56^{dim}$ $CD16^{+}$ cells, predominantly found in peripheral blood, are well-known for their potent cytotoxicity and their ability to induce apoptosis in target cells.

NK cells function by integrating signals from two types of surface receptors: activating and inhibitory receptors (Figure 1). Activating receptors, such as NKG2D, NKp30, NKp46, and DNAM-1, trigger NK cell activation by recognizing stress-induced ligands or pathogen-associated molecules on abnormal cells, enabling the elimination of cancer cells and virus-infected cells. In contrast, inhibitory receptors, including CD94/NKG2A and LILRs, recognize self-molecules like major histocompatibility complex (MHC) class I proteins on healthy cells, delivering "don't kill" signals to prevent damage to normal tissues. The "missing self" theory[77-79] further explains that NK cells can recognize and target cells that lack these self-MHC class I molecules, as their absence removes the inhibitory signals, prompting NK cell activation. This balance between activating and inhibitory signals ensures that NK cells efficiently target diseased cells while maintaining immune tolerance and preventing autoimmunity[9,80,81] (Table 1). Tumor cells often evade T cells by downregulating MHC class I, making them more susceptible to NK cell-mediated killing. Once activated, NK cells release cytotoxic granules containing perforin, which forms pores in the target cell membrane, and granzymes, which enter the cell to induce apoptosis. Additionally, NK cells can trigger apoptosis through death receptor signaling, where TNF-related apoptosis-inducing ligand (TRAIL) or Fas ligand (FasL) expressed on NK cells (Figure 1) binds to corresponding receptors on target cells, activating apoptotic pathways. NK cells also secrete cytokines like IFN-γ, which recruit other immune cells and modulate adaptive immunity. Another key mechanism is antibody-dependent cellular cytotoxicity (ADCC), where NK cells, via their Fcγ receptor CD16, bind to the Fc region of IgG antibodies on target cells, triggering a cascade that leads to cytotoxic molecule release and cell elimination.

The tumor is not just a mass of cancer cells but rather a complex ecosystem referred to as the tumor microenvironment (TME). This environment comprises cancer cells, cancer-associated fibroblasts, immune cells, endothelial cells, pericytes, the extracellular matrix (ECM), and various secreted molecules. The ECM provides structural support, while interactions between cancer cells and surrounding cells occur through direct contact or signaling molecules such as cytokines, chemokines, and extracellular vesicles[82-87]. These interactions can contribute to immune suppression, allowing

tumors to evade detection. Immune-suppressive cells within the TME, including myeloid-derived suppressor cells (MDSCs) and regulatory T cells (Tregs), are crucial to tumor progression by dampening anti-tumor immune responses. MDSCs suppress T cell and NK cell activation and proliferation through multiple pathways, including the generation of reactive oxygen species and the secretion of immunosuppressive cytokines like TGF-β and IL-10[88,89]. Likewise, Tregs can inhibit the activity of effector T cells and NK cells by producing these same cytokines[90,91]. Exosomes, small membrane-bound extracellular vesicles (30 - 150 nm) secreted by various cell types including cancer cells, are another important component of the TME[92,93]. These exosomes facilitate intercellular communication by transferring bioactive molecules, such as lipids, nucleic acids, metabolites, and proteins, that reflect the cell of origin. Exosomes released by tumor cells have potent immunosuppressive properties[94], altering the immune landscape within the TME. These exosomes can carry immunosuppressive molecules, such as PD-L1 and TGF-β, which suppress the activity of effector T cells and NK cells while promoting the expansion of immunosuppressive populations such as Tregs, MDSCs, and regulatory B cells (Bregs). Additionally, tumor-derived exosomes interfere with the maturation of myeloid progenitor cells, promote the polarization of macrophages into the tumor-supportive M2 phenotype, and hinder neutrophil recruitment, collectively enabling tumors to escape immune detection and surveillance[95-97]. Together, these cellular and molecular interactions within the TME create a supportive environment for tumor growth and immune evasion.

Despite their potent anti-cancer abilities, NK cells in the TME face various challenges that severely impair their function. First, the ECM can create physical barriers that prevent NK cell infiltration into tumor tissue, limiting their ability to reach and eliminate tumor cells, especially in poorly vascularized regions. Another major obstacle is the presence of immunosuppressive factors. TGF-β suppresses the expression of key NK cell activating receptors such as NKG2D and reduces cytokine production such as IFN-γ, which is vital for anti-tumor immunity[98-100]. Prostaglandin E2 (PGE2), an immunosuppressive molecule in the TME, downregulates activating receptors and promotes inhibitory pathways in NK cells[101-103]. Hypoxia, or low oxygen levels, alters the expression of activating receptors such as NKp44, NKp46, NKp30, and NKG2D, and reduces NK cell cytokine secretion, thus impairing their ability to recognize and kill tumor cells[104,105]. Tumor cells can also exploit NK cell inhibitory receptors to evade killing by upregulating ligands for these receptors. Similar to T cells, NK cells can express the checkpoint receptor PD-1. When PD-1 engages with its ligand PD-L1 on tumor cells, NK cells become exhausted, losing their cytotoxic capabilities. Table 2 provides an overview of key mechanisms by which tumors evade NK cell-mediated immunosurveillance. Addressing these challenges is a major goal of advancing NK cell immunotherapy, with the focus on restoring or enhancing NK cell function to more effectively target and eliminate tumors.

NK Cell-Based Cancer Therapies

NK cell-based cancer therapies leverage the killing function of NK cells to target and destroy tumor cells, presenting a promising approach in cancer immunotherapy. These strategies include CAR-NK cells, NK cell engagers, NK exosomes, and other innovative strategies (Figure 2).

Cell Sources The source of NK cells is a critical factor in determining their clinical effectiveness and scalability in NK cell therapy (Figure 3). Primary NK cells, sourced from peripheral blood, umbilical cord blood, or hematopoietic stem cell progenitors, are pivotal in cancer immunotherapy due to their natural ability to recognize and eliminate tumor cells through activating receptors such as NKG2D and CD16, the latter of which facilitates ADCC. Autologous NK cells, derived from the patient's own immune system, are activated using cytokines like IL-2, IL-12, IL-15, and type I IFNs to enhance their antitumor function. While offering personalized therapy, their effectiveness is often diminished in cancer patients due to the immunosuppressive TME. In contrast, allogeneic NK cells from healthy donors exhibit stronger cytotoxicity, particularly in patients undergoing hematopoietic stem cell transplantation, where donor NK cells can counteract TME-induced immunosuppression. Immortalized NK cell lines, such as NK-92[106,107] , KHYG-1[108,109], and NKL[110,111] , offer a scalable and consistent

therapeutic source, with NK-92 being the only cell line in clinical trials. However, NK-92 cells lack CD16 expression, limiting their ADCC potential, and require irradiation to prevent tumorigenicity. Induced pluripotent stem cell (iPSC)-derived NK cells provide a scalable and renewable option, enabling off-the-shelf therapies with consistent quality and enhanced engineering potential[112,113], though their production is time-intensive and costly, and their long-term safety remains under investigation. In terms of anti-tumor activity, NK-92 cells display robust efficacy, particularly when engineered with CARs; however, their lack of CD16 limits their ability to synergize with monoclonal antibodies. Primary NK cells exhibit strong cytotoxicity due to their mature phenotype and high ADCC potential. While iPSC-derived NK cells have lower baseline cytotoxicity compared to primary NK cells and NK cell lines, their ability to be engineered for enhanced functionality highlights their promise in advancing NK cell-based cancer immunotherapy.

CAR-NK Cells CAR-NK cell technology harnesses the innate cytotoxicity of NK cells while integrating the tumor-targeting precision of engineered receptors. Like CAR-T cells, CAR-NK cells are engineered to express CARs that recognize specific antigens on cancer cells. Conventional CAR-NK cells consist of a CAR structure with an extracellular single-chain variable fragment (scFv), a transmembrane domain, and intracellular signaling domains. Next-generation CAR-NK cells incorporate innovative features, such as dual-targeting receptors for multiple antigens, armored CARs that secrete cytokines like IL-15 to enhance persistence, and modules to block immune checkpoints such as PD-L1, counteracting tumor-induced immune suppression[114-116]. Combining CAR-NK cells with monoclonal antibodies or checkpoint inhibitors has shown potential to improve therapeutic outcomes[117,118]. When comparing CAR-T and CAR-NK therapies, both demonstrate distinct strengths and challenges. CAR-T cells have shown remarkable efficacy in hematologic malignancies due to their antigen-specific cytotoxicity, long persistence, and memory T cell formation. However, they encounter significant limitations in solid tumors, including susceptibility to the TME and risks of cytokine release syndrome (CRS), neurotoxicity, and graft-versus-host disease (GVHD)[119,120] in allogeneic settings. In contrast, CAR-NK cells combine innate and CAR-mediated cytotoxicity, enabling them to target tumors with heterogeneous antigen expression and function more effectively within the TME. They also exhibit a superior safety profile, with minimal risks of CRS and neurotoxicity, and can be produced as off-the-shelf therapies using sources like cord blood and iPSCs. Despite these advantages, CAR-NK cells face challenges such as limited persistence in vivo, difficulties infiltrating solid tumors, immunosuppressive TME, and antigen downregulation, which can reduce therapeutic efficacy. Additionally, systemic administration for central nervous system cancers, such as HGGs, must overcome physical barriers like the blood-brain barrier (BBB), posing a significant hurdle for CAR-NK therapies.

NK Cell Engagers (NKCEs) NKCEs are engineered, antibody-based molecules designed to target cancer or infected cells. These molecules come in two main forms: bispecific killer cell engagers (biKEs) and trispecific killer cell engagers (triKEs)[121,122]. NKCEs function by simultaneously binding to NK cells and tumor-specific antigens on cancer cells, bringing them into close proximity and activating NK cells to more effectively kill the target cells. Ideally, the tumor antigen should be a cell surface protein overexpressed in cancer cells, maximizing the ability of NKCE to recognize and target the cancer cell. Equally important is the selection of NK cell receptors. One of the most common NK cell receptors used in NKCEs is CD16, known for its ability to fully activate NK cells without needing coactivation from other receptors[123]. Other receptors, such as NKG2D[124,125], NKp30[126,127], and NKp46[128,129], can also be used depending on the specific advantages they offer. In triKEs, the NK receptor and tumor cell antigen are linked via an IL-15 component, which boosts NK cell growth, activation, and survival (Figure S1). Preclinical studies have shown promising results for triKEs in treating cancers such as ovarian cancer[130], high-risk myelodysplastic syndromes[131], and advanced systemic mastocytosis[132]. While NKCEs offer many advantages, such as enhanced specificity and immune activation, they also present challenges. These include a short half-life in the body and potential size limitations that may hinder their ability to penetrate dense tumors.

NK Cell Exosomes The formation of NK cell-derived exosomes begins with the invagination of the plasma membrane of the NK cells, creating an early endosome that encapsulates various

macromolecules during this process[133]. As the early endosome matures into a late endosome, it undergoes further sorting and packaging of its macromolecular contents[134]. Subsequently, the late endosome fuses with the plasma membrane of the NK cells, releasing exosomes into the extracellular matrix[133]. Once secreted, NK cell exosomes interact with target cells, delivering their molecular cargo to exert their functions. NK cell exosomes retain characteristics of their parent NK cells, including the expression of surface markers such as CD16, CD69, NKp44, and NKG2D[135-137]. They carry cytotoxic molecules like TNF-α, granzyme A/B, and perforin, and they display transmembrane proteins such as FasL and TRAIL, which can induce both caspase-dependent and caspase-independent apoptosis in tumor cells. A major advantage of NK cell exosomes in cancer therapy is their small size, which allows them to navigate tumor vasculature effectively, along with their resilience in acidic environments. Recent studies have highlighted their effectiveness against various cancers, demonstrating significant cytotoxic activity against melanoma[138] and breast cancer[139]. However, despite their promise, preclinical and clinical validation of NK cell exosomes in brain tumors remains in its early stages, necessitating further studies to evaluate their long-term safety and efficacy.

NK Cell-Based Therapies for Targeting GSCs in HGG

GSCs play a critical role in the initiation, progression, and therapeutic resistance of HGGs, making them a key target for innovative treatment approaches. Recent studies have shown that NK cells can effectively target and destroy GSC tumorspheres in vitro, offering hope for improved therapeutic outcomes [140-142]. However, the success of NK cells in eliminating GSCs is not always assured and often depends on their activation within the TME[141,143]. HGGs create a "cold" TME by promoting hypoxia, upregulating immunosuppressive molecules such as TGF-β and IL-10, and recruiting regulatory cells, including MDSCs and tumor-associated macrophages. These factors impair the ability of NK cells to recognize and destroy GSCs. Additionally, GSCs downregulate activating ligands, further reducing NK cell efficacy (Figure 4)[144]. The heterogeneity of GSCs compounds these challenges, as these cells express varying surface markers at different stages of differentiation, making it difficult for NK cells to consistently recognize and target them.

To enhance NK cell efficacy against HGGs, researchers have been investigating multiple strategies. The NKG2D ligand family consists of several stress-induced proteins, including MICA, MICB, and the ULBP (UL16-binding proteins) subfamily, which are expressed on the surface of cancer cells. These ligands bind to the NKG2D receptor on NK cells, triggering NK cell activation and cytotoxicity, thereby facilitating the elimination of cancer cells. However, GSCs in GBM have been shown to downregulate NKG2D ligands, which confers resistance to NK cell-mediated killing[144]. Thus, targeting NKG2D ligands represents a promising strategy to enhance NK cell-based therapies for GSCs in HGG. Epigenetic modifications play a vital role in regulating gene expression, including the expression of NKG2D ligands[145-148]. Previous studies have demonstrated that targeting EZH2-92aa, a protein encoded by circular EZH2, can promote NK cell-mediated eradication of GSCs both in vitro and in vivo by activating NKG2D ligands[144]. Another study highlighted that the epigenetic regulator, histone deacetylase HDAC8, regulates the expression of NKG2D ligands in glioma cells, and inhibiting HDAC8 increases NKG2D ligand expression, thereby enhancing NK cell-mediated cytotoxicity[149]. MicroRNAs (miRNAs), small non-coding RNA molecules, represent a crucial epigenetic regulatory mechanism by targeting mRNA and modulating gene expression. They play a pivotal role in regulating NK cell functions, such as cytotoxicity, proliferation, and cytokine production. For instance, overexpression of miR-362-5p in human primary NK cells enhances the expression of cytotoxic molecules like IFN-γ, perforin, granzyme-B, and CD107a by targeting the cylindromatosis (CYLD) gene, a negative regulator of NF-κB signaling[150]. Similarly, miR-155 enhances NK cell activation in response to IL-2, IL-15, and IL-21 stimulation[151]. Nanoparticles offer a promising platform for miRNA delivery, with their ability to precisely target tumor sites, cross the BBB, and penetrate tumor vasculature, making them particularly suitable for HGG therapy. Incorporating miRNA-loaded nanoparticles into NK cell-based therapies is an emerging field with immense potential. For example, NK cell-derived exosomes carrying miR-186 have demonstrated cytotoxic effects against MYCN-

amplified neuroblastoma by preventing TGFβ1-mediated NK cell inhibition[152]. These findings underscore the potential of miR-186-loaded nanoparticles as a therapeutic strategy to enhance NK cell-mediated immunotherapy.

Furthermore, the development of CAR-NK cells, genetically engineered to recognize specific antigens expressed on GSCs such as EGFRvIII and GD2, represents a cutting-edge strategy with significant therapeutic potential[153-155]. EGFRvIII, a tumor-specific antigen resulting from an in-frame deletion of certain exons in the *EGFR* gene, promotes tumor growth through constitutive activation while remaining absent in normal tissues, making it an ideal target. Studies have shown that dual-specific CAR-NK cells targeting both EGFR and EGFRvIII using NK-92 cells effectively inhibited tumor growth and prolonged survival in mice with intracranial GBM xenografts[153]. Additionally, EGFRvIII-specific CAR-NK cells engineered to overexpress the chemokine receptor CXCR4 demonstrated enhanced chemotaxis to CXCL12/SDF-1α-secreting GBM cells, achieving complete tumor remission in some mice and significantly improved survival compared to controls[156]. Ganglioside GD2, a glycolipid highly expressed on various tumors, including GBM[155] and DIPG[157], but with limited expression in normal tissues, is another promising target. GSCs in GBM are known to overexpress GD2[158], which plays a role in tumor progression by promoting cell proliferation, migration, and immune evasion. GD2-specific CAR-NK cells have demonstrated the ability to effectively kill DIPG cells with high GD2 expression in both in vitro and in vivo patient-derived cell models[157].

Oncolytic viruses can reshape the TME to enhance NK cell activity. By directly targeting tumor cells and triggering an anti-tumor immune response, viruses engineered to express the IL15/IL15Rα complex have been shown to significantly boost the efficacy of EGFR-CAR NK cells against GBM[159]. Additionally, studies have reported that autophagy inhibitors[19] and STING agonists[160] possess the ability to modify the TME, further enhancing NK cell-mediated anti-tumor immune responses in GBM. Targeting galectin-1, a β-galactoside-binding lectin overexpressed in GBM cells[161], induces the release of exosomes containing miRNA-1983 into the TME. These exosomes bind to toll-like receptor 7 (TLR7) on plasmacytoid dendritic cells (pDCs) and conventional dendritic cells (cDCs), activating TLR7 and triggering the release of IFNβ, which enhances NK cell-mediated cytotoxicity against GBM cells[162]. Another promising approach is targeting the αv integrin/TGF-β axis, which has shown potential to improve the infiltration and function of adoptively transferred NK cells in attacking GSCs[141]. Cytokine-based therapies, including IL-2 and IL-15, have been used to boost NK cell proliferation and activation[163-166]. Immunocytokines, which are monoclonal antibodies conjugated with cytokines[167,168], represent another innovative approach to boosting NK cell activity. In preclinical GBM models, therapy involving the L19 antibody linked to cytokine IL-2 has successfully increased NK infiltration, promoting tumor regression and improving survival rates when used alongside radiotherapy[169]. Furthermore, targeting the recruitment of immunosuppressive MDSCs and tumor-associated macrophages may improve NK cell infiltration and functionality in HGGs. Other strategies to improve NK cell efficacy against HGGs are outlined in Table 3.

Table 4 highlights a selection of recent clinical trials employing NK cell therapies for GBM patients. A phase I/IIa clinical trial (KCT0003815) evaluated the safety and efficacy of adoptive, ex vivo-expanded, and activated NK cells and T lymphocytes derived from peripheral blood mononuclear cells of patients with recurrent GBM. The results showed a median overall survival (OS) of 22.5 months and a median progression-free survival of 10 months. Notably, five patients demonstrated durable responses, remaining alive for over two years, with enhanced immune-reaction transcriptomic signatures and no signs of clinical decline at their last follow-up after completing therapy[170]. Additionally, a phase I trial (NCT01588769) investigated the tolerability and efficacy of autologous lymphoid effector cells specific against tumor cells (ALECSAT) in patients with GBM. Activated CD4+ T helper cells, treated with a DNA-demethylating agent, were induced to express a broad spectrum of endogenous cancer/testis antigens, enabling them to serve as antigen-presenting cells and facilitate the generation of autologous cytotoxic T lymphocytes and NK cells. Tumor regression was observed in three patients, with responses sustained for up to 27 months, and no treatment-related adverse events reported[171]. Furthermore, ongoing clinical trials are actively exploring NK cell-based therapies for HGGs. For example, NCT03383978 is evaluating intracranial injection of NK-92/5.28.z CAR NK cells combined with

intravenous ezabenlimab in patients with recurrent HER2-positive GBM. The Phase II trial NCT06687681 is investigating intrathecal injections of active allogeneic NK cells in newly diagnosed patients with grade 3 or 4 brain tumors, including GBM and DIPG. Patients will receive active NK cell injections via lumbar puncture. Additionally, the Phase I trial NCT04991870 aims to determine the optimal dose, benefits, and side effects of engineered NK cells with deleted TGF-betaR2 and NR3C1 (CB-NK-TGF-betaR2-/NR3C1-) in treating recurrent GBM. This study focuses on evaluating the safety and tolerability of escalating doses of off-the-shelf CB-NK-TGF-betaR2-/NR3C1- in these patients. These diverse strategies reflect a comprehensive effort to optimize NK cell-based therapies for HGGs, offering hope for more potent and durable treatment options against these aggressive and deadly brain cancers.

Discussion and Future Perspectives

HGGs are some of the most lethal cancers, with GBM in adults and DIPG in children being particularly aggressive forms. Despite affecting different patient populations and regions of the brain, these tumors share key characteristics: resistance to standard treatments, high invasiveness, poor prognosis, and the presence of GSCs, which drive tumor progression and relapse.

Despite advancements in NK cell-based therapies, significant challenges remain in treating HGGs. Autologous and allogeneic primary NK cells have limited lifespans and struggle to persist after infusion, with their infiltration into solid tumors hindered by physical barriers such as the BBB and the ECM in TME. As tumors progress, their targeting efficacy diminishes. NK cell lines offer scalable, off-the-shelf options for therapy but are difficult to manipulate or engineer for enhanced effectiveness. While CAR-NK cells and NKCEs provide more precise targeting, they still face issues with physical barriers, poor persistence, and short half-lives within the TME of HGGs. NK cell-derived exosomes, considered a next-generation therapy, show promise in bypassing these barriers due to their smaller size, which allows for easier crossing of physical obstacles. However, the immunosuppressive TME continues to undermine their efficacy. Given these limitations, it is crucial to explore new approaches to enhance NK cell-based therapies for HGGs. We propose three key directions for improving and clinically advancing these therapies.

1. Promoting NK cell trafficking and infiltration into HGGs. The BBB is a significant obstacle for NK cells as well as the NKCEs administered systemically, limiting their access to the tumors. Temporarily disrupting the BBB can improve NK cells and NKCEs delivery, with methods like focused ultrasound showing promise by creating transient openings in the BBB[172-174]. Another approach involves modulating chemokines to enhance NK cell trafficking. For example, overexpressing chemokines like CXCL10, which binds CXCR3 on NK cells[175,176], can improve NK cell homing to the HGGs. Radiation therapy may also increase chemokine expression in TME and disrupt physical barriers, making tumors more accessible to NK cells[177-179]. Tumor Treating Fields (TTFields), which received FDA approval in 2011 for GBM treatment, can activate IFN signaling within the tumor[180,181]. This activation may enhance NK cell homing to HGGs and improve their capacity to target and eliminate GSCs. Furthermore, switching from systemic to local delivery of NK cells can significantly improve infiltration. Administering NK cells intraventricularly into the ventricles of brain or intratumorally directly into the tumor bypasses the BBB, resulting in higher concentrations of NK cells at the tumor site and enhanced therapeutic outcomes. Additionally, using BBB-penetrating NK cell exosomes may allow better access to the tumor. NK cell exosomes, which retain cytotoxic properties by carrying perforin and granzymes, can target GSCs in HGGs without the need for direct NK cell infiltration. For instance, considering the therapeutic potential of miRNA-1983 in GBM treatment[161,162], developing NK cell-derived exosomes loaded with miRNA-1983 could be a promising approach for targeting GSCs within the HGG TME.

2. Enhancing NK cell persistence and efficacy. Precision-engineering tools such as CRISPR/Cas9 can enhance NK cell activity in the immunosuppressive TME by deleting genes associated with NK cell exhaustion or inhibitory pathways. Combining NK cells with IgG antibodies amplifies the ADCC effect,

strengthening NK cell killing efficacy[182-186]. Modulating the TME itself, by targeting immunosuppressive signals like TGF-β or reprogramming tumor-associated macrophages and MDSCs, can also enhance NK cell survival and function. Another promising approach involves combining NK cell-based therapies with other treatments, such as radiotherapy, chemotherapy, TTFields, oncolytic viruses, immune checkpoint antibodies (e.g., PD-1/PD-L1 or CTLA-4 antibodies), or cytokines (e.g., IL-12 or IL-15). For example, radiation and chemotherapeutics like TMZ, the standard of care for GBM, could induce the upregulation of stress ligands, such as MICA/B and ULBPs, on GSCs, which are recognized by the activating receptor NKG2D on NK cells[187]. Furthermore, the immune-modulatory effects of radiation and chemotherapy may improve NK cell infiltration and persistence within the TME, amplifying their direct cytotoxic effects. Certain chemotherapeutics also reduce the immunosuppressive nature of the TME by depleting immunosuppressive cells, including MDSCs and Tregs, thereby further enhancing NK cell functionality. Oncolytic viruses selectively infect and replicate within tumor cells, releasing chemokines like CXCL10 that recruit NK cells to the TME[188,189]. They also enhance the expression of stress ligands on tumor cells, facilitating improved NK cell recognition, and induce immunogenic cell death, which activates dendritic cells and promotes NK cell activation within the TME[190]. Immune checkpoints, including PD-1/PD-L1, TIGIT/CD96/CD155, and LAG-3/TIM-3, play a significant role in suppressing NK cell activity in the HGG TME[191,192]. Targeting these checkpoints through immune checkpoint blockade, either individually or in combination, offers a promising strategy to restore NK cell functionality and enhance their cytotoxicity against GSCs.

3. Enhancing clinical translation of NK cell-based therapies. The transition from preclinical success to scalable, reliable therapies for HGG patients faces many challenges, including regulatory, manufacturing, and logistical hurdles[193]. Establishing robust manufacturing platforms for NK cell therapies and ensuring they are scalable will be crucial for their clinical adoption. By overcoming these challenges, we can facilitate the broader implementation of effective NK cell-based treatments for HGGs in clinical settings.

ACKNOWLEDGMENTS

This study was supported by the National Cancer Institute of the National Institutes of Health under Award Number P30CA030199. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health. Additional funding was provided by ACS-IRG Grant Mechanism (22-147-37; J. Shen), the Schwarz Family and Friends Cancer Research Fund (J. Shen), the Indiana University School of Medicine Start-Up Fund (J. Shen), and Indiana University EMPOWER grant (J. Shen). Figures were created with BioRender.com.

AUTHOR CONTRIBUTIONS

Writing – Original Draft, P.P., J.M., B.C., and J.S.; Writing – Review & Editing, M.V.S., K.E.P., and J.S.; Project administration, J.S.; Funding acquisition, J.S.

DECLARATION OF INTERESTS

The authors declared no competing interests.

KEY WORDS

HGG, GBM, DIPG, GSC, NK, CAR-NK, NK cell engagers, NK cell exosomes

REFERENCES

1. Ceccarelli, M., Barthel, F.P., Malta, T.M., Sabedot, T.S., Salama, S.R., Murray, B.A., Morozova, O., Newton, Y., Radenbaugh, A., Pagnotta, S.M., Anjum, S., et al. (2016). Molecular Profiling Reveals Biologically Discrete Subsets and Pathways of Progression in Diffuse Glioma. Cell *164*, 550-563. 10.1016/j.cell.2015.12.028.
2. Aggarwal, P., Luo, W., Pehlivan, K.C., Hoang, H., Rajappa, P., Cripe, T.P., Cassady, K.A., Lee, D.A., and Cairo, M.S. (2022). Pediatric versus adult high grade glioma: Immunotherapeutic and genomic considerations. Frontiers in immunology *13*, 1038096. 10.3389/fimmu.2022.1038096.
3. Stitzlein, L.M., Adams, J.T., Stitzlein, E.N., Dudley, R.W., and Chandra, J. (2024). Current and future therapeutic strategies for high-grade gliomas leveraging the interplay between epigenetic regulators and kinase signaling networks. Journal of experimental & clinical cancer research : CR *43*, 12. 10.1186/s13046-023-02923-7.
4. Schaff, L.R., and Mellinghoff, I.K. (2023). Glioblastoma and Other Primary Brain Malignancies in Adults: A Review. Jama *329*, 574-587. 10.1001/jama.2023.0023.
5. Ostrom, Q.T., Cioffi, G., Waite, K., Kruchko, C., and Barnholtz-Sloan, J.S. (2021). CBTRUS Statistical Report: Primary Brain and Other Central Nervous System Tumors Diagnosed in the United States in 2014-2018. Neuro-oncology *23*, iii1-iii105. 10.1093/neuonc/noab200.
6. Miller, K.D., Ostrom, Q.T., Kruchko, C., Patil, N., Tihan, T., Cioffi, G., Fuchs, H.E., Waite, K.A., Jemal, A., Siegel, R.L., and Barnholtz-Sloan, J.S. (2021). Brain and other central nervous system tumor statistics, 2021. CA: a cancer journal for clinicians *71*, 381-406. 10.3322/caac.21693.
7. Stupp, R., Mason, W.P., van den Bent, M.J., Weller, M., Fisher, B., Taphoorn, M.J., Belanger, K., Brandes, A.A., Marosi, C., Bogdahn, U., Curschmann, J., et al. (2005). Radiotherapy plus concomitant and adjuvant temozolomide for glioblastoma. The New England journal of medicine *352*, 987-996. 10.1056/NEJMoa043330.
8. Vredenburgh, J.J., Desjardins, A., Herndon, J.E., 2nd, Dowell, J.M., Reardon, D.A., Quinn, J.A., Rich, J.N., Sathornsumetee, S., Gururangan, S., Wagner, M., Bigner, D.D., et al. (2007). Phase II trial of bevacizumab and irinotecan in recurrent malignant glioma. Clinical cancer research : an official journal of the American Association for Cancer Research *13*, 1253-1259. 10.1158/1078-0432.CCR-06-2309.
9. Du, Y., Pollok, K.E., and Shen, J. (2023). Unlocking Glioblastoma Secrets: Natural Killer Cell Therapy against Cancer Stem Cells. Cancers *15*. 10.3390/cancers15245836.
10. Estevez-Ordonez, D., Gary, S.E., Atchley, T.J., Maleknia, P.D., George, J.A., Laskay, N.M.B., Gross, E.G., Devulapalli, R.K., and Johnston, J.M. (2023). Immunotherapy for Pediatric Brain and Spine Tumors: Current State and Future Directions. Pediatric neurosurgery *58*, 313-336. 10.1159/000528792.
11. Veldhuijzen van Zanten, S.E., Jansen, M.H., Sanchez Aliaga, E., van Vuurden, D.G., Vandertop, W.P., and Kaspers, G.J. (2015). A twenty-year review of diagnosing and treating children with diffuse intrinsic pontine glioma in The Netherlands. Expert review of anticancer therapy *15*, 157-164. 10.1586/14737140.2015.974563.
12. Auffinger, B., Spencer, D., Pytel, P., Ahmed, A.U., and Lesniak, M.S. (2015). The role of glioma stem cells in chemotherapy resistance and glioblastoma multiforme recurrence. Expert review of neurotherapeutics *15*, 741-752. 10.1586/14737175.2015.1051968.
13. Suva, M.L., and Tirosh, I. (2020). The Glioma Stem Cell Model in the Era of Single-Cell Genomics. Cancer cell *37*, 630-636. 10.1016/j.ccell.2020.04.001.
14. El-Khouly, F.E., Veldhuijzen van Zanten, S.E.M., Santa-Maria Lopez, V., Hendrikse, N.H., Kaspers, G.J.L., Loizos, G., Sumerauer, D., Nysom, K., Pruunsild, K., Pentikainen, V., Thorarinsdottir, H.K., et al. (2019). Diagnostics and treatment of diffuse intrinsic pontine glioma: where do we stand? Journal of neuro-oncology *145*, 177-184. 10.1007/s11060-019-03287-9.

15. Da-Veiga, M.A., Rogister, B., Lombard, A., Neirinckx, V., and Piette, C. (2022). Glioma Stem Cells in Pediatric High-Grade Gliomas: From Current Knowledge to Future Perspectives. Cancers *14*. 10.3390/cancers14092296.
16. Prager, B.C., Bhargava, S., Mahadev, V., Hubert, C.G., and Rich, J.N. (2020). Glioblastoma Stem Cells: Driving Resilience through Chaos. Trends in cancer *6*, 223-235. 10.1016/j.trecan.2020.01.009.
17. Shin, E., Bak, S.H., Park, T., Kim, J.W., Yoon, S.R., Jung, H., and Noh, J.Y. (2023). Understanding NK cell biology for harnessing NK cell therapies: targeting cancer and beyond. Frontiers in immunology *14*, 1192907. 10.3389/fimmu.2023.1192907.
18. Ames, E., Canter, R.J., Grossenbacher, S.K., Mac, S., Chen, M., Smith, R.C., Hagino, T., Perez-Cunningham, J., Sckisel, G.D., Urayama, S., Monjazeb, A.M., et al. (2015). NK Cells Preferentially Target Tumor Cells with a Cancer Stem Cell Phenotype. J Immunol *195*, 4010-4019. 10.4049/jimmunol.1500447.
19. Wang, J., Toregrosa-Allen, S., Elzey, B.D., Utturkar, S., Lanman, N.A., Bernal-Crespo, V., Behymer, M.M., Knipp, G.T., Yun, Y., Veronesi, M.C., Sinn, A.L., et al. (2021). 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 *118*. 10.1073/pnas.2107507118.
20. Klemm, F., Maas, R.R., Bowman, R.L., Kornete, M., Soukup, K., Nassiri, S., Brouland, J.P., Iacobuzio-Donahue, C.A., Brennan, C., Tabar, V., Gutin, P.H., et al. (2020). Interrogation of the Microenvironmental Landscape in Brain Tumors Reveals Disease-Specific Alterations of Immune Cells. Cell *181*, 1643-1660 e1617. 10.1016/j.cell.2020.05.007.
21. Wang, L., Jung, J., Babikir, H., Shamardani, K., Jain, S., Feng, X., Gupta, N., Rosi, S., Chang, S., Raleigh, D., Solomon, D., et al. (2022). A single-cell atlas of glioblastoma evolution under therapy reveals cell-intrinsic and cell-extrinsic therapeutic targets. Nature cancer *3*, 1534-1552. 10.1038/s43018-022-00475-x.
22. Zhang, L., Yu, H., Xue, Y., and Liu, Y. (2020). Decreased natural killer cells in diffuse intrinsic pontine glioma patients. Child's nervous system : ChNS : official journal of the International Society for Pediatric Neurosurgery *36*, 1345-1346. 10.1007/s00381-020-04665-9.
23. Savage, W.M., Yeary, M.D., Tang, A.J., Sperring, C.P., Argenziano, M.G., Adapa, A.R., Yoh, N., Canoll, P., and Bruce, J.N. (2024). Biomarkers of immunotherapy in glioblastoma. Neuro-oncology practice *11*, 383-394. 10.1093/nop/npae028.
24. Mumtaz, S.M., Bhardwaj, G., Goswami, S., Tonk, R.K., Goyal, R.K., Abu-Izneid, T., and Pottoo, F.H. (2021). Management of Glioblastoma Multiforme by Phytochemicals: Applications of Nanoparticle-Based Targeted Drug Delivery System. Current drug targets *22*, 429-442. 10.2174/1389450121666200727115454.
25. Wu, F., Chai, R.C., Wang, Z., Liu, Y.Q., Zhao, Z., Li, G.Z., and Jiang, H.Y. (2019). Molecular classification of IDH-mutant glioblastomas based on gene expression profiles. Carcinogenesis *40*, 853-860. 10.1093/carcin/bgz032.
26. Agnihotri, S., Aldape, K.D., and Zadeh, G. (2014). Isocitrate dehydrogenase status and molecular subclasses of glioma and glioblastoma. Neurosurgical focus *37*, E13. 10.3171/2014.9.FOCUS14505.
27. Gimple, R.C., Bhargava, S., Dixit, D., and Rich, J.N. (2019). Glioblastoma stem cells: lessons from the tumor hierarchy in a lethal cancer. Genes & development *33*, 591-609. 10.1101/gad.324301.119.
28. Neftel, C., Laffy, J., Filbin, M.G., Hara, T., Shore, M.E., Rahme, G.J., Richman, A.R., Silverbush, D., Shaw, M.L., Hebert, C.M., Dewitt, J., et al. (2019). An Integrative Model of Cellular States, Plasticity, and Genetics for Glioblastoma. Cell *178*, 835-849 e821. 10.1016/j.cell.2019.06.024.
29. Verhaak, R.G., Hoadley, K.A., Purdom, E., Wang, V., Qi, Y., Wilkerson, M.D., Miller, C.R., Ding, L., Golub, T., Mesirov, J.P., Alexe, G., et al. (2010). Integrated genomic analysis identifies clinically relevant subtypes of glioblastoma characterized by abnormalities in PDGFRA, IDH1, EGFR, and NF1. Cancer cell *17*, 98-110. 10.1016/j.ccr.2009.12.020.
30. Wang, Q., Hu, B., Hu, X., Kim, H., Squatrito, M., Scarpace, L., deCarvalho, A.C., Lyu, S., Li, P., Li, Y., Barthel, F., et al. (2017). Tumor Evolution of Glioma-Intrinsic Gene Expression

Subtypes Associates with Immunological Changes in the Microenvironment. Cancer cell *32*, 42-56 e46. 10.1016/j.ccell.2017.06.003.

31. Gisina, A., Kholodenko, I., Kim, Y., Abakumov, M., Lupatov, A., and Yarygin, K. (2022). Glioma Stem Cells: Novel Data Obtained by Single-Cell Sequencing. International journal of molecular sciences *23*. 10.3390/ijms232214224.
32. Patel, A.P., Tirosh, I., Trombetta, J.J., Shalek, A.K., Gillespie, S.M., Wakimoto, H., Cahill, D.P., Nahed, B.V., Curry, W.T., Martuza, R.L., Louis, D.N., et al. (2014). Single-cell RNA-seq highlights intratumoral heterogeneity in primary glioblastoma. Science *344*, 1396-1401. 10.1126/science.1254257.
33. Bao, S., Wu, Q., McLendon, R.E., Hao, Y., Shi, Q., Hjelmeland, A.B., Dewhirst, M.W., Bigner, D.D., and Rich, J.N. (2006). Glioma stem cells promote radioresistance by preferential activation of the DNA damage response. Nature *444*, 756-760. 10.1038/nature05236.
34. Cheng, L., Huang, Z., Zhou, W., Wu, Q., Donnola, S., Liu, J.K., Fang, X., Sloan, A.E., Mao, Y., Lathia, J.D., Min, W., et al. (2013). Glioblastoma stem cells generate vascular pericytes to support vessel function and tumor growth. Cell *153*, 139-152. 10.1016/j.cell.2013.02.021.
35. Lathia, J.D., Mack, S.C., Mulkearns-Hubert, E.E., Valentim, C.L., and Rich, J.N. (2015). Cancer stem cells in glioblastoma. Genes & development *29*, 1203-1217. 10.1101/gad.261982.115.
36. Ludwig, K., and Kornblum, H.I. (2017). Molecular markers in glioma. Journal of neuro-oncology *134*, 505-512. 10.1007/s11060-017-2379-y.
37. Brown, D.V., Filiz, G., Daniel, P.M., Hollande, F., Dworkin, S., Amiridis, S., Kountouri, N., Ng, W., Morokoff, A.P., and Mantamadiotis, T. (2017). Expression of CD133 and CD44 in glioblastoma stem cells correlates with cell proliferation, phenotype stability and intra-tumor heterogeneity. PloS one *12*, e0172791. 10.1371/journal.pone.0172791.
38. Hamerlik, P., Lathia, J.D., Rasmussen, R., Wu, Q., Bartkova, J., Lee, M., Moudry, P., Bartek, J., Jr., Fischer, W., Lukas, J., Rich, J.N., et al. (2012). Autocrine VEGF-VEGFR2-Neuropilin-1 signaling promotes glioma stem-like cell viability and tumor growth. The Journal of experimental medicine *209*, 507-520. 10.1084/jem.20111424.
39. Annovazzi, L., Mellai, M., Caldera, V., Valente, G., and Schiffer, D. (2011). SOX2 expression and amplification in gliomas and glioma cell lines. Cancer genomics & proteomics *8*, 139-147.
40. Alonso, M.M., Diez-Valle, R., Manterola, L., Rubio, A., Liu, D., Cortes-Santiago, N., Urquiza, L., Jauregi, P., Lopez de Munain, A., Sampron, N., Aramburu, A., et al. (2011). Genetic and epigenetic modifications of Sox2 contribute to the invasive phenotype of malignant gliomas. PloS one *6*, e26740. 10.1371/journal.pone.0026740.
41. Liu, K., Lin, B., Zhao, M., Yang, X., Chen, M., Gao, A., Liu, F., Que, J., and Lan, X. (2013). The multiple roles for Sox2 in stem cell maintenance and tumorigenesis. Cellular signalling *25*, 1264-1271. 10.1016/j.cellsig.2013.02.013.
42. Neradil, J., and Veselska, R. (2015). Nestin as a marker of cancer stem cells. Cancer science *106*, 803-811. 10.1111/cas.12691.
43. Jin, X., Jung, J.E., Beck, S., and Kim, H. (2013). Cell surface Nestin is a biomarker for glioma stem cells. Biochemical and biophysical research communications *433*, 496-501. 10.1016/j.bbrc.2013.03.021.
44. Mangiola, A., Lama, G., Giannitelli, C., De Bonis, P., Anile, C., Lauriola, L., La Torre, G., Sabatino, G., Maira, G., Jhanwar-Uniyal, M., and Sica, G. (2007). Stem cell marker nestin and c-Jun NH2-terminal kinases in tumor and peritumor areas of glioblastoma multiforme: possible prognostic implications. Clinical cancer research : an official journal of the American Association for Cancer Research *13*, 6970-6977. 10.1158/1078-0432.CCR-07-1229.
45. Wang, C., Wang, Z., Chen, C., Fu, X., Wang, J., Fei, X., Yan, X., and Xu, R. (2020). A low MW inhibitor of CD44 dimerization for the treatment of glioblastoma. British journal of pharmacology *177*, 3009-3023. 10.1111/bph.15030.
46. Rimkus, T.K., Carpenter, R.L., Sirkisoon, S., Zhu, D., Pasche, B.C., Chan, M.D., Lesser, G.J., Tatter, S.B., Watabe, K., Debinski, W., and Lo, H.W. (2018). Truncated Glioma-Associated Oncogene Homolog 1 (tGLI1) Mediates Mesenchymal Glioblastoma via Transcriptional Activation of CD44. Cancer research *78*, 2589-2600. 10.1158/0008-5472.CAN-17-2933.

47. Juric, V., Dussmann, H., Lamfers, M.L.M., Prehn, J.H.M., Rehm, M., and Murphy, B.M. (2021). Transcriptional CDK Inhibitors CYC065 and THZ1 Induce Apoptosis in Glioma Stem Cells Derived from Recurrent GBM. Cells *10*. 10.3390/cells10051182.
48. Cheng, P., Wang, J., Waghmare, I., Sartini, S., Coviello, V., Zhang, Z., Kim, S.H., Mohyeldin, A., Pavlyukov, M.S., Minata, M., Valentim, C.L., et al. (2016). FOXD1-ALDH1A3 Signaling Is a Determinant for the Self-Renewal and Tumorigenicity of Mesenchymal Glioma Stem Cells. Cancer research *76*, 7219-7230. 10.1158/0008-5472.CAN-15-2860.
49. Mao, P., Joshi, K., Li, J., Kim, S.H., Li, P., Santana-Santos, L., Luthra, S., Chandran, U.R., Benos, P.V., Smith, L., Wang, M., et al. (2013). Mesenchymal glioma stem cells are maintained by activated glycolytic metabolism involving aldehyde dehydrogenase 1A3. Proceedings of the National Academy of Sciences of the United States of America *110*, 8644-8649. 10.1073/pnas.1221478110.
50. Trepant, A.L., Bouchart, C., Rorive, S., Sauvage, S., Decaestecker, C., Demetter, P., and Salmon, I. (2015). Identification of OLIG2 as the most specific glioblastoma stem cell marker starting from comparative analysis of data from similar DNA chip microarray platforms. Tumour biology : the journal of the International Society for Oncodevelopmental Biology and Medicine *36*, 1943-1953. 10.1007/s13277-014-2800-5.
51. Bouchart, C., Trepant, A.L., Hein, M., Van Gestel, D., and Demetter, P. (2020). Prognostic impact of glioblastoma stem cell markers OLIG2 and CCND2. Cancer medicine *9*, 1069-1078. 10.1002/cam4.2592.
52. Wang, X., Yang, K., Wu, Q., Kim, L.J.Y., Morton, A.R., Gimple, R.C., Prager, B.C., Shi, Y., Zhou, W., Bhargava, S., Zhu, Z., et al. (2019). Targeting pyrimidine synthesis accentuates molecular therapy response in glioblastoma stem cells. Science translational medicine *11*. 10.1126/scitranslmed.aau4972.
53. Lu, F., Chen, Y., Zhao, C., Wang, H., He, D., Xu, L., Wang, J., He, X., Deng, Y., Lu, E.E., Liu, X., et al. (2016). Olig2-Dependent Reciprocal Shift in PDGF and EGF Receptor Signaling Regulates Tumor Phenotype and Mitotic Growth in Malignant Glioma. Cancer cell *29*, 669-683. 10.1016/j.ccell.2016.03.027.
54. Hoffman, L.M., Veldhuijzen van Zanten, S.E.M., Colditz, N., Baugh, J., Chaney, B., Hoffmann, M., Lane, A., Fuller, C., Miles, L., Hawkins, C., Bartels, U., et al. (2018). Clinical, Radiologic, Pathologic, and Molecular Characteristics of Long-Term Survivors of Diffuse Intrinsic Pontine Glioma (DIPG): A Collaborative Report From the International and European Society for Pediatric Oncology DIPG Registries. Journal of clinical oncology : official journal of the American Society of Clinical Oncology *36*, 1963-1972. 10.1200/JCO.2017.75.9308.
55. Vitanza, N.A., and Monje, M. (2019). Diffuse Intrinsic Pontine Glioma: From Diagnosis to Next-Generation Clinical Trials. Current treatment options in neurology *21*, 37. 10.1007/s11940-019-0577-y.
56. Aziz-Bose, R., and Monje, M. (2019). Diffuse intrinsic pontine glioma: molecular landscape and emerging therapeutic targets. Current opinion in oncology *31*, 522-530. 10.1097/CCO.0000000000000577.
57. van den Bent, M., Saratsis, A.M., Geurts, M., and Franceschi, E. (2024). H3 K27M-altered glioma and diffuse intrinsic pontine glioma: Semi-systematic review of treatment landscape and future directions. Neuro-oncology *26*, S110-S124. 10.1093/neuonc/noad220.
58. Kluiver, T.A., Alieva, M., van Vuurden, D.G., Wehrens, E.J., and Rios, A.C. (2020). Invaders Exposed: Understanding and Targeting Tumor Cell Invasion in Diffuse Intrinsic Pontine Glioma. Frontiers in oncology *10*, 92. 10.3389/fonc.2020.00092.
59. Thomas, B.C., Staudt, D.E., Douglas, A.M., Monje, M., Vitanza, N.A., and Dun, M.D. (2023). CAR T cell therapies for diffuse midline glioma. Trends in cancer *9*, 791-804. 10.1016/j.trecan.2023.07.007.
60. Hashizume, R. (2017). Epigenetic Targeted Therapy for Diffuse Intrinsic Pontine Glioma. Neurologia medico-chirurgica *57*, 331-342. 10.2176/nmc.ra.2017-0018.
61. Langmoen, I.A., Lundar, T., Storm-Mathisen, I., Lie, S.O., and Hovind, K.H. (1991). Management of pediatric pontine gliomas. Child's nervous system : ChNS : official journal of the International Society for Pediatric Neurosurgery *7*, 13-15. 10.1007/BF00263825.

62. Kim, H.J., and Suh, C.O. (2023). Radiotherapy for Diffuse Intrinsic Pontine Glioma: Insufficient but Indispensable. Brain tumor research and treatment *11*, 79-85. 10.14791/btrt.2022.0041.
63. Louis, D.N., Perry, A., Wesseling, P., Brat, D.J., Cree, I.A., Figarella-Branger, D., Hawkins, C., Ng, H.K., Pfister, S.M., Reifenberger, G., Soffietti, R., et al. (2021). The 2021 WHO Classification of Tumors of the Central Nervous System: a summary. Neuro-oncology *23*, 1231-1251. 10.1093/neuonc/noab106.
64. Miguel Llordes, G., Medina Perez, V.M., Curto Simon, B., Castells-Yus, I., Vazquez Sufuentes, S., and Schuhmacher, A.J. (2023). Epidemiology, Diagnostic Strategies, and Therapeutic Advances in Diffuse Midline Glioma. Journal of clinical medicine *12*. 10.3390/jcm12165261.
65. Liu, I., Jiang, L., Samuelsson, E.R., Marco Salas, S., Beck, A., Hack, O.A., Jeong, D., Shaw, M.L., Englinger, B., LaBelle, J., Mire, H.M., et al. (2022). The landscape of tumor cell states and spatial organization in H3-K27M mutant diffuse midline glioma across age and location. Nature genetics *54*, 1881-1894. 10.1038/s41588-022-01236-3.
66. Filbin, M.G., Tirosh, I., Hovestadt, V., Shaw, M.L., Escalante, L.E., Mathewson, N.D., Neftel, C., Frank, N., Pelton, K., Hebert, C.M., Haberler, C., et al. (2018). Developmental and oncogenic programs in H3K27M gliomas dissected by single-cell RNA-seq. Science *360*, 331-335. 10.1126/science.aao4750.
67. Da-Veiga, M.A., Coppieters, N., Lombard, A., Rogister, B., Neirinckx, V., and Piette, C. (2023). Comprehensive profiling of stem-like features in pediatric glioma cell cultures and their relation to the subventricular zone. Acta neuropathologica communications *11*, 96. 10.1186/s40478-023-01586-x.
68. Abou-Antoun, T.J., Hale, J.S., Lathia, J.D., and Dombrowski, S.M. (2017). Brain Cancer Stem Cells in Adults and Children: Cell Biology and Therapeutic Implications. Neurotherapeutics : the journal of the American Society for Experimental NeuroTherapeutics *14*, 372-384. 10.1007/s13311-017-0524-0.
69. Friedman, G.K., Raborn, J., Kelly, V.M., Cassady, K.A., Markert, J.M., and Gillespie, G.Y. (2013). Pediatric glioma stem cells: biologic strategies for oncolytic HSV virotherapy. Frontiers in oncology *3*, 28. 10.3389/fonc.2013.00028.
70. Vivier, E., Tomasello, E., Baratin, M., Walzer, T., and Ugolini, S. (2008). Functions of natural killer cells. Nature immunology *9*, 503-510. 10.1038/ni1582.
71. Abel, A.M., Yang, C., Thakar, M.S., and Malarkannan, S. (2018). Natural Killer Cells: Development, Maturation, and Clinical Utilization. Frontiers in immunology *9*, 1869. 10.3389/fimmu.2018.01869.
72. Sayegh, M., Ma, S., and Yu, J. (2023). Application of natural killer immunotherapy in blood cancers and solid tumors. Current opinion in oncology *35*, 446-452. 10.1097/CCO.0000000000000968.
73. Morvan, M.G., and Lanier, L.L. (2016). NK cells and cancer: you can teach innate cells new tricks. Nature reviews. Cancer *16*, 7-19. 10.1038/nrc.2015.5.
74. Rodriguez-Mogeda, C., van Ansenwoude, C.M.J., van der Molen, L., Strijbis, E.M.M., Mebius, R.E., and de Vries, H.E. (2024). The role of CD56(bright) NK cells in neurodegenerative disorders. Journal of neuroinflammation *21*, 48. 10.1186/s12974-024-03040-8.
75. Nielsen, N., Odum, N., Urso, B., Lanier, L.L., and Spee, P. (2012). Cytotoxicity of CD56(bright) NK cells towards autologous activated CD4+ T cells is mediated through NKG2D, LFA-1 and TRAIL and dampened via CD94/NKG2A. PloS one *7*, e31959. 10.1371/journal.pone.0031959.
76. Laroni, A., Armentani, E., Kerlero de Rosbo, N., Ivaldi, F., Marcenaro, E., Sivori, S., Gandhi, R., Weiner, H.L., Moretta, A., Mancardi, G.L., and Uccelli, A. (2016). Dysregulation of regulatory CD56(bright) NK cells/T cells interactions in multiple sclerosis. Journal of autoimmunity *72*, 8-18. 10.1016/j.jaut.2016.04.003.
77. Ljunggren, H.G., and Karre, K. (1990). In search of the 'missing self': MHC molecules and NK cell recognition. Immunology today *11*, 237-244. 10.1016/0167-5699(90)90097-s.
78. Karre, K. (2002). NK cells, MHC class I molecules and the missing self. Scandinavian journal of immunology *55*, 221-228. 10.1046/j.1365-3083.2002.01053.x.

79. Karre, K., Ljunggren, H.G., Piontek, G., and Kiessling, R. (1986). Selective rejection of H-2-deficient lymphoma variants suggests alternative immune defence strategy. Nature *319*, 675-678. 10.1038/319675a0.
80. Sivori, S., Vacca, P., Del Zotto, G., Munari, E., Mingari, M.C., and Moretta, L. (2019). Human NK cells: surface receptors, inhibitory checkpoints, and translational applications. Cellular & molecular immunology *16*, 430-441. 10.1038/s41423-019-0206-4.
81. Paul, S., and Lal, G. (2017). The Molecular Mechanism of Natural Killer Cells Function and Its Importance in Cancer Immunotherapy. Frontiers in immunology *8*, 1124. 10.3389/fimmu.2017.01124.
82. Neophytou, C.M., Panagi, M., Stylianopoulos, T., and Papageorgis, P. (2021). The Role of Tumor Microenvironment in Cancer Metastasis: Molecular Mechanisms and Therapeutic Opportunities. Cancers *13*. 10.3390/cancers13092053.
83. Elhanani, O., Ben-Uri, R., and Keren, L. (2023). Spatial profiling technologies illuminate the tumor microenvironment. Cancer cell *41*, 404-420. 10.1016/j.ccell.2023.01.010.
84. Hinshaw, D.C., and Shevde, L.A. (2019). The Tumor Microenvironment Innately Modulates Cancer Progression. Cancer research *79*, 4557-4566. 10.1158/0008-5472.CAN-18-3962.
85. Bayik, D., and Lathia, J.D. (2021). Cancer stem cell-immune cell crosstalk in tumour progression. Nature reviews. Cancer *21*, 526-536. 10.1038/s41568-021-00366-w.
86. van Niel, G., Carter, D.R.F., Clayton, A., Lambert, D.W., Raposo, G., and Vader, P. (2022). Challenges and directions in studying cell-cell communication by extracellular vesicles. Nature reviews. Molecular cell biology *23*, 369-382. 10.1038/s41580-022-00460-3.
87. Reale, A., Khong, T., and Spencer, A. (2022). Extracellular Vesicles and Their Roles in the Tumor Immune Microenvironment. Journal of clinical medicine *11*. 10.3390/jcm11236892.
88. Groth, C., Hu, X., Weber, R., Fleming, V., Altevogt, P., Utikal, J., and Umansky, V. (2019). Immunosuppression mediated by myeloid-derived suppressor cells (MDSCs) during tumour progression. British journal of cancer *120*, 16-25. 10.1038/s41416-018-0333-1.
89. Ma, T., Renz, B.W., Ilmer, M., Koch, D., Yang, Y., Werner, J., and Bazhin, A.V. (2022). Myeloid-Derived Suppressor Cells in Solid Tumors. Cells *11*. 10.3390/cells11020310.
90. Vignali, D.A., Collison, L.W., and Workman, C.J. (2008). How regulatory T cells work. Nature reviews. Immunology *8*, 523-532. 10.1038/nri2343.
91. Lee, K.Y., Mei, Y., Liu, H., and Schwarz, H. (2025). CD137-expressing regulatory T cells in cancer and autoimmune diseases. Molecular therapy : the journal of the American Society of Gene Therapy *33*, 51-70. 10.1016/j.ymthe.2024.12.010.
92. Kalluri, R., and LeBleu, V.S. (2020). The biology, function, and biomedical applications of exosomes. Science *367*. 10.1126/science.aau6977.
93. Zhang, M., Hu, S., Liu, L., Dang, P., Liu, Y., Sun, Z., Qiao, B., and Wang, C. (2023). Engineered exosomes from different sources for cancer-targeted therapy. Signal transduction and targeted therapy *8*, 124. 10.1038/s41392-023-01382-y.
94. Ruan, S., Greenberg, Z., Pan, X., Zhuang, P., Erwin, N., and He, M. (2022). Extracellular Vesicles as an Advanced Delivery Biomaterial for Precision Cancer Immunotherapy. Advanced healthcare materials *11*, e2100650. 10.1002/adhm.202100650.
95. Greening, D.W., Gopal, S.K., Xu, R., Simpson, R.J., and Chen, W. (2015). Exosomes and their roles in immune regulation and cancer. Seminars in cell & developmental biology *40*, 72-81. 10.1016/j.semcdb.2015.02.009.
96. Wen, C., Seeger, R.C., Fabbri, M., Wang, L., Wayne, A.S., and Jong, A.Y. (2017). Biological roles and potential applications of immune cell-derived extracellular vesicles. Journal of extracellular vesicles *6*, 1400370. 10.1080/20013078.2017.1400370.
97. Lu, Y., Zheng, J., Lin, P., Lin, Y., Zheng, Y., Mai, Z., Chen, X., Xia, T., Zhao, X., and Cui, L. (2024). Tumor Microenvironment-Derived Exosomes: A Double-Edged Sword for Advanced T Cell-Based Immunotherapy. ACS nano *18*, 27230-27260. 10.1021/acsnano.4c09190.
98. Lazarova, M., and Steinle, A. (2019). Impairment of NKG2D-Mediated Tumor Immunity by TGF-beta. Frontiers in immunology *10*, 2689. 10.3389/fimmu.2019.02689.
99. Lee, J.C., Lee, K.M., Kim, D.W., and Heo, D.S. (2004). Elevated TGF-beta1 secretion and down-modulation of NKG2D underlies impaired NK cytotoxicity in cancer patients. J Immunol *172*, 7335-7340. 10.4049/jimmunol.172.12.7335.

100. Wang, Z., Guo, L., Song, Y., Zhang, Y., Lin, D., Hu, B., Mei, Y., Sandikin, D., and Liu, H. (2017). Augmented anti-tumor activity of NK-92 cells expressing chimeric receptors of TGF-betaR II and NKG2D. Cancer immunology, immunotherapy : CII *66*, 537-548. 10.1007/s00262-017-1959-1.
101. Knudsen, N.H., and Manguso, R.T. (2020). Tumor-Derived PGE2 Gives NK Cells a Headache. Immunity *53*, 1131-1132. 10.1016/j.immuni.2020.11.018.
102. Leung, K.H. (1989). Inhibition of human NK cell and LAK cell cytotoxicity and differentiation by PGE2. Cellular immunology *123*, 384-395. 10.1016/0008-8749(89)90298-0.
103. Li, T., Yang, Y., Hua, X., Wang, G., Liu, W., Jia, C., Tai, Y., Zhang, Q., and Chen, G. (2012). Hepatocellular carcinoma-associated fibroblasts trigger NK cell dysfunction via PGE2 and IDO. Cancer letters *318*, 154-161. 10.1016/j.canlet.2011.12.020.
104. Teng, R., Wang, Y., Lv, N., Zhang, D., Williamson, R.A., Lei, L., Chen, P., Wang, B., Fu, J., Liu, X., He, A., et al. (2020). Hypoxia Impairs NK Cell Cytotoxicity through SHP-1-Mediated Attenuation of STAT3 and ERK Signaling Pathways. Journal of immunology research *2020*, 4598476. 10.1155/2020/4598476.
105. Solocinski, K., Padget, M.R., Fabian, K.P., Wolfson, B., Cecchi, F., Hembrough, T., Benz, S.C., Rabizadeh, S., Soon-Shiong, P., Schlom, J., and Hodge, J.W. (2020). Overcoming hypoxia-induced functional suppression of NK cells. Journal for immunotherapy of cancer *8*. 10.1136/jitc-2019-000246.
106. Klingemann, H. (2023). The NK-92 cell line-30 years later: its impact on natural killer cell research and treatment of cancer. Cytotherapy *25*, 451-457. 10.1016/j.jcyt.2022.12.003.
107. Suck, G., Odendahl, M., Nowakowska, P., Seidl, C., Wels, W.S., Klingemann, H.G., and Tonn, T. (2016). NK-92: an 'off-the-shelf therapeutic' for adoptive natural killer cell-based cancer immunotherapy. Cancer immunology, immunotherapy : CII *65*, 485-492. 10.1007/s00262-015-1761-x.
108. Stikvoort, A., van der Schans, J., Sarkar, S., Poels, R., Ruiter, R., Naik, J., Yuan, H., de Bruijn, J.D., van de Donk, N., Zweegman, S., Themeli, M., et al. (2021). CD38-specific Chimeric Antigen Receptor Expressing Natural Killer KHYG-1 Cells: A Proof of Concept for an "Off the Shelf" Therapy for Multiple Myeloma. HemaSphere *5*, e596. 10.1097/HS9.0000000000000596.
109. Suck, G., Branch, D.R., Smyth, M.J., Miller, R.G., Vergidis, J., Fahim, S., and Keating, A. (2005). KHYG-1, a model for the study of enhanced natural killer cell cytotoxicity. Experimental hematology *33*, 1160-1171. 10.1016/j.exphem.2005.06.024.
110. Choi, J.W., Lee, E.S., Kim, S.Y., Park, S.I., Oh, S., Kang, J.H., Ryu, H.A., and Lee, S. (2019). Cytotoxic effects of ex vivo-expanded natural killer cell-enriched lymphocytes (MYJ1633) against liver cancer. BMC cancer *19*, 817. 10.1186/s12885-019-6034-1.
111. Jiang, W., Zhang, C., Tian, Z., and Zhang, J. (2018). hIL-15-gene modified human natural killer cells (NKL-IL15) exhibit anti-human leukemia functions. Journal of cancer research and clinical oncology *144*, 1279-1288. 10.1007/s00432-018-2654-0.
112. Arias, J., Yu, J., Varshney, M., Inzunza, J., and Nalvarte, I. (2021). Hematopoietic stem cell-and induced pluripotent stem cell-derived CAR-NK cells as reliable cell-based therapy solutions. Stem cells translational medicine *10*, 987-995. 10.1002/sctm.20-0459.
113. Karagiannis, P., and Kim, S.I. (2021). iPSC-Derived Natural Killer Cells for Cancer Immunotherapy. Molecules and cells *44*, 541-548. 10.14348/molcells.2021.0078.
114. Xie, G., Dong, H., Liang, Y., Ham, J.D., Rizwan, R., and Chen, J. (2020). CAR-NK cells: A promising cellular immunotherapy for cancer. EBioMedicine *59*, 102975. 10.1016/j.ebiom.2020.102975.
115. Wang, W., Liu, Y., He, Z., Li, L., Liu, S., Jiang, M., Zhao, B., Deng, M., Mi, X., Sun, Z., and Ge, X. (2024). Breakthrough of solid tumor treatment: CAR-NK immunotherapy. Cell death discovery *10*, 40. 10.1038/s41420-024-01815-9.
116. Dagher, O.K., and Posey, A.D., Jr. (2023). Forks in the road for CAR T and CAR NK cell cancer therapies. Nature immunology *24*, 1994-2007. 10.1038/s41590-023-01659-y.
117. Wang, F., Wu, L., Yin, L., Shi, H., Gu, Y., and Xing, N. (2022). Combined treatment with anti-PSMA CAR NK-92 cell and anti-PD-L1 monoclonal antibody enhances the antitumour

efficacy against castration-resistant prostate cancer. Clinical and translational medicine *12*, e901. 10.1002/ctm2.901.

118. Zhong, Y., and Liu, J. (2024). Emerging roles of CAR-NK cell therapies in tumor immunotherapy: current status and future directions. Cell death discovery *10*, 318. 10.1038/s41420-024-02077-1.
119. Choe, H., and Ferrara, J.L.M. (2021). New therapeutic targets and biomarkers for acute graft-versus-host disease (GVHD). Expert opinion on therapeutic targets *25*, 761-771. 10.1080/14728222.2021.1992383.
120. Jiang, H., Fu, D., Bidgoli, A., and Paczesny, S. (2021). T Cell Subsets in Graft Versus Host Disease and Graft Versus Tumor. Frontiers in immunology *12*, 761448. 10.3389/fimmu.2021.761448.
121. Phung, S.K., Miller, J.S., and Felices, M. (2021). Bi-specific and Tri-specific NK Cell Engagers: The New Avenue of Targeted NK Cell Immunotherapy. Molecular diagnosis & therapy *25*, 577-592. 10.1007/s40291-021-00550-6.
122. Felices, M., Lenvik, T.R., Davis, Z.B., Miller, J.S., and Vallera, D.A. (2016). Generation of BiKEs and TriKEs to Improve NK Cell-Mediated Targeting of Tumor Cells. Methods Mol Biol *1441*, 333-346. 10.1007/978-1-4939-3684-7_28.
123. Demaria, O., Gauthier, L., Debroas, G., and Vivier, E. (2021). Natural killer cell engagers in cancer immunotherapy: Next generation of immuno-oncology treatments. European journal of immunology *51*, 1934-1942. 10.1002/eji.202048953.
124. Kiefer, A., Prufer, M., Roder, J., Pfeifer Serrahima, J., Bodden, M., Kuhnel, I., Oberoi, P., and Wels, W.S. (2024). Dual Targeting of Glioblastoma Cells with Bispecific Killer Cell Engagers Directed to EGFR and ErbB2 (HER2) Facilitates Effective Elimination by NKG2D-CAR-Engineered NK Cells. Cells *13*. 10.3390/cells13030246.
125. Pfeifer Serrahima, J., Schoenfeld, K., Kuhnel, I., Harwardt, J., Macarron Palacios, A., Prufer, M., Kolaric, M., Oberoi, P., Kolmar, H., and Wels, W.S. (2024). Bispecific killer cell engagers employing species cross-reactive NKG2D binders redirect human and murine lymphocytes to ErbB2/HER2-positive malignancies. Frontiers in immunology *15*, 1457887. 10.3389/fimmu.2024.1457887.
126. Klausz, K., Pekar, L., Boje, A.S., Gehlert, C.L., Krohn, S., Gupta, T., Xiao, Y., Krah, S., Zaynagetdinov, R., Lipinski, B., Toleikis, L., et al. (2022). Multifunctional NK Cell-Engaging Antibodies Targeting EGFR and NKp30 Elicit Efficient Tumor Cell Killing and Proinflammatory Cytokine Release. J Immunol *209*, 1724-1735. 10.4049/jimmunol.2100970.
127. Boje, A.S., Pekar, L., Koep, K., Lipinski, B., Rabinovich, B., Evers, A., Gehlert, C.L., Krohn, S., Xiao, Y., Krah, S., Zaynagetdinov, R., et al. (2024). Impact of antibody architecture and paratope valency on effector functions of bispecific NKp30 x EGFR natural killer cell engagers. mAbs *16*, 2315640. 10.1080/19420862.2024.2315640.
128. Demaria, O., Gauthier, L., Vetizou, M., Blanchard Alvarez, A., Vagne, C., Habif, G., Batista, L., Baron, W., Belaid, N., Girard-Madoux, M., Cesari, C., et al. (2022). Antitumor immunity induced by antibody-based natural killer cell engager therapeutics armed with not-alpha IL-2 variant. Cell reports. Medicine *3*, 100783. 10.1016/j.xcrm.2022.100783.
129. Gauthier, L., Virone-Oddos, A., Beninga, J., Rossi, B., Nicolazzi, C., Amara, C., Blanchard-Alvarez, A., Gourdin, N., Courta, J., Basset, A., Agnel, M., et al. (2023). Control of acute myeloid leukemia by a trifunctional NKp46-CD16a-NK cell engager targeting CD123. Nature biotechnology *41*, 1296-1306. 10.1038/s41587-022-01626-2.
130. Vallera, D.A., Oh, F., Kodal, B., Hinderlie, P., Geller, M.A., Miller, J.S., and Felices, M. (2021). A HER2 Tri-Specific NK Cell Engager Mediates Efficient Targeting of Human Ovarian Cancer. Cancers *13*. 10.3390/cancers13163994.
131. Sarhan, D., Brandt, L., Felices, M., Guldevall, K., Lenvik, T., Hinderlie, P., Curtsinger, J., Warlick, E., Spellman, S.R., Blazar, B.R., Weisdorf, D.J., et al. (2018). 161533 TriKE stimulates NK-cell function to overcome myeloid-derived suppressor cells in MDS. Blood advances *2*, 1459-1469. 10.1182/bloodadvances.2017012369.
132. Yun, H.D., Felices, M., Vallera, D.A., Hinderlie, P., Cooley, S., Arock, M., Gotlib, J., Ustun, C., and Miller, J.S. (2018). Trispecific killer engager CD16xIL15xCD33 potently induces NK

cell activation and cytotoxicity against neoplastic mast cells. Blood advances *2*, 1580-1584. 10.1182/bloodadvances.2018018176.

133. Batista, I.A., Quintas, S.T., and Melo, S.A. (2021). The Interplay of Exosomes and NK Cells in Cancer Biology. Cancers *13*. 10.3390/cancers13030473.

134. Hushmandi, K., Saadat, S.H., Raei, M., Aref, A.R., Reiter, R.J., Nabavi, N., Taheriazam, A., and Hashemi, M. (2024). The science of exosomes: Understanding their formation, capture, and role in cellular communication. Pathology, research and practice *259*, 155388. 10.1016/j.prp.2024.155388.

135. Enomoto, Y., Li, P., Jenkins, L.M., Anastasakis, D., Lyons, G.C., Hafner, M., and Leonard, W.J. (2022). Cytokine-enhanced cytolytic activity of exosomes from NK Cells. Cancer gene therapy *29*, 734-749. 10.1038/s41417-021-00352-2.

136. Jong, A.Y., Wu, C.H., Li, J., Sun, J., Fabbri, M., Wayne, A.S., and Seeger, R.C. (2017). Large-scale isolation and cytotoxicity of extracellular vesicles derived from activated human natural killer cells. Journal of extracellular vesicles *6*, 1294368. 10.1080/20013078.2017.1294368.

137. Lugini, L., Cecchetti, S., Huber, V., Luciani, F., Macchia, G., Spadaro, F., Paris, L., Abalsamo, L., Colone, M., Molinari, A., Podo, F., et al. (2012). Immune surveillance properties of human NK cell-derived exosomes. J Immunol *189*, 2833-2842. 10.4049/jimmunol.1101988.

138. Zhu, L., Kalimuthu, S., Gangadaran, P., Oh, J.M., Lee, H.W., Baek, S.H., Jeong, S.Y., Lee, S.W., Lee, J., and Ahn, B.C. (2017). Exosomes Derived From Natural Killer Cells Exert Therapeutic Effect in Melanoma. Theranostics *7*, 2732-2745. 10.7150/thno.18752.

139. Tao, B., Du, R., Zhang, X., Jia, B., Gao, Y., Zhao, Y., and Liu, Y. (2023). Engineering CAR-NK cell derived exosome disguised nano-bombs for enhanced HER2 positive breast cancer brain metastasis therapy. Journal of controlled release : official journal of the Controlled Release Society *363*, 692-706. 10.1016/j.jconrel.2023.10.007.

140. Breznik, B., Ko, M.W., Tse, C., Chen, P.C., Senjor, E., Majc, B., Habic, A., Angelillis, N., Novak, M., Zupunski, V., Mlakar, J., et al. (2022). Infiltrating natural killer cells bind, lyse and increase chemotherapy efficacy in glioblastoma stem-like tumorospheres. Communications biology *5*, 436. 10.1038/s42003-022-03402-z.

141. Shaim, H., Shanley, M., Basar, R., Daher, M., Gumin, J., Zamler, D.B., Uprety, N., Wang, F., Huang, Y., Gabrusiewicz, K., Miao, Q., et al. (2021). Targeting the alphav integrin/TGF-beta axis improves natural killer cell function against glioblastoma stem cells. The Journal of clinical investigation *131*. 10.1172/JCI142116.

142. Du, Y., Metcalfe, S., Akunapuram, S., Ghosh, S., Spruck, C., Richardson, A.M., Cohen-Gadol, A.A., and Shen, J. (2024). Image-based assessment of natural killer cell activity against glioblastoma stem cells. FEBS open bio *14*, 1028-1034. 10.1002/2211-5463.13818.

143. Guan, X., Lu, Y., Zhang, Y., Zhan, P., Chen, Z., Wang, C., and Yin, Z. (2023). Tumor-associated NK cells facilitate tumor growth via NKp46 in immunocompetent murine hepatocellular carcinoma. Immunology letters *258*, 8-19. 10.1016/j.imlet.2023.04.009.

144. Zhong, J., Yang, X., Chen, J., He, K., Gao, X., Wu, X., Zhang, M., Zhou, H., Xiao, F., An, L., Wang, X., et al. (2022). Circular EZH2-encoded EZH2-92aa mediates immune evasion in glioblastoma via inhibition of surface NKG2D ligands. Nature communications *13*, 4795. 10.1038/s41467-022-32311-2.

145. Fernandez-Sanchez, A., Baragano Raneros, A., Carvajal Palao, R., Sanz, A.B., Ortiz, A., Ortega, F., Suarez-Alvarez, B., and Lopez-Larrea, C. (2013). DNA demethylation and histone H3K9 acetylation determine the active transcription of the NKG2D gene in human CD8+ T and NK cells. Epigenetics *8*, 66-78. 10.4161/epi.23115.

146. Bugide, S., Green, M.R., and Wajapeyee, N. (2018). Inhibition of Enhancer of zeste homolog 2 (EZH2) induces natural killer cell-mediated eradication of hepatocellular carcinoma cells. Proceedings of the National Academy of Sciences of the United States of America *115*, E3509-E3518. 10.1073/pnas.1802691115.

147. Bugide, S., Gupta, R., Green, M.R., and Wajapeyee, N. (2021). EZH2 inhibits NK cell-mediated antitumor immunity by suppressing CXCL10 expression in an HDAC10-dependent manner. Proceedings of the National Academy of Sciences of the United States of America *118*. 10.1073/pnas.2102718118.

148. Kumar, R., and Gupta, R. (2024). Epigenetic regulation of NKG2D ligand and the rise of NK cell-based immunotherapy for cancer treatment. Frontiers in oncology *14*, 1456631. 10.3389/fonc.2024.1456631.
149. Mormino, A., Cocozza, G., Fontemaggi, G., Valente, S., Esposito, V., Santoro, A., Bernardini, G., Santoni, A., Fazi, F., Mai, A., Limatola, C., et al. (2021). Histone-deacetylase 8 drives the immune response and the growth of glioma. Glia *69*, 2682-2698. 10.1002/glia.24065.
150. Ni, F., Guo, C., Sun, R., Fu, B., Yang, Y., Wu, L., Ren, S., Tian, Z., and Wei, H. (2015). MicroRNA transcriptomes of distinct human NK cell populations identify miR-362-5p as an essential regulator of NK cell function. Scientific reports *5*, 9993. 10.1038/srep09993.
151. Liu, X., Wang, Y., Sun, Q., Yan, J., Huang, J., Zhu, S., and Yu, J. (2012). Identification of microRNA transcriptome involved in human natural killer cell activation. Immunology letters *143*, 208-217. 10.1016/j.imlet.2012.02.014.
152. Neviani, P., Wise, P.M., Murtadha, M., Liu, C.W., Wu, C.H., Jong, A.Y., Seeger, R.C., and Fabbri, M. (2019). Natural Killer-Derived Exosomal miR-186 Inhibits Neuroblastoma Growth and Immune Escape Mechanisms. Cancer research *79*, 1151-1164. 10.1158/0008-5472.CAN-18-0779.
153. Genssler, S., Burger, M.C., Zhang, C., Oelsner, S., Mildenberger, I., Wagner, M., Steinbach, J.P., and Wels, W.S. (2016). Dual targeting of glioblastoma with chimeric antigen receptor-engineered natural killer cells overcomes heterogeneity of target antigen expression and enhances antitumor activity and survival. Oncoimmunology *5*, e1119354. 10.1080/2162402X.2015.1119354.
154. Murakami, T., Nakazawa, T., Natsume, A., Nishimura, F., Nakamura, M., Matsuda, R., Omoto, K., Tanaka, Y., Shida, Y., Park, Y.S., Motoyama, Y., et al. (2018). Novel Human NK Cell Line Carrying CAR Targeting EGFRvIII Induces Antitumor Effects in Glioblastoma Cells. Anticancer research *38*, 5049-5056. 10.21873/anticanres.12824.
155. Mount, C.W., Majzner, R.G., Sundaresh, S., Arnold, E.P., Kadapakkam, M., Haile, S., Labanieh, L., Hulleman, E., Woo, P.J., Rietberg, S.P., Vogel, H., et al. (2018). Potent antitumor efficacy of anti-GD2 CAR T cells in H3-K27M(+) diffuse midline gliomas. Nature medicine *24*, 572-579. 10.1038/s41591-018-0006-x.
156. Muller, N., Michen, S., Tietze, S., Topfer, K., Schulte, A., Lamszus, K., Schmitz, M., Schackert, G., Pastan, I., and Temme, A. (2015). Engineering NK Cells Modified With an EGFRvIII-specific Chimeric Antigen Receptor to Overexpress CXCR4 Improves Immunotherapy of CXCL12/SDF-1alpha-secreting Glioblastoma. J Immunother *38*, 197-210. 10.1097/CJI.0000000000000082.
157. Zuo, P., Li, Y., He, C., Wang, T., Zheng, X., Liu, H., Wu, Z., Zhang, J., Liao, X., and Zhang, L. (2023). Anti-tumor efficacy of anti-GD2 CAR NK-92 cells in diffuse intrinsic pontine gliomas. Frontiers in immunology *14*, 1145706. 10.3389/fimmu.2023.1145706.
158. Woo, S.R., Oh, Y.T., An, J.Y., Kang, B.G., Nam, D.H., and Joo, K.M. (2015). Glioblastoma specific antigens, GD2 and CD90, are not involved in cancer stemness. Anatomy & cell biology *48*, 44-53. 10.5115/acb.2015.48.1.44.
159. Ma, R., Lu, T., Li, Z., Teng, K.Y., Mansour, A.G., Yu, M., Tian, L., Xu, B., Ma, S., Zhang, J., Barr, T., et al. (2021). An Oncolytic Virus Expressing IL15/IL15Ralpha Combined with Off-the-Shelf EGFR-CAR NK Cells Targets Glioblastoma. Cancer research *81*, 3635-3648. 10.1158/0008-5472.CAN-21-0035.
160. Berger, G., Knelson, E.H., Jimenez-Macias, J.L., Nowicki, M.O., Han, S., Panagioti, E., Lizotte, P.H., Adu-Berchie, K., Stafford, A., Dimitrakakis, N., Zhou, L., et al. (2022). STING activation promotes robust immune response and NK cell-mediated tumor regression in glioblastoma models. Proceedings of the National Academy of Sciences of the United States of America *119*, e2111003119. 10.1073/pnas.2111003119.
161. Baker, G.J., Chockley, P., Yadav, V.N., Doherty, R., Ritt, M., Sivaramakrishnan, S., Castro, M.G., and Lowenstein, P.R. (2014). Natural killer cells eradicate galectin-1-deficient glioma in the absence of adaptive immunity. Cancer research *74*, 5079-5090. 10.1158/0008-5472.CAN-14-1203.
162. Shah, D., Comba, A., Faisal, S.M., Kadiyala, P., Baker, G.J., Alghamri, M.S., Doherty, R., Zamler, D., Nunez, G., Castro, M.G., and Lowenstein, P.R. (2021). A novel miR1983-TLR7-

IFNbeta circuit licenses NK cells to kill glioma cells, and is under the control of galectin-1. Oncoimmunology *10*, 1939601. 10.1080/2162402X.2021.1939601.

163. Sharifzad, F., Mardpour, S., Fakharian, E., Taghikhani, A., Sharifzad, A., Kiani, S., Heydarian, Y., Los, M.J., Azizi, Z., Ghavami, S., Hamidieh, A.A., et al. (2020). HSP70/IL-2 Treated NK Cells Effectively Cross the Blood Brain Barrier and Target Tumor Cells in a Rat Model of Induced Glioblastoma Multiforme (GBM). International journal of molecular sciences *21*. 10.3390/ijms21072263.
164. Pawlowski, K.D., Duffy, J.T., Gottschalk, S., and Balyasnikova, I.V. (2023). Cytokine Modification of Adoptive Chimeric Antigen Receptor Immunotherapy for Glioblastoma. Cancers *15*. 10.3390/cancers15245852.
165. Woan, K.V., Kim, H., Bjordahl, R., Davis, Z.B., Gaidarova, S., Goulding, J., Hancock, B., Mahmood, S., Abujarour, R., Wang, H., Tuininga, K., et al. (2021). Harnessing features of adaptive NK cells to generate iPSC-derived NK cells for enhanced immunotherapy. Cell stem cell *28*, 2062-2075 e2065. 10.1016/j.stem.2021.08.013.
166. Chu, Y., Nayyar, G., Jiang, S., Rosenblum, J.M., Soon-Shiong, P., Safrit, J.T., Lee, D.A., and Cairo, M.S. (2021). Combinatorial immunotherapy of N-803 (IL-15 superagonist) and dinutuximab with ex vivo expanded natural killer cells significantly enhances in vitro cytotoxicity against GD2(+) pediatric solid tumors and in vivo survival of xenografted immunodeficient NSG mice. Journal for immunotherapy of cancer *9*. 10.1136/jitc-2020-002267.
167. Pabani, A., and Gainor, J.F. (2023). Facts and Hopes: Immunocytokines for Cancer Immunotherapy. Clinical cancer research : an official journal of the American Association for Cancer Research *29*, 3841-3849. 10.1158/1078-0432.CCR-22-1837.
168. Gout, D.Y., Groen, L.S., and van Egmond, M. (2022). The present and future of immunocytokines for cancer treatment. Cellular and molecular life sciences : CMLS *79*, 509. 10.1007/s00018-022-04514-9.
169. Olivo Pimentel, V., Marcus, D., van der Wiel, A.M., Lieuwes, N.G., Biemans, R., Lieverse, R.I., Neri, D., Theys, J., Yaromina, A., Dubois, L.J., and Lambin, P. (2021). Releasing the brakes of tumor immunity with anti-PD-L1 and pushing its accelerator with L19-IL2 cures poorly immunogenic tumors when combined with radiotherapy. Journal for immunotherapy of cancer *9*. 10.1136/jitc-2020-001764.
170. Lim, J., Park, Y., Ahn, J.W., Sim, J., Kang, S.J., Hwang, S., Chun, J., Choi, H., Kim, S.H., Chun, D.H., Sung, K.S., et al. (2021). Autologous adoptive immune-cell therapy elicited a durable response with enhanced immune reaction signatures in patients with recurrent glioblastoma: An open label, phase I/IIa trial. PloS one *16*, e0247293. 10.1371/journal.pone.0247293.
171. Kirkin, A.F., Dzhandzhugazyan, K.N., Guldberg, P., Fang, J.J., Andersen, R.S., Dahl, C., Mortensen, J., Lundby, T., Wagner, A., Law, I., Broholm, H., et al. (2018). Adoptive cancer immunotherapy using DNA-demethylated T helper cells as antigen-presenting cells. Nature communications *9*, 785. 10.1038/s41467-018-03217-9.
172. Paun, L., Moiraghi, A., Jannelli, G., Nouri, A., DiMeco, F., Pallud, J., Meling, T.R., Momjian, S., Schaller, K., Prada, F., and Migliorini, D. (2021). From Focused Ultrasound Tumor Ablation to Brain Blood Barrier Opening for High Grade Glioma: A Systematic Review. Cancers *13*. 10.3390/cancers13225614.
173. Bunevicius, A., McDannold, N.J., and Golby, A.J. (2020). Focused Ultrasound Strategies for Brain Tumor Therapy. Oper Neurosurg (Hagerstown) *19*, 9-18. 10.1093/ons/opz374.
174. Chen, K.T., Huang, C.Y., Pai, P.C., Yang, W.C., Tseng, C.K., Tsai, H.C., Li, J.C., Chuang, C.C., Hsu, P.W., Lee, C.C., Toh, C.H., et al. (2023). Focused ultrasound combined with radiotherapy for malignant brain tumor: a preclinical and clinical study. Journal of neuro-oncology *165*, 535-545. 10.1007/s11060-023-04517-x.
175. Wennerberg, E., Kremer, V., Childs, R., and Lundqvist, A. (2015). CXCL10-induced migration of adoptively transferred human natural killer cells toward solid tumors causes regression of tumor growth in vivo. Cancer immunology, immunotherapy : CII *64*, 225-235. 10.1007/s00262-014-1629-5.

176. Zang, J., Ye, J., Zhang, C., Sha, M., and Gao, J. (2019). Senescent hepatocytes enhance natural killer cell activity via the CXCL-10/CXCR3 axis. Experimental and therapeutic medicine *18*, 3845-3852. 10.3892/etm.2019.8037.

177. Walle, T., Kraske, J.A., Liao, B., Lenoir, B., Timke, C., von Bohlen Und Halbach, E., Tran, F., Griebel, P., Albrecht, D., Ahmed, A., Suarez-Carmona, M., et al. (2022). Radiotherapy orchestrates natural killer cell dependent antitumor immune responses through CXCL8. Science advances *8*, eabh4050. 10.1126/sciadv.abh4050.

178. Wang, J., Saung, M.T., Li, K., Fu, J., Fujiwara, K., Niu, N., Muth, S., Xu, Y., Rozich, N., Zlomke, H., Chen, S., et al. (2022). CCR2/CCR5 inhibitor permits the radiation-induced effector T cell infiltration in pancreatic adenocarcinoma. The Journal of experimental medicine *219*. 10.1084/jem.20211631.

179. Li, P.J., Lai, S.Z., Jin, T., Ying, H.J., Chen, Y.M., Zhang, P., Hang, Q.Q., Deng, H., Wang, L., Feng, J.G., Chen, X.Z., et al. (2023). Radiotherapy opens the blood-brain barrier and synergizes with anlotinib in treating glioblastoma. Radiotherapy and oncology : journal of the European Society for Therapeutic Radiology and Oncology *183*, 109633. 10.1016/j.radonc.2023.109633.

180. Chen, D., Le, S.B., Hutchinson, T.E., Calinescu, A.A., Sebastian, M., Jin, D., Liu, T., Ghiaseddin, A., Rahman, M., and Tran, D.D. (2022). Tumor Treating Fields dually activate STING and AIM2 inflammasomes to induce adjuvant immunity in glioblastoma. The Journal of clinical investigation *132*. 10.1172/JCI149258.

181. Hong, P., Kudulaiti, N., Wu, S., Nie, J., and Zhuang, D. (2022). Tumor treating fields: a comprehensive overview of the underlying molecular mechanism. Expert review of molecular diagnostics *22*, 19-28. 10.1080/14737159.2022.2017283.

182. Rezvani, K., Rouce, R., Liu, E., and Shpall, E. (2017). Engineering Natural Killer Cells for Cancer Immunotherapy. Molecular therapy : the journal of the American Society of Gene Therapy *25*, 1769-1781. 10.1016/j.ymthe.2017.06.012.

183. Wang, W., Erbe, A.K., Hank, J.A., Morris, Z.S., and Sondel, P.M. (2015). NK Cell-Mediated Antibody-Dependent Cellular Cytotoxicity in Cancer Immunotherapy. Frontiers in immunology *6*, 368. 10.3389/fimmu.2015.00368.

184. Park, J.E., Kim, S.E., Keam, B., Park, H.R., Kim, S., Kim, M., Kim, T.M., Doh, J., Kim, D.W., and Heo, D.S. (2020). Anti-tumor effects of NK cells and anti-PD-L1 antibody with antibody-dependent cellular cytotoxicity in PD-L1-positive cancer cell lines. Journal for immunotherapy of cancer *8*. 10.1136/jitc-2020-000873.

185. Chin, D.S., Lim, C.S.Y., Nordin, F., Arifin, N., and Jun, T.G. (2022). Antibody-Dependent Cell-Mediated Cytotoxicity Through Natural Killer (NK) Cells: Unlocking NK Cells for Future Immunotherapy. Current pharmaceutical biotechnology *23*, 552-578. 10.2174/1389201022666210820093608.

186. Chan, W.K., Kung Sutherland, M., Li, Y., Zalevsky, J., Schell, S., and Leung, W. (2012). Antibody-dependent cell-mediated cytotoxicity overcomes NK cell resistance in MLL-rearranged leukemia expressing inhibitory KIR ligands but not activating ligands. Clinical cancer research : an official journal of the American Association for Cancer Research *18*, 6296-6305. 10.1158/1078-0432.CCR-12-0668.

187. Weiss, T., Schneider, H., Silginer, M., Steinle, A., Pruschy, M., Polic, B., Weller, M., and Roth, P. (2018). NKG2D-Dependent Antitumor Effects of Chemotherapy and Radiotherapy against Glioblastoma. Clinical cancer research : an official journal of the American Association for Cancer Research *24*, 882-895. 10.1158/1078-0432.CCR-17-1766.

188. Silk, A.W., O'Day, S.J., Kaufman, H.L., Bryan, J., Norrell, J.T., Imbergamo, C., Portal, D., Zambrano-Acosta, E., Palmeri, M., Fein, S., Wu, C., et al. (2023). A phase 1b single-arm trial of intratumoral oncolytic virus V937 in combination with pembrolizumab in patients with advanced melanoma: results from the CAPRA study. Cancer immunology, immunotherapy : CII *72*, 1405-1415. 10.1007/s00262-022-03314-1.

189. Ma, R., Li, Z., Chiocca, E.A., Caligiuri, M.A., and Yu, J. (2023). The emerging field of oncolytic virus-based cancer immunotherapy. Trends in cancer *9*, 122-139. 10.1016/j.trecan.2022.10.003.

190. Janssens, S., Rennen, S., and Agostinis, P. (2024). Decoding immunogenic cell death from a dendritic cell perspective. Immunological reviews *321*, 350-370. 10.1111/imr.13301.

191. Khan, M., Arooj, S., and Wang, H. (2020). NK Cell-Based Immune Checkpoint Inhibition. Frontiers in immunology *11*, 167. 10.3389/fimmu.2020.00167.
192. Kim, N., and Kim, H.S. (2018). Targeting Checkpoint Receptors and Molecules for Therapeutic Modulation of Natural Killer Cells. Frontiers in immunology *9*, 2041. 10.3389/fimmu.2018.02041.
193. Wang, X., Byrne, M.E., Liu, C., Ma, M.T., and Liu, D. (2024). Scalable process development of NK and CAR-NK expansion in a closed bioreactor. Frontiers in immunology *15*, 1412378. 10.3389/fimmu.2024.1412378.
194. Dubrot, J., Du, P.P., Lane-Reticker, S.K., Kessler, E.A., Muscato, A.J., Mehta, A., Freeman, S.S., Allen, P.M., Olander, K.E., Ockerman, K.M., Wolfe, C.H., et al. (2022). In vivo CRISPR screens reveal the landscape of immune evasion pathways across cancer. Nature immunology *23*, 1495-1506. 10.1038/s41590-022-01315-x.
195. Hrbac, T., Kopkova, A., Siegl, F., Vecera, M., Ruckova, M., Kazda, T., Jancalek, R., Hendrych, M., Hermanova, M., Vybihal, V., Fadrus, P., et al. (2022). HLA-E and HLA-F Are Overexpressed in Glioblastoma and HLA-E Increased After Exposure to Ionizing Radiation. Cancer genomics & proteomics *19*, 151-162. 10.21873/cgp.20311.
196. Baginska, J., Viry, E., Paggetti, J., Medves, S., Berchem, G., Moussay, E., and Janji, B. (2013). The critical role of the tumor microenvironment in shaping natural killer cell-mediated anti-tumor immunity. Frontiers in immunology *4*, 490. 10.3389/fimmu.2013.00490.
197. Zhang, Y., Guo, F., and Wang, Y. (2024). Hypoxic tumor microenvironment: Destroyer of natural killer cell function. Chinese journal of cancer research = Chung-kuo yen cheng yen chiu *36*, 138-150. 10.21147/j.issn.1000-9604.2024.02.04.
198. Susek, K.H., Schwietzer, Y.A., Karvouni, M., Gilljam, M., Keszei, M., Hussain, A., Lund, J., Kashif, M., Lundqvist, A., Ljunggren, H.G., Nahi, H., et al. (2023). Generation of NK cells with chimeric-switch receptors to overcome PD1-mediated inhibition in cancer immunotherapy. Cancer immunology, immunotherapy : CII *72*, 1153-1167. 10.1007/s00262-022-03317-y.
199. Xie, Q.H., Zheng, J.Q., Ding, J.Y., Wu, Y.F., Liu, L., Yu, Z.L., and Chen, G. (2022). Exosome-Mediated Immunosuppression in Tumor Microenvironments. Cells *11*. 10.3390/cells11121946.
200. Guan, Z., Lan, H., Cai, X., Zhang, Y., Liang, A., and Li, J. (2021). Blood-Brain Barrier, Cell Junctions, and Tumor Microenvironment in Brain Metastases, the Biological Prospects and Dilemma in Therapies. Frontiers in cell and developmental biology *9*, 722917. 10.3389/fcell.2021.722917.
201. Tondepu, C., and Karumbaiah, L. (2022). Glycomaterials to Investigate the Functional Role of Aberrant Glycosylation in Glioblastoma. Advanced healthcare materials *11*, e2101956. 10.1002/adhm.202101956.
202. Liao, Y.M., Hung, T.H., Tung, J.K., Yu, J., Hsu, Y.L., Hung, J.T., and Yu, A.L. (2021). Low Expression of IL-15 and NKT in Tumor Microenvironment Predicts Poor Outcome of MYCN-Non-Amplified Neuroblastoma. Journal of personalized medicine *11*. 10.3390/jpm11020122.
203. Liu, R.B., Engels, B., Schreiber, K., Ciszewski, C., Schietinger, A., Schreiber, H., and Jabri, B. (2013). IL-15 in tumor microenvironment causes rejection of large established tumors by T cells in a noncognate T cell receptor-dependent manner. Proceedings of the National Academy of Sciences of the United States of America *110*, 8158-8163. 10.1073/pnas.1301022110.
204. Romee, R., Rosario, M., Berrien-Elliott, M.M., Wagner, J.A., Jewell, B.A., Schappe, T., Leong, J.W., Abdel-Latif, S., Schneider, S.E., Willey, S., Neal, C.C., et al. (2016). Cytokine-induced memory-like natural killer cells exhibit enhanced responses against myeloid leukemia. Science translational medicine *8*, 357ra123. 10.1126/scitranslmed.aaf2341.

List of Figure Captions

Figure 1. NK cell inhibitory and activating receptors. The inhibitory receptors on NK cells include CD94/NKG2A, TIGIT, and PD-1, while the activating receptors comprise NKG2D, NKp44, and NKp46, among others. TNF-related apoptosis-inducing ligand (TRAIL) and Fas ligand (FasL) on NK cells is also illustrated.

Figure 2. NK cell-based cancer therapies. This diagram showcases various NK cell-based immunotherapies, including NK cell engagers such as bispecific killer engager (BiKE) and trispecific killer engager (TriKE), which are designed to enhance NK cell targeting and activation. NK exosomes, depicted as extracellular vesicles released by NK cells, contain cytotoxic molecules. Chimeric antigen receptor (CAR)-NK cells are illustrated across different generations, with next-generation versions exhibiting enhanced features such as cytokine production and dual-targeting capabilities. Additionally, antibody-dependent cellular cytotoxicity (ADCC) highlights the role of NK cells in targeting antibody-coated cancer cells.

Figure 3. NK Cell Sources for Cancer Immunotherapy. This diagram presents the four primary sources of NK cells for immunotherapy. In the upper left, allogeneic NK cells are depicted, derived from peripheral blood (PB) or cord blood (CB), expanded in vitro, and then infused into the patient. The upper right shows autologous PB-NK cells, where the patient's own NK cells are collected from PB, expanded, and reinfused. The lower left illustrates NK cell lines, consisting of immortalized NK cells grown in culture for therapeutic application. The lower right highlights induced pluripotent stem cell (iPSCs)-derived NK cells for clinical use.

Figure 4. GSC-NK interactions in the TME of HGGs. Two key signaling pathways are highlighted, while others are either not depicted here or remain unidentified. In signaling 1, the downregulation of NKG2D ligands (NKG2D-L) on GSCs reduces NK cell recognition, resulting in diminished sensitivity to NK cell-mediated killing of GSCs. In signaling 2, GSCs enhance the secretion of TGF-β within the TME, which binds to TGF-β receptors on NK cells, inhibiting their cytotoxic activity against GSCs.

Table 1. NK cell receptors and their corresponding tumor cell ligands

NK cell receptors	Tumor cell ligands	Function
NKG2D	MICA/MICB, ULBP1-6	Activating
NKp30	B7-H6, PCNA	Activating
NKp46	Heparan sulfate	Activating
CD16 (FcγRIII)	Fc region of IgG	Activating, facilitates ADCC
DNAM-1	PVR, Nectin-2	Activating
2B4 (CD244)	CD48	Activating or inhibitory, depending on signaling partners
CD94/NKG2C	HLA-E	Activating
CD2	LFA-3	Activating
KIRs	HLA-C	Inhibitory (mostly)
CD94/NKG2A	HLA-E	Inhibitory
LILRs	HLA-G	Inhibitory
TIGIT	PVR, Nectin-2	Inhibitory
SIGLEC-7	Sialylated ligands	Inhibitory
PD-1	PD-L1, PD-L2	Inhibitory
ILT2 (lir-1)	HLA class I	Inhibitory
TRAIL	Death receptors DR4/DR5	Induces apoptosis in tumor cells
FasL	Fas	Induces apoptosis in tumor cells

Table 2. Mechanisms of tumor evasion from NK cell immunosurveillance

Mechanisms	Description	Examples/Key molecules
Interactions of inhibitory ligands and receptors[194,195]	Tumor cells overexpress ligands that bind NK cell inhibitory receptors, blocking NK activation	MHC class I molecules (e.g., HLA-E) bind NK cell inhibitory receptors like KIR and NKG2A HLA-E/NKG2A interaction is a key pathway for tumor evasion
Secretion of immunosuppressive cytokines[98-100]	Tumors secrete cytokines that suppress NK cell activation, proliferation, and function These cytokines create an immunosuppressive microenvironment	TGF-β reduces NK cell activating receptors (e.g., NKG2D) IL-10 inhibits NK cell cytokine production PGE2 diminishes NK cell cytotoxicity
Hypoxia[196,197]	Hypoxia in the TME impairs NK cell function	Hypoxia downregulates NK cell activating receptors (e.g., NKp30, NKp44, NKp46)
Downregulation of NK cell activating ligands[9,144]	Tumor cells downregulate or shed ligands that bind to NK cell activating receptors Soluble ligands can block NK cell function	NKG2D ligands (MICA, MICB, ULBP) are downregulated or shed in soluble forms (e.g., sMICA) This "decoy" mechanism neutralizes NK cell responses
Expression of checkpoint proteins[191,198]	Tumors upregulate immune checkpoint ligands that bind inhibitory receptors on NK cells This leads to immune exhaustion and reduced NK cell activity	Tumors express PD-L1, which binds to PD-1 on NK cells, reducing NK activity The PD-1/PD-L1 pathway is a key immune evasion mechanism
Exosomal suppression[199]	Tumor cells release exosomes containing immunosuppressive molecules that inhibit NK cell activity	Tumor-derived exosomes with TGF-β and NKG2D ligands suppress NK cells by reducing their activation and recognition of cancer cells
Physical barriers in the TME[200,201]	Tumors create physical barriers (e.g., fibrous tissue, ECM) that prevent NK cell infiltration	Fibrous matrix and BBB shield tumors, especially in brain cancers
Loss of NK cell activating cytokines[202-204]	Tumors decrease cytokine levels necessary for NK cell activation and survival	IL-15 and IL-12 levels are often reduced in the TME, impairing NK cell proliferation and cytotoxicity

1282 **Table 3. Preclinical strategies for enhancing NK cell-based therapies for HGGs**

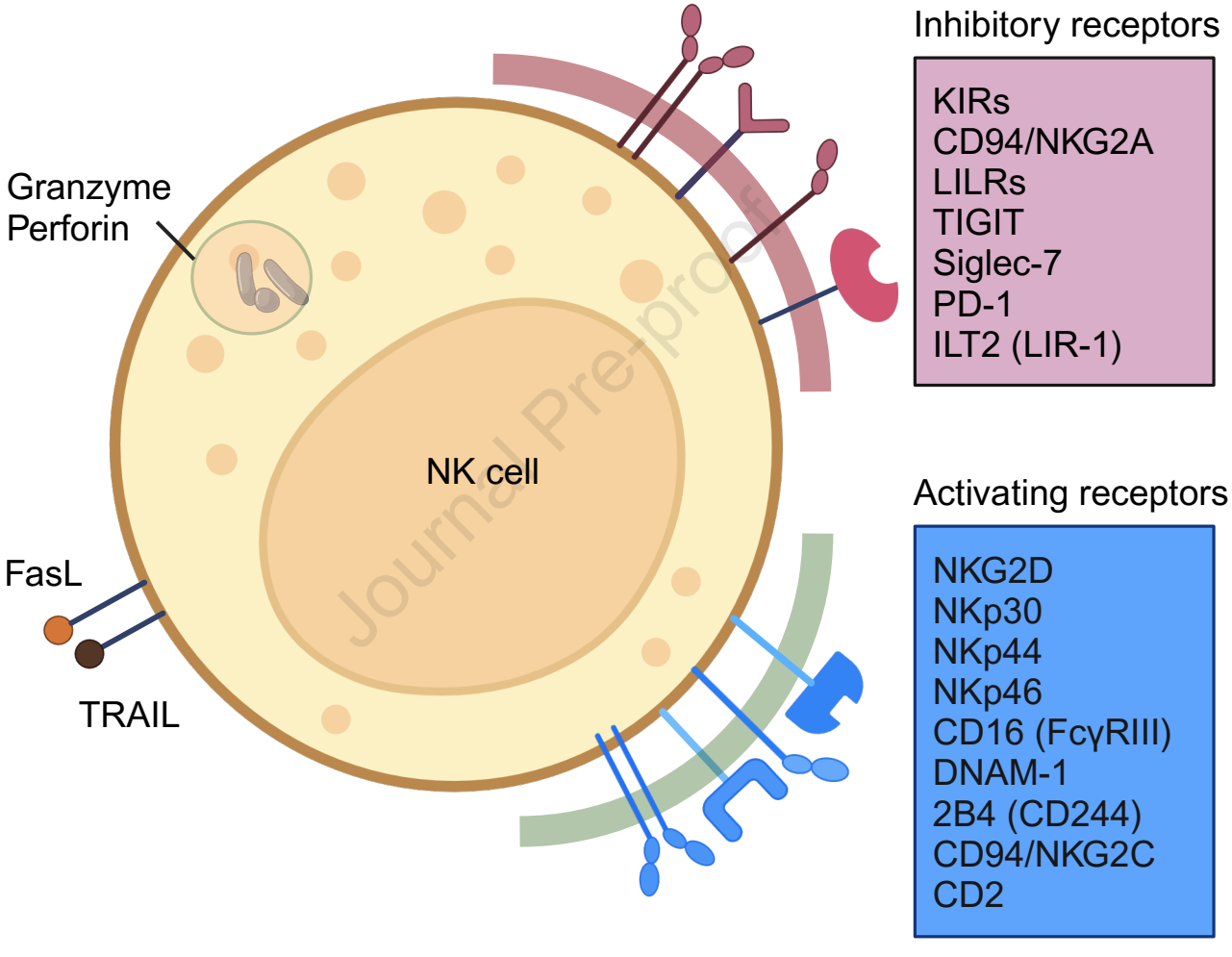
Strategies	Description	Key features	Application
CAR-NK cells	NK cells engineered with CAR	Target specific tumor antigens (e.g., EGFRvIII, GD2) Less toxicity than CAR-T cells CAR constructs with intracellular signaling domains	CAR-NK cells have shown promise in targeting glioma-specific antigens like EGFRvIII and GD2, both of which are overexpressed in GBM and DIPG Their reduced risk of cytokine release syndrome (CRS) makes them safer than CAR-T cells in glioma treatments, especially in pediatric patients with DIPG where safety is critical
NKCEs	BiKEs, or TriKEs that enhance NK cell activation	BiKEs: Two arms (one for tumor antigen, one for NK cell receptor) TriKEs: Three arms for enhanced NK activation	BiKEs and TriKEs can enhance NK cell-mediated cytotoxicity against GSCs, which are highly resistant to conventional therapies in GBM and DIPG TriKEs may engage NK cells with multiple tumor antigens or immune receptors, providing a multifaceted approach to overcoming HGG immune evasion Can be combined with radiotherapy to enhance tumor destruction
ADCC enhancement	Antibody-dependent cellular cytotoxicity via NK cell engagement	Maximize the effectiveness of NK cells in antibody-dependent tumor cell destruction	ADCC-enhancing monoclonal antibodies can increase NK cell-mediated killing of glioma cells that overexpress antigens such as CD133, a marker associated with GSCs ADCC therapies can be particularly useful when combined with immune checkpoint inhibitors to further increase NK cell activation in HGGs
Immunocytokines	Fusion proteins of cytokines and tumor-targeting antibodies	Combines cytokines (e.g., IL-2, IL-12) with antibodies Increased NK cell infiltration into the TME	Immunocytokines like IL-2 and IL-12 linked to tumor-targeting antibodies could enhance NK cell infiltration in the immunologically "cold" TME of HGGs Delivering immunocytokines directly to the tumor site may improve NK cell survival, proliferation, and cytotoxic activity

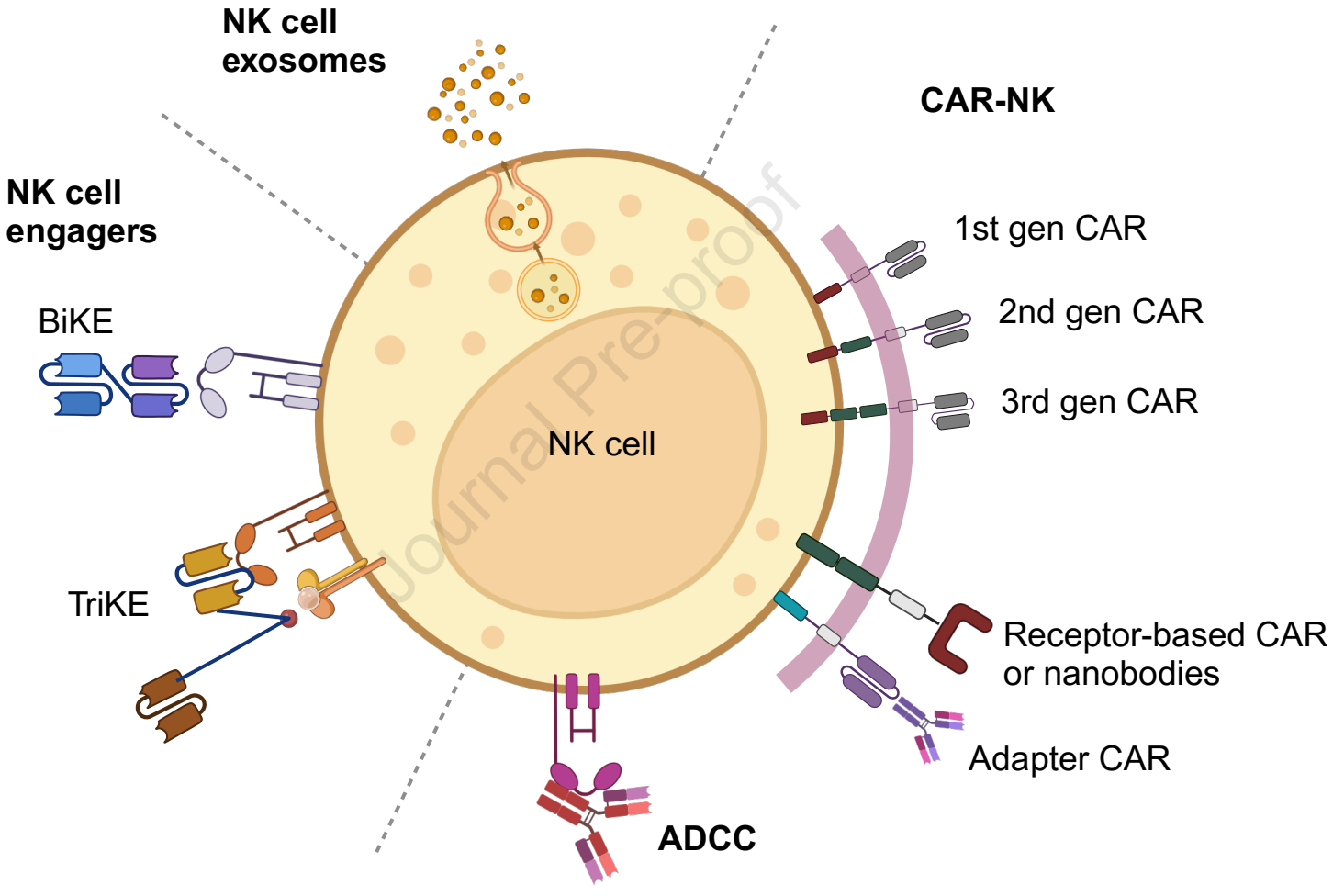
			Could synergize with NK cell engagers and CAR-NK cells for better outcomes
CRISPR/Cas9-edited NK cells	Genetically edited NK cells for enhanced functionality	CRISPR/Cas9 can be used Enhanced NK cell resistance to tumor suppression	Gene knockouts of inhibitory checkpoint molecules (e.g., PD-1) in NK cells makes them resistant to the immunosuppressive TME in HGGs This approach has the potential to restore NK cell function compromised by the TME through inhibition of the TGF-β pathway
NK cell-derived exosomes	Exosomes released by NK cells engineered for therapeutic purposes	Small vesicles loaded with proteins, genetic material Can be engineered to deliver therapeutic agents directly to tumor cells	NK cell-derived exosomes can carry cytotoxic proteins or RNA molecules targeting GSCs These exosomes can cross the BBB, making them an ideal candidate for targeting GSCs in HGGs Engineered NK exosomes can be loaded with molecules that inhibit HGG growth, such as pro-apoptotic factors or tumor-suppressive RNA

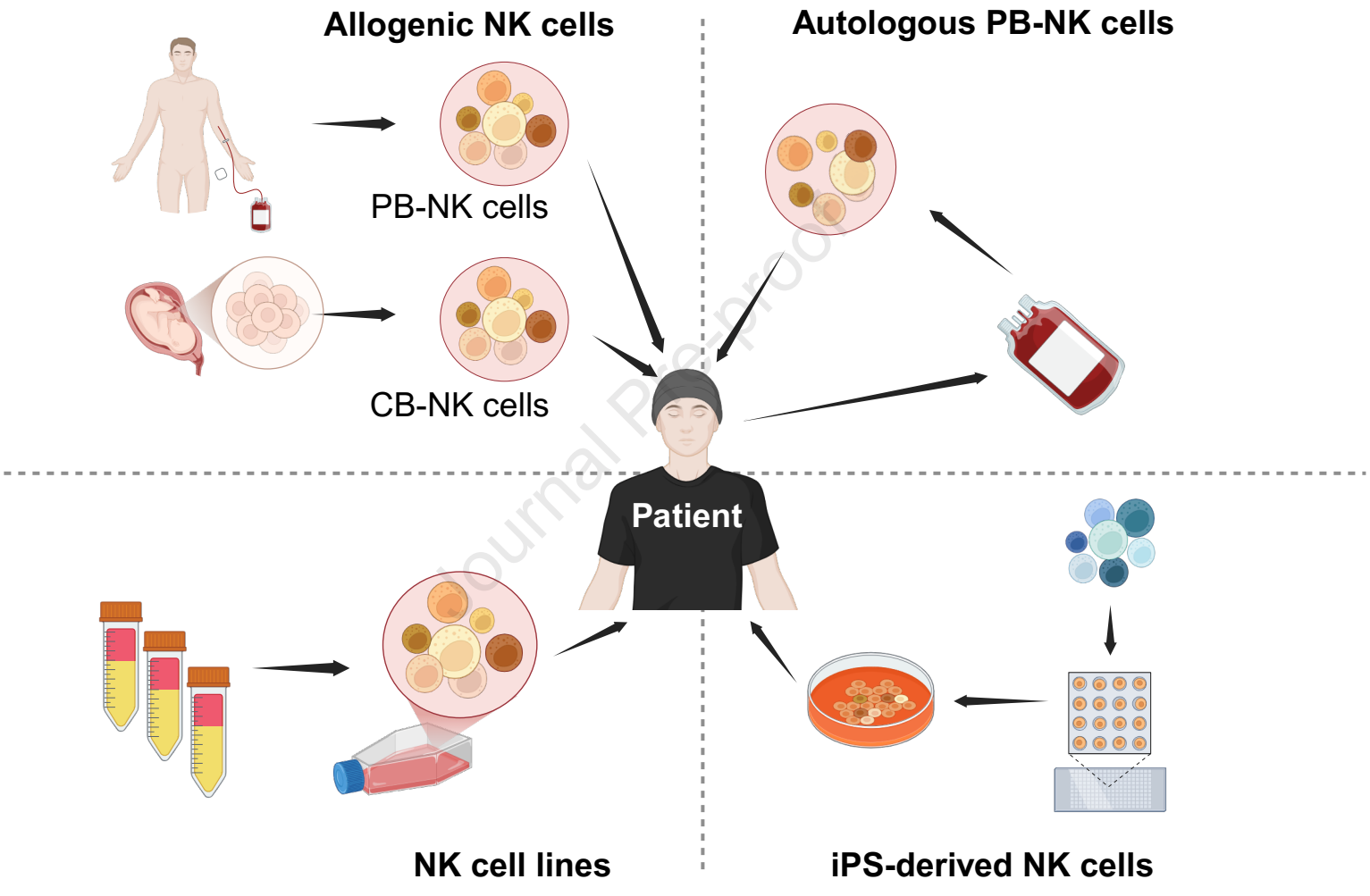
1300 **Table 4. Clinical trials of NK cell-based therapies for GBM**

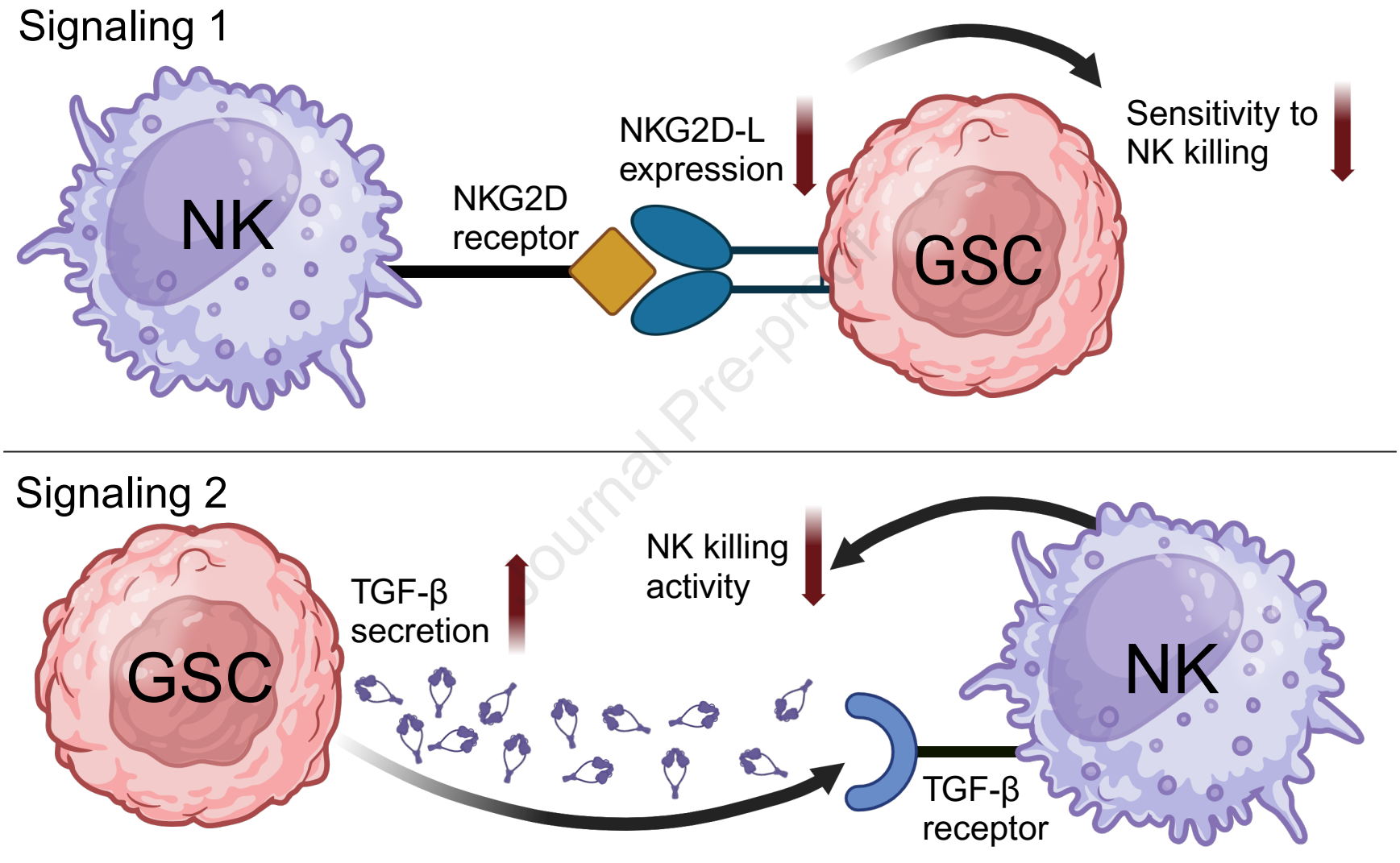
NCT #	Trials	Phase	Enrolled	Outcome	Ref
KCT0003815	A phase I/IIa clinical trial to investigate the safety and efficacy of adoptive, ex-vivo-expanded, and activated NK cells and T lymphocytes from peripheral blood mononuclear cells of patients with recurrent GBM	I/IIa	14	The median overall survival (OS) was 22.5 months, with a median progression-free survival of 10 months. 5 patients remained alive for over 2 years, demonstrating a durable response accompanied by enhanced immune-reaction transcriptomic signatures and no signs of clinical decline at their last follow-up after completing therapy	[170]
NCT01588769	A phase I study to investigate tolerability and efficacy of autologous lymphoid effector cells specific against tumour-cells (ALECSAT) administered to patients with GBM	I	25	Activated CD4+ T helper cells, following treatment with a DNA-demethylating agent, express a wide range of endogenous cancer/testis antigens, enabling them to function as antigen-presenting cells to generate autologous cytotoxic T lymphocytes and NK cells. These cells successfully targeted the tumor, leading to tumor regression in 3 patients, sustained for 14, 22, and 27 months, respectively	[171]
NCT03383978	Intracranial injection of NK-92/5.28.z cells in combination with intravenous ezabenlimab in patients with recurrent HER2-positive GBM	I	30	Not available yet	
NCT04991870	Engineered NK cells containing deleted TGF-betaR2 and NR3C1 for the treatment of recurrent GBM	I	25	Not available yet	
NCT06147505	NK cells (XS005) injection combined with stupp regimen for adjuvant chemotherapy in subjects with primary GBM	I/II	30	Not available yet	

1301









Shen and colleagues review the role of glioma stem cells in high-grade gliomas across adults and children, and highlight advancements and future strategies to enhance natural killer cell-based therapies in treating high-grade gliomas.