



NK Cell-Based Immunotherapy and Therapeutic Perspective in Gliomas

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Glioma is the most common malignant primary brain tumor diagnosed in adults. Current therapies are unable to improve its clinical prognosis, imposing the need for innovative therapeutic approaches. The main reason for the poor prognosis is the great cell heterogeneity of the tumor and its immunosuppressive microenvironment. Development of new therapies that avoid this immune evasion could improve the response to the current treatments. Natural killer (NK) cells are an intriguing candidate for the next wave of therapies because of several unique features that they possess. For example, NK cell-based immunotherapy causes minimal graft-versus-host disease. Cytokine release syndrome is less likely to occur during chimeric antigen receptor (CAR)-NK therapy, and CAR-NK cells can kill targets in a CAR-independent manner. However, NK cell-based therapy in treating glioma faces several difficulties. For example, CAR molecules are not sufficiently well designed so that they will thoroughly release functioning NK cells. Compared to hematological malignancies, the application of many potential NK cell-based therapies in glioma lags far behind. Here, we review several issues of NK cells and propose several strategies that will improve the efficacy of NK cell-based cancer immunotherapy in the treatment of glioma.

Keywords: natural killer cells, alloreactivity, chimeric antigen receptor, adoptive cell immunotherapy, glioma

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INTRODUCTION

Gliomas are the most common intracranial primary malignant tumor (1). The incidence of gliomas is approximately of six cases per 100,000 individuals worldwide. Glioblastoma (GBM), the most common glioma histology, has a 5-year relative survival of ~5%. While the majority of cases are sporadic, a small portion of these tumors are associated with neurofibromatosis type I, tuberous sclerosis, and Li-Fraumeni syndrome. Standard medical care, including the most extensive tumor resection followed by radiotherapy and chemotherapy. Surgery is commonly performed with both diagnostic and therapeutic intent. The therapeutic goal of surgery is to remove as much tumor tissue while preserving neurological function. Even for diffuse gliomas, a biopsy is recommended to acquire tissue specimens for molecular profiling (IDH mutations, 1p/19q codeletion, MGMT promoter methylation, EGFR amplification et al) (2). Most patients receive chemotherapy. Classic schemes including Stupp (NCT00006353) and PCV (Procarbazine, CCNU, and Vincristine) (3). The strategies of radiotherapy are determined by the disease subtype and prognostic factors, including residual tumor volume, age, KPS. The details of novel strategies

including tumor-treating fields (TTFields), checkpoint inhibitor, vaccine and oncolytic virus are described in **Table 1**. In general, the prognosis of high-grade glioma is still unpleasant, which calls for more efficient approaches.

Adoptive cell therapy (ACT), especially CAR-armed cell therapy, has great potential due to its high cytotoxicity and precise strikes. ACT consists of a series of infusions of autologous or allogeneic immune cells to kill targets, and T cell-based immunotherapy is an example of a mainstream form of ACT that is well-studied. Chimeric antigen receptor (CAR) T cells targeting CD19 is one therapy that has resulted in encouraging success in patients with B cell malignancies and has been approved by the US Food and Drug Administration (FDA) (5–7). However, there are numerous logistic and clinical limitations to the use of autologous CAR-modified T cells. Personalized CAR-T products are time-consuming and expensive to produce. Allogeneic T cell-based therapy can cause substantial toxic effects, such as graft-versus-host disease (GvHD) and cytokine release syndrome (CRS) (8). Furthermore, the results of CAR-T cell therapy for solid tumors are suboptimal. These shortcomings of CAR-T cells have called for interest in other candidate.

NK cells are a subpopulation of the innate immune system (9, 10). NK cells can be identified by CD3(-) CD56(+). Depending on the level of CD56 and CD16 expression, NK cells can be divided into CD16⁺CD56^{dim} and CD16⁻CD56^{bright} cells. CD16⁺CD56^{dim} NK cells predominate in peripheral blood while CD16⁻CD56^{bright} NK cells are distributed into secondary lymphoid organs (11). CD16⁻CD56^{bright} NK cells are robust cytokine producers and are weakly cytotoxic while the CD16⁺CD56^{dim} NK cell population can mediate serial killing of infected and/or malignant cells. NK cell receptors are germline-encoded without a requirement for ‘V(D)J’ recombination. Natural killer (NK) cells have gained attention as a promising alternative candidate for ACT owing to their unique biological attributes.

NK cells do not require any prior antigen and can rapidly recognize and kill cells for which major histocompatibility

complex (MHC) class I molecular expression is compromised by infection or transformation (9). Once activated, NK cells can release perforin and granzyme, contributing to target cell lysis. NK cells upregulate death ligands on their surface, such as FAS ligand and TRAIL, and initiate the caspase pathway of tumor cells and induce apoptosis when binding to death receptors on target cells. NK cells can eradicate cancer cells through antibody-dependent cellular cytotoxicity (ADCC) mediated by FcγRIIIA/CD16a. Furthermore, NK cells produce interferon gamma (IFN-γ), regulating and activating the adaptive immune response.

NK cell-based therapy is safe and has potential generated as off-the-shelf cellular therapy products. Autologous NK cells exert limited cytotoxicity against autologous tumors, while allogeneic NK cells are highly cytotoxic and cause minimal risk of GvHD (12–16). Thus, NK cells can originate from different sources, such as peripheral blood NK cells (PBNK), induced pluripotent stem cells (iPSCs), umbilical cord blood (UCB) and NK-92 cells, and this eliminates the need to produce a personalized CAR-NK product. However, the claim that allogeneic NK cells cause no or minimal GvHD and CRS is controversial and originated from observations obtained during clinical trials, especially in the setting of hematopoietic cell transplantation (HCT), the mechanism of which has not been thoroughly discussed. Here, we review important issues regarding NK cells and glioma and discuss several options that can be used to improve the efficacy of CAR-NK in glioma treatment.

THE SAFETY OF NK CELL-BASED IMMUNOTHERAPY

NK Cell Alloreactivity

All NK cells are non-responsive towards healthy autologous cells, which involves the interaction of at least inhibitory killer immunoglobulin-like receptors (KIRs) or CD94-NKG2A with one autologous MHC class I molecule. KIRs can be classified based on two factors: the number of immunoglobulin-like

TABLE 1 | Applications of novel strategies in glioma.

Strategies	Interventions	Results	Tumors	Phase	Reference/NCT
TTFields	TTFields plus temozolomide VS temozolomide alone	TTFields plus temozolomide improve PFS and OS significantly	Glioblastoma	III	NCT00916409
Checkpoint Inhibitor	nivolumab VS bevacizumab	Nivolumab failed to improve OS	Recurrent Glioblastoma	III	NCT02017717
	Neoadjuvant pembrolizumab	Neoadjuvant extended OS and enhanced both the local and systemic antitumor immune response	Recurrent Glioblastoma	/	Cloughesy et al. (4)
Vaccine	Rindopepimut (CDX-110), a vaccine targeting EGFRvIII	Rindopepimut did not increase OS	Glioblastoma	III	NCT01480479
	peptide vaccine (IDH1-vac) targeting mutant IDH1	IDH1-vac increased PFS and immune responses, but accompanied by a high frequency of pseudoprogression	Grade III and IV Astrocytomas	I	NCT02454634
	autologous dendritic cell vaccine ICT-107	ICT-107 significantly improved PFS	Glioblastoma	II	NCT01280552
Oncolytic Virus	intratumoral infusion of polio-rhinovirus chimera (PVSRIPO)	PVSRIPO therapy increased OS	Recurrent malignant Glioma	I	NCT01491893
	intratumoral injection of oncolytic adenovirus (DNX-2401)	DNX-2401 resulted in dramatic responses with long-term survival	Recurrent malignant Glioma	I	NCT00805376

domains (2D and 3D) and the length of the intracytoplasmic tail (L or S). Inhibitory KIRs usually possess a long cytoplasmic tail (KIR2DL), whereas activating KIR possess a short one (KIR2DS), except for the activating KIR2DL4, which has a long cytoplasmic tail. Inhibitory KIRs contain immunoreceptor tyrosine-based inhibition motif (ITIM) sequences responsible for the inhibitory signal. Unlike cytotoxic CD8+ T cells, which are highly specific for antigens, NK cells express clonally distributed inhibitory receptors termed KIRs that recognize determinants (KIR ligands) shared by subsets of HLA-B or -C allotypes (17–20). More than fifty KIR family members have been identified, and each of these genes is highly polymorphic and has thousands of alleles (21). Three subfamilies and associated inhibitory specificities are well determined (Table 2). The CD94-NKG2A heterodimer, belonging to C-type lectins, is specific for HLA-E (24–26).

Major models used to predict NK cell alloreactivity include ‘missing self’ and ‘missing ligand’ (Figure 1). Missing self-recognition (the ‘ligand–ligand’ model) was proposed by Karre

et al. and occurs under HLA haplotype-mismatched transplants in the graft-*versus*-host direction (27). Donor NK cells express a KIR for the self HLA class I group that is absent in the recipient, which mediates alloreactions (28–30). HLA testing is required to predict NK cell alloreactivity due to the missing self-model.

Because the genes for KIR, HLA, and CD94–NKG2 are located on different chromosomes (31–33), KIR genes segregate independently of the HLA genes, and thus, KIR mismatches can exist in two HLA-matched individuals. Also, it was found that many individuals have 3 inhibitory KIRs (for HLA-C1 and -C2 and for HLA-Bw4 alleles), while their own cells only express 1 or 2 HLA KIR ligands (22, 34, 35). KIR expression is donor specific, but not related to the donor or recipient HLA and is not affected by the recipient’s HLA groups (36). The missing ligand model (the ‘receptor–ligand’ model) was based on these. According to this model, NK cell alloreactivity occurs not only in HLA haplotype-mismatched transplants, but also in HLA haplotype-matched transplants from donors possessing ‘extra’ KIR(s), for which neither donor nor recipient possess HLA ligand(s) (35–37). The donor’s potentially self-reactive NK cells can trigger an alloreactive effect in the recipient while maintaining anergy in the donor. Analysis of the KIR expression on the donor’s NK cells and HLA testing of the recipient’s cells are required to predict NK cell alloreactivity due to the missing ligand model.

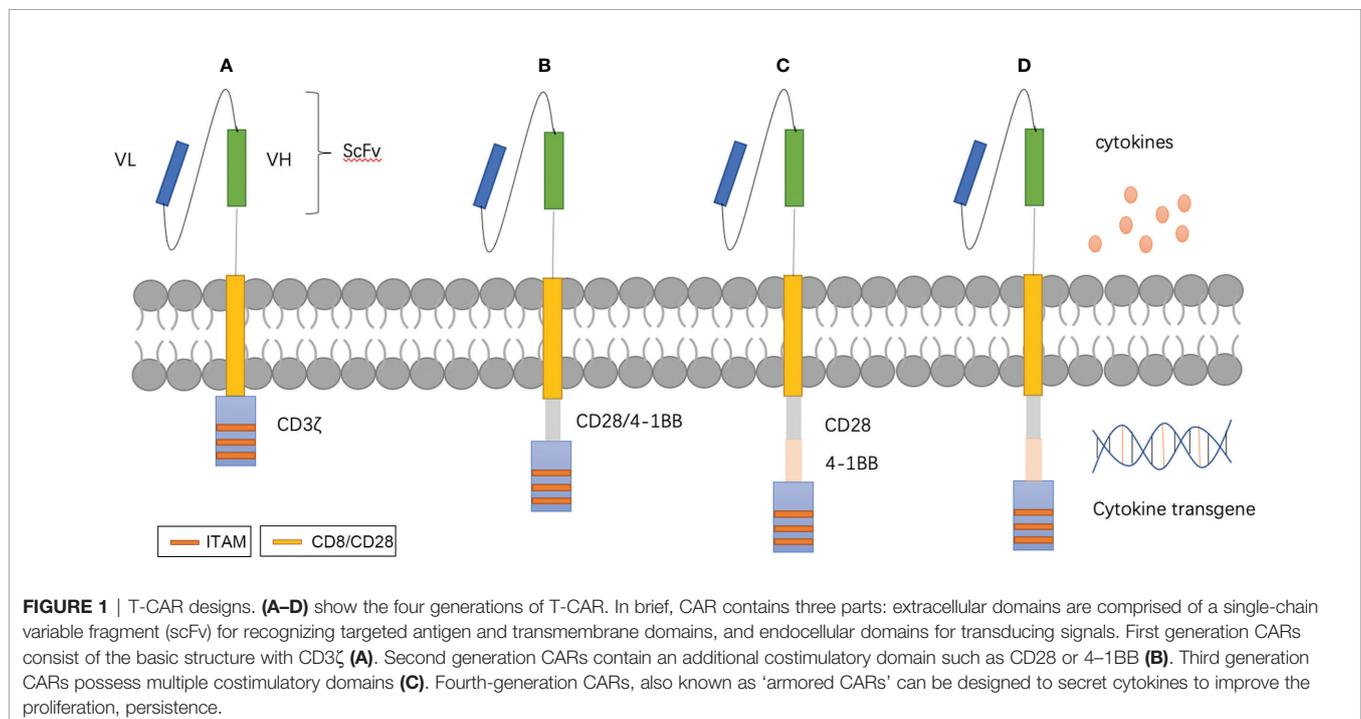
The missing self and missing ligand models can be used to predict NK cell alloreactivity. Although alloreactive NK cells can eradicate tumor cells, the anti-tumor effect is not confined to alloreactive NK cells. NK cell activity depends on the balance between inhibitory and stimulatory receptors. An anti-tumor effect can be mediated by NK cells expressing stimulatory receptors, such as activating KIR and NKG2D (38–40).

TABLE 2 | Three subfamilies of KIRs and specific ligands.

Group ^a	HLA-class I specificity ^b
KIR2DL1(CD158a)	C2(-Cw2, -Cw4, -Cw5, -Cw6)
KIR2DL2/3(CD158b1/b2)	C1(-Cw1, -Cw3, -Cw7, -Cw8)
KIR3DL1(CD158e1)	Bw4(-B27, -B51)

^aEach group comprises different numbers of alleles, which differ by 1–9 nucleotide substitutions (22).

^bTwo groups of HLA-C alleles are distinguished by dimorphic positions Ser 77–Asn 80 (C1) and Asn 77–Lys 80 (C2) of the $\alpha 1$ helix (23). HLA-B allotypes share the Bw4 sequence motif at positions 77–83 of the $\alpha 1$ helix (22).



NK CELL-BASED IMMUNOTHERAPY CAUSES MINIMAL GvHD AND CRS

GvHD refers to a condition resulting from the systemic attack of allogeneic T cells on recipient tissues after allogeneic hematopoietic stem cell transplantation or infusion of allogeneic T cells (41–43). The effects of GvHD are commonly manifested in the gastrointestinal tract, liver, and skin (44), and severe GvHD can be fatal. The role of alloreactive NK cells on GvHD in the setting of HCT varies among studies. Some investigations found that alloreactive NK cells were related to decreased GvHD (36, 45, 46), which was partially attributed to the observations that allogeneic NK cells would be expected to kill host dendritic cells (DCs) and donor T cells (46, 47). Miller et al. analyzed 2,062 patients undergoing unrelated donor HCT (48). They found that one or more KIR ligands were missing *versus* the presence of all ligands, which is associated with a low relapse rate in patients with early myeloid leukemia. This omission predicted a greater risk of developing grade 3–4 GvHD in the setting of chronic myeloid leukemia (CML) patients. Miller et al. attributed the higher rate of acute GvHD in CML to the expanded myeloid pool with more host antigen-presenting cells (APCs) capable of presenting alloantigen to donor T cells (48).

HCT after ablation of bone marrow is used to cure hematological malignancies and results in less cancer relapse compared to chemoradiotherapy (49). T cells of allogeneic hematopoietic grafts for treating leukemia mediate the antileukemia effect as well as lethal GvHD. In many studies, it was attempted to prevent GvHD by depleting the T cells from the graft and infusing large numbers of hematopoietic stem cells to overcome rejection (50), which was at the expense of immunity reconstitution failure and infection. Later, NK cells from alloreactive donors were found to protect patients against rejection and GvHD in the setting of HCT (46). Interestingly, we found the idea that NK cell-based therapy caused GvHD mostly happened in the setting of HCT. But we should not evaluate the effects of alloreactive NK cells on GvHD in the setting of HCT because the effect of T cells in the grafts is negligible. It is likely that T cell interference is the most important controversial element with respect to the alloreactive NK cell effects on GvHD.

In fact, NK cell-based immunotherapy is safe and causes minimal GvHD. GvHD most likely occurs when NK cells from donors with several KIR subfamilies are infused into recipients possessing one group HLA ligand. Valiante et al. analyzed NK cell receptor repertoires in the peripheral blood of two human donors (donor PP only possessed group 1 HLA-C ligand, and donor NV possessed group 1 and 2 HLA-C ligands and the Bw4 HLA-B ligand, both of which have three KIR subfamilies as demonstrated in **Table 2**) (51). They found that more than 98% of NK clones were inhibited self-HLA class I allotypes, and no NK cell from either donor was able to lyse the autologous B cell line (51). Interestingly, NV possessed approximately 15% of the analyzed NK cell clones, did not express KIR2DL2 or CD94: NKG2a, and was able to lyse the B cell line from PP, whereas the

NK cell clones from PP failed to lyse the B cell line from NV (51). Ruggeri adopted functional analysis to evaluate the NK cell alloreactivity in more than 200 NK clones (46). Alloreactivity was defined as positive when the frequency of lytic clones was no less than 1 in 50 (46). In addition, the expression of CD94: NKG2a is inversely related to KIR levels (51). Approximately, 50% of NK cells in an individual express CD94: NKG2a (51, 52). Cell-surface HLA-E expression depends on many peptides, including the leader peptides of HLA-A, -B, or -C, and downregulation of HLA-E expression requires the elimination of three types of HLA molecules (53, 54). Thus, NK cells expressing CD94–NKG2A display no alloreactivity because all individuals express HLA-E molecules. Therefore, NK cell-based immunotherapy is safe most of the time and will cause minimal GvHD because alloreactive NK cells only account for a small proportion. In addition, healthy cells express high levels of MHC class I molecules, but they express no or minimal level of ligands for NK cell activating receptors. Conversely, tumorigenic cells downregulate MHC class I expression but upregulate the expression of ligands for NK cell activating receptors. For example, MICA/MICB and ULBP, ligands for NKG2D, are often induced by stress or transformation (55, 56). The integration of the activating and inhibitory signals from the ligand/receptor determines NK cell activity. Some studies indicated that the positive signal delivered by NKG2D could override inhibition. Therefore, NK cells become alloreactive prior to killing tumor cells.

CRS involves elevated levels of circulating cytokines, especially interferons and immune-cell hyperactivation, which manifests as an influenza-like syndrome, organ failure, and even death (57). CAR-NK is less likely to induce CRS and neurotoxicity partially because of a different spectrum of secreted cytokines consisting of activated NK cells that produce IFN- γ and GM-CSF, and CAR-T cells that predominantly release tumor necrosis factor (TNF)- α and interleukins, such as IL-1, IL-2, and IL-6 (57, 58). The mechanism was validated by clinical trials. Liu et al. launched a clinical trial (NCT03056339) that administered HLA-mismatched anti-CD19 CAR-NK cells to 11 patients with high-risk lymphoid malignancies (16). The administration of CAR-NK cells was not associated with the development of cytokine release syndrome and there was no increase in the levels of inflammatory cytokines, including interleukin-6, over baseline (16).

NK EXPANSION TECHNIQUES

Large numbers of cells are essential for successful adoptive transfer cell therapy. It has been proved that high doses of NK cells from 10^7 cells/kg to 4.7×10^{10} total NK cells can be well tolerated (16, 59, 60). NK cells only account for approximately 10% of peripheral blood mononuclear cells. The NK92 cell line is used in current clinical trials with CAR-NK because of their unlimited proliferation ability *in vitro*. However, the NK92 cell line is tumorigenic and lacks CD16 and NKp44 expression, and additionally, these cells will lose their proliferation ability due to

lethal irradiation infusion (61, 62). Because of these drawbacks, it is unlikely that they will be an ideal cell source for CAR-NK cell therapy.

Expansion protocols possess considerable heterogeneity. The expansion process often takes 2-3 weeks of culture in the presence of mitogenic cytokines, and engineered feeder cells can optimize the expansion process. K562 cells engineered to express membrane-bound IL-15 or IL-21 along with the adhesion molecule 4-1BBL are adopted as feeder cells in many clinical trials (63, 64). There have been reports of 300-fold expansions combined with IL-2 and IL-15. IL-15 is important to NK cell survival and function (65, 66), and 4-1BBL provides a cell to cell contact-dependent co-stimulatory signal (67). We observed that low density less than 10^5 cells/mL is not ideal for NK expansion. We recommend that the ratio of engineered K562 feeder cells and NK cells is 1:1 to 2:1. The K562 cell line possesses unique properties and lacks HLA expression. As described above, inhibitory KIRs recognizing corresponding HLA ligands can inactivate NK cells. This can be proved by our laboratory findings that other cell lines engineered to express IL-21 and 4-1BBL failed to achieve high-fold NK expansion. Some studies used RetroNectin-stimulated T (RN-T) cells as feeder cells (68, 69). Briefly, the procedure for this is to culture T cells from autologous peripheral blood mononuclear cells (PBMCs) with RetroNectin and anti-CD3 monoclonal antibody. In approximately 2 weeks, RN-T cells can serve as feeder cells after irradiation. RetroNectin plays a role in cell adhesion (70). The anti-CD3 monoclonal antibody leads to T-cell activation and cytokine secretion, and activated T cells express ligands of NKG2D (47, 71). Irradiated PBMCs as feeder cells were utilized by Parkhurst et al. and share a similar mechanism with RN-T in NK cell expansion (60, 72). The CD14⁺ monocyte fraction of irradiated PBMCs function in cell-cell contact (73). Feeder cells derived from autologous PBMCs eliminate the need for infusion of viable malignant feeder cells into the NK cell product. Feeder-free expansion approaches have also been tested. Li et al. cultured NK cells in an anti-CD16 (Beckman Coulter)-coated flask (74). Antibody-coated beads targeting CD2 and NKp46 (CD335) are commercially available. However, not everyone or each NK cell expresses these stimulatory receptors, and feeder-free expansion protocols lose cell-to-cell contact effects. For example, only a part of NK cells of an individual expresses CD16 on blood NK cells (75, 76).

ADOPTIVE CELL THERAPY AND CAR-NK CELLS

There are several developmental stages in ACT. Lymphokine-activated killer (LAK) cells, which consist of a mixture of NK cells, NKT cells, and T cells, were adopted to overcome insufficient quantities of immune cells (77). An unwanted side effect of a high dose of IL-2 is that it induces capillary leak syndrome and neuropsychiatric diseases in a manner similar to that of CRS. To obtain immune cells that can effectively respond to tumors, Rosenberg introduced the concept of tumor-

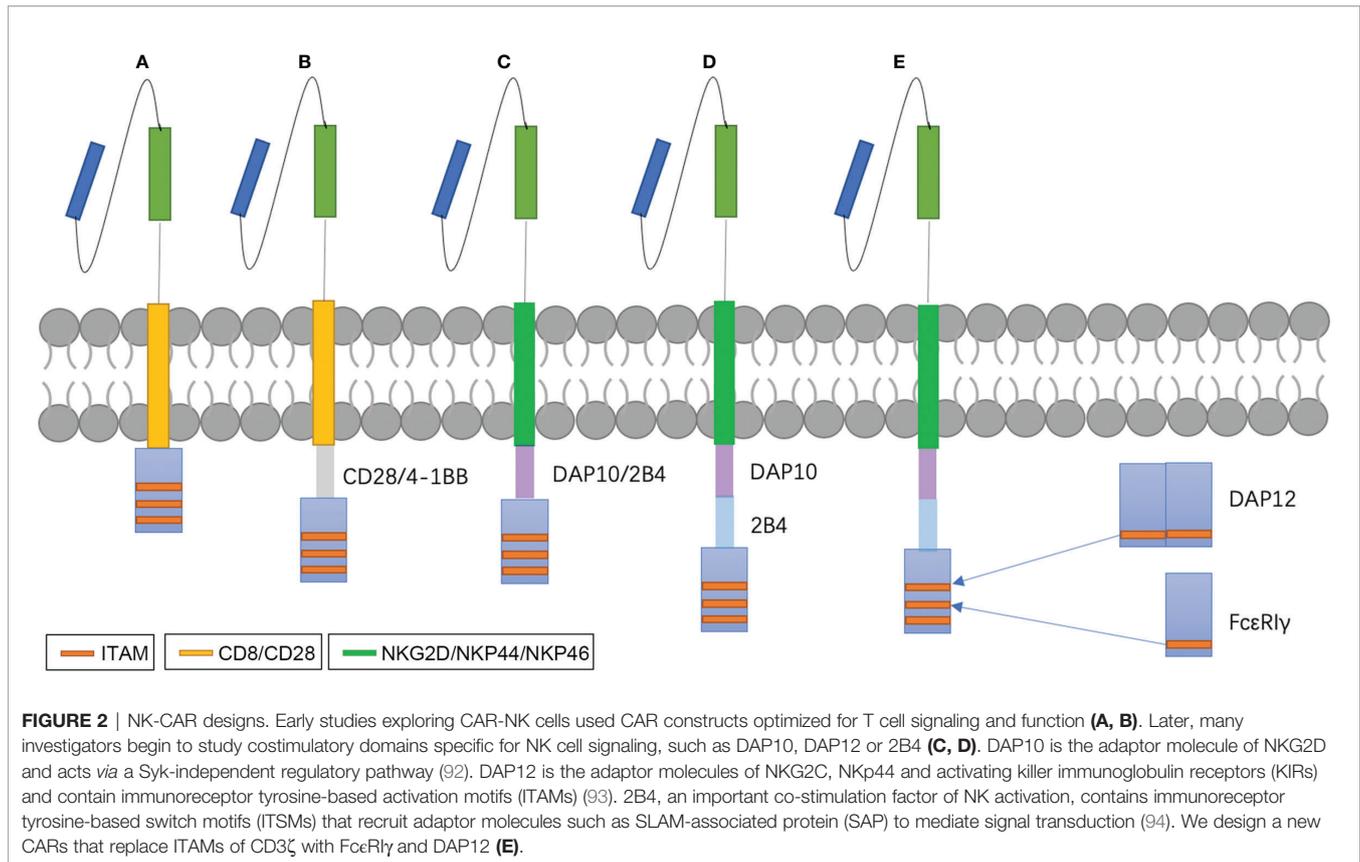
infiltrating lymphocytes (TILs) (78), which have many similarities with LAK cells except the origin of lymphocytes. The former is isolated from the stroma of tumors, while the latter is acquired from PBMCs. Success with TILs has been achieved in many solid tumors, including breast cancer tumors (79, 80). With TCR engineering, tumor-specific TCR α and β chains are identified and integrated with T cells *via* viral vectors (81). There must be specificity with T-cell engineering and CAR-T. Compared to CAR-T therapy, there are limited choices for physiological receptors with TCR engineering. Effective CAR-T cell therapy relies on optimal CAR molecular design. The CAR construct is becoming increasingly sophisticated with the understanding of T cell activation and tumor-specific and -associated antigens (82). Four generations of CAR designs have been developed that are mainly different in categories and number of co-stimulation factors (Figure 1). For safety and effectiveness, many novel designs have been tested, such as 'inverted CAR,' 'off-switch CAR' and 'logic-gate CAR' (83-85).

NK cell-based immunotherapy is a subset of ACT and is similar to adoptive T therapy, especially with respect to CAR therapy. CAR-NK-related clinical trials show that the most adopted CAR design corresponds with first and second generation T-CAR (86). Most NK-CARs use CD28 and 4-1BB, which are more specific to T cells, as their transmembrane and intracellular domain, respectively (87, 88). Later studies began to design CARs specific to NK cells. For example, intracellular domains replaced CD28 with 2B4, DAP12, or DAP10 (89, 90). Li et al. proved that the signaling domains of CAR-NK, such as NKG2D-2B4, exhibited superior *in vitro* and *in vivo* anti-tumor activities compared to that which contains CD28-4-1BB (91). The revolution of CAR-NK therapy is described in Figures 2A-D, and it is clear that the CAR construct is becoming increasingly sophisticated with the growing understanding of T cell activation and tumor-specific and -associated antigens that are also suitable for NK cells.

IMMUNOSUPPRESSIVE MECHANISMS OF GLIOMA

Glioma, especially GBM, shows an extreme cell heterogeneity, diffuse growth patterns and high invasiveness. Gliomas were thought to be "immune cold" tumors with low infiltration of lymphocytes (95). Furthermore, microenvironment of the glioma has the ability to suppress immune response systematically and locally.

Mahaley et al. first reported the presence of lymphopenia in GBM (96). Studies have found patient-derived peripheral blood lymphocytes shows immune defects that exhibited varying levels of proliferative unresponsiveness to the T-cell mitogens concanavalin A (ConA), phytohemagglutinin (PHA) and anti-CD3 monoclonal antibody as well as with the T-dependent B-cell mitogen, pokeweed mitogen (PWM) (97, 98). A selective impairment of the IL-2 system and T cell receptor-mediated signaling in lymphocytes of patients with glioblastomas may contribute to the unresponsiveness (99, 100). Compared to healthy individuals,



accumulation of myeloid-derived suppressor cells (MDSCs) in the peripheral blood of patients with glioma was found (101, 102). MDSCs impair tumor immunity by interacting with macrophages to increase IL-10 and decrease IL-12 production, driving a tumor-promoting type 2 response (103). Inhibitory soluble factors secreted by glioma can suppress lymphocyte's function. Transforming growth factor beta (TGF-β) can impair peripheral blood NK cell function by downregulating NKG2D (104–107). Therefore, the immune responses are suppressed systematically in glioma microenvironment.

Gliomas often overexpress phosphorylated signal transducer and activator of transcription 3 (p-STAT3) that induces a variety of immunosuppressive factors including IL-10, prostaglandin E2 (PGE2), vascular endothelial growth factor (VEGF) and TGF-β (108). These soluble factors can suppress cytotoxic T lymphocytes activity and proliferation (108). TGF-β and IL-10 can induce Tregs that inversely modulate immune response (109). Chemokines and cytokines, such as CX3CL1 and CCL5 can recruit tumor-associated macrophages to GBM microenvironment and contribute to abnormal angiogenesis (110, 111). Most GBM cells express high levels of MHC class I molecules that can inhibit NK cells by interacting with inhibitory KIRs (112). Absolute survival advantages of tumor depriving of nutrition and oxygen might suppress NK cell metabolism and antitumor activity (113). N6-methyladenosine (m6A) modification is an emerging field in the study of tumorigenicity and therapy resistance of glioma (114, 115). The relationship between m6A states and immune

infiltration and function in glioma is still unclear. Studies found higher m6Ascore was associated with T cells exhaustion and lower NK cells in the m6Ascore-high pancreatic ductal adenocarcinoma (116). So, the immune responses are suppressed locally in glioma microenvironment.

APPLICATIONS OF NK CELLS FOR GLIOMA TREATMENT

Except for unique advantages of NK cells, there is still a potential of NK cells in treating glioma. Cózar et al. analyzed RNA-seq datasets from the TCGA database and found NK-cell infiltration in both low grade glioma and GBM and even had higher scores compared to T-cell infiltration, which paved the way for the use of treatments targeting NK cells in glioma (117). Similar to adoptive T therapy, NK cell-based immunotherapy mainly concentrates on hematological malignancies. Thus far, the therapeutic utility of NK cell-based immunotherapy for the treatment of glioma has mainly been investigated in preclinical studies (Table 3). These trials most utilize either PBNK cells or NK92 cells, as well as first and second generation CARs designed for T cells and not optimized for NK cell signaling. Furthermore, clinical trials pay more attention to evaluate the safety of CAR-NK therapy. Although preclinical studies began to test the efficiency of DAP12 specific for NK cell signaling. Most of

TABLE 3 | Applications of NK cells in glioma.

Targets	NK Source	CAR construct	Tumors	Phase	Reference/NCT
EGFRvIII	NK92 cell line	scFv-CD28TM+IC-CD3 ζ	Glioblastoma	/	GenBler et al. (118)
EGFRvIII	YTS NK cell line	scFv-DAP12 TM+IC	Glioblastoma	/	Müller et al. (90)
EGFRvIII	KHYG-1 NK cell line	scFv-CD28 TM+IC-CD137-CD3 ζ	Glioblastoma	/	Murakami et al. (119)
EGFRvIII	NK92 cell line	scFv-CD28 TM+IC-CD3 ζ	Glioblastoma	/	Han et al. (120)
HER2	NK92 cell line	scFv-CD3 TM+IC	Glioblastoma	/	Alkins et al. (121)
HER2	NK92 cell line	scFv-CD28 TM+IC-CD3 ζ	Glioblastoma	/	Zhang et al. (122)
HER2	NK92 cell line	scFv-CD28-CD3 ζ	Glioblastoma	I	NCT03383978
HER2	NK92 cell line	Unknown	Glioblastoma	I	NCT03383978
MUC1	Unknown	Unknown	High Grade Glioma	I/II	NCT02839954
None	PBMCs	None	High Grade Glioma	I	NCT04254419
None	PBMCs	None	Glioma	I	NCT00909558
None	Placenta	None	High Grade Glioma	I	NCT04489420

them adopted similar CARs as used in clinical trials. Compared to hematological malignancies, both in the quantity and CARs design, the application of NK cell-based therapies in glioma lags far behind.

FUTURE PERSPECTIVES

Compared to T cell-based therapy, the development of NK cell-based therapy falls behind to some degree in the treatment of glioma. Adoptive NK cell therapy is lack of *in vivo* persistence without cytokine support, which may limit the efficacy of the NK cell immunotherapy. System administration of cytokines is associated with undesirable toxicities as described above. Trafficking to tumor beds is critical for the efficacy of adoptive cellular therapy. Müller et al. observed an infiltration increase of anti-EGFRvIII CAR-NK cells engineered to express CXCR4 to CXCL12/SDF-1 α secreting glioblastoma cells, leading to improved tumor regression and survival in a mouse model of glioblastoma (90). The difficulty may be resolved by intratumoral administration of NK cells products. In general, there is much room for the development of CAR-NK therapy in the field of glioma treatment. Much more pressing for NK cell-based therapy is designing more efficient products in treating glioma. Therefore, we try to put up several strategies to achieve the goal on the base of the knowledge of NK cells and glioma.

NK cell therapy is the lack of *in vivo* persistence in the absence of cytokine support. IL-15 is essential for NK cell function and homeostasis and can be added to CAR molecules to mimic the fourth generation of T-CAR. TGF- β plays an essential role in impairing NK cell function. Inverted CAR may be applied to reverse the situation by fusing the ectodomain of the TGF- β receptor to the endodomain of an activating receptor. CD3 ζ is found in the intracellular domains of T-CAR and NK-CAR. Furthermore, CD3 ζ , containing three immunoreceptor tyrosine-based activation motifs (ITAMs; YxxL/Ix6-8YxxL/I, with 29 amino acids), has a limited impact on the effectiveness of CAR-NK (123).

Modification targeting CD3 ζ has been tested in CAR-T therapy. Wu et al. found that CD3 ϵ recruits Csk and p85 *via* its mono-phosphorylated ITAM and BRS motif, respectively (124). Incorporation of the ITAM of CD3 ϵ into a second-

generation CAR increased the antitumor activity of CAR-T cells by reducing the cytokine production and promoting the persistence of CAR-T (124). NK cells possess many types of stimulatory receptors, such as CD16, NKP46, and NKG2D, and these stimulatory receptors do not act separately. In fact, apart from CD16, which is sufficient for activation of resting NK cells, it is necessary for all activating receptors to cooperate and synergize with one another for NK activation (125). Interestingly, the ITAM of many stimulatory receptors or their related adaptors, such as Fc ϵ RI γ and DAP12, also consists of 29 amino acids with different sequences. Therefore, an exchange may produce more effective CAR-NK cells (**Figure 2E**) with mild changes in CD3 ζ structure.

CAR-NK cells can kill targets in a CAR-independent manner. Combination therapy with monoclonal antibodies is promising, and it was observed that trifunctional antibodies recognized targets and simultaneously engaged NKP46 and CD16, which controlled tumor growth in mouse models (126). A team used antibodies to prevent the loss of cell surface MICA and MICB in human cancer cells, which stabilizes the bond between NKG2D and its ligands. These antibodies inhibit tumor growth in mouse models, and the antitumor effect is mediated mainly by the activation of NKG2D and CD16 (127). Apart from the activating receptors, antibodies blocking the inhibitory receptors of NK cells, such as KIR, NKG2A, TIM3, and TIGIT have been studied (52, 128–130). NK cells can express Fc γ RIIA/CD16a and/or Fc γ RIIC, which bind to the Fc portion of human immunoglobulins. Once antibodies bind to targets, NK cells are able to recognize the Fc portion and lyse target cells through antibody-dependent cell-mediated cytotoxicity (ADCC) (131). So, the combination of immune checkpoint inhibitors and NK cell-based therapy may be potential. Although, Nivolumab failed to improve overall survival of patients with recurrent glioblastoma. Studies have found that cancer type 1 or 2 susceptibility gene (BRCA1/2) alteration was associated with higher tumor mutation burden (TMB) and may serve as a novel indicator associated with better treatment outcomes of immune checkpoint inhibitors (132). The function of BRCA1/2 and other DNA mismatch repair gene alteration are worth being investigated in glioma. Most GBM cells express high levels of MHC class I molecules (112). Thus, blockade of such KIRs with antibodies may enhance NK-cell mediated killing.

NK cells and T cells originate from a common ancestor and share many similarities. Both interact with MHC class I molecules, contributing to innate and adaptive immunity. They have similar cell-surface phenotypes and cellular functions, such as cytotoxicity, secretion of cytokines, and interaction with DCs (133). NK cells also play a role in regulating T cell response. For example, NK cells can produce IFN- γ , which promotes CD4⁺ T cell differentiation into TH1 helper cells (134). The latter contributes to an enhanced CD8⁺ T cell response (135). NK cells produce IFN- γ , leading to DC maturation and IL-12 secretion, which is sufficient for CD8⁺ T cell activation independent of CD4⁺ T cell (136). As an important constituent of the innate immunity response, NK cells can kill target cells and release antigen for cross-presentation and activation of T cells (137). NK cells can also negatively regulate a T cell response as described above or in the setting of acute viral infections (138, 139). A combination of NK cells and T cells comprise an ideal potential therapy for tumor treatment. A study has reported that CAR-NK cells can eliminate myeloid-derived suppressor cells and rescue impaired CAR-T cell activity against solid tumors (140). Moreover, CAR-NK cells can improve the infiltration and functions of subsequently infused CAR-T cells by secreting proinflammatory cytokines and chemokines (140). Cózar et al. found marked NK-cell infiltration in solid tumors were also infiltrated with T cells (117). Anti-IL13R α , anti-HER2, and anti-EGFRvIII CAR-T have been tested in glioma (141–143). Utilizing the safety of CAR-NK cells and the high efficacy of CAR-T cells is worth exploring in gliomas.

Oncolytic virus (OV) OVs have a double oncolytic action by both directly attacking the cancer cells and inspiring a tumor specific immune response. OVs can be engineered to repress antibodies targeting tumor antigen and/or secrete cytokines activating immune response. Xilin Chen et al. observed that the combination of EGFR-CAR NK-92 cells with oHSV-1

resulted in more efficient killing of MDA-MB-231 tumor cells and significantly longer survival of tumor-bearing mice (144). Rui Ma et al. (145) generated a therapy that combined off-the-shelf EGFR-CAR NK cells and an Oncolytic virus OV called OV-IL15C. OV-IL15C-infected GBM cells can secrete soluble IL15/IL15R α complex. GBM-bearing mice models exhibited that the therapy synergistically suppressed tumor growth. These potential therapies are anticipated to be further investigated in clinical trials. Combination therapies are based on the knowledge of NK cell biology. An evolving understanding of gliomas can inspire treatment strategies targeting the basic elements of these malignant cells and their microenvironments. We believe NK cell-based immunotherapy will have a better performance in treating glioma in the future.

AUTHOR CONTRIBUTIONS

CP conceived the article. YZ compiled the review and prepared the draft of the manuscript. GL, TJ, and WZ reviewed and edited the manuscript. All authors contributed to the article and approved the submitted version.

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