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Review Article

CAR-armed cell therapy for gliomas

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Abstract: Chimeric antigen receptor (CAR)-armed cell therapy has developed rapidly in recent years, especially in the treatment of leukemia. However, the treatment methods for solid tumors represented by glioma have not achieved the ideal therapeutic effect. This situation necessitates learning from chimeric antigen receptor T cell (CAR-T) treatment in other malignancies and discovering the differences between gliomas and other solid tumors. The current design idea is to enhance the targeting, regulatory effects, and adaptation of CAR-armed cells. This review traced not only clinical trials, but also several animal experiments, which might promote the development of CAR-T treatment in glioma. Furthermore, we have discussed the obstacles to CAR-T in the treatment of glioma and the current possible solutions.

Keywords: Glioma, CAR-T, tumor microenvironment

Introduction

Gliomas are the most common intracranial malignant tumors that are associated with disappointing prognosis. They are thought to originate from the neuroepithelium, and more than 70% of newly diagnosed cases are classified as glioblastomas (GBMs), which are highly aggressive and lethal [1]. But so far, medical care for these lethal conditions is limited. In brief, although patients receive timely and the most extensive tumor resection, followed by standard radiotherapy and chemotherapy [2], they still cannot escape the recurrence of glioma. The ineffectiveness of current treatments and the absence of novel treatments are the main obstacles to improving the prognosis.

Immunotherapy is a promising strategy. At present, targeted therapy against the aberrant expression of molecule in GBM is disappointing [3]. This can be perfectly exemplified by the hopeless results obtained by the clinical trials of EGFR kinase inhibitors and PD-1 inhibitors against GBM [4]. These disappointments prompted us to think whether this aberrant expression is attributed to the properties of GBM, or there are other barriers to drug delivery apart from the blood brain barrier [5, 6]. Taken together, inhibiting the function of a given molecule does not seem to be an ideal strategy for tumor elimination. But compared to the molecule-targeting treatment, cell therapy, such as chimeric antigen receptor T cell (CAR-T), provides a cell-targeting strategy regardless of the flexible molecular pathways. By recognizing the tumor specific antigen ideally or the tumor-associated antigen alternatively, engineered T cells are able to destroy the tumor cells precisely. After its great success in treating Acute B-lymphoblastic leukemia (B-ALL), CAR-T has become a universal immunotherapy method. Considering several inspiring outcomes in some solid tumors, engineered T cells can be a novel strategy for treating newly diagnosed and recurrent gliomas. Here, we review several novel approaches to cellular immunotherapy for glioblastoma and analyze the strengths and weaknesses of these strategies. Further, we will discuss the directions and obstacles in developing this promising new treatment option.

CAR-armed lymphocyte therapy

Chimeric antigen receptor (CAR) is a genetically engineered molecule expressed on leukocytes

(T, NK, and NKT cells). The construction of CAR is becoming increasingly sophisticated with an in-depth understanding of leukocyte activation and tumor-specific antigen. Taken together, the CAR molecule consists of endocellular, transmembrane, and extracellular domains. The endocellular domain contains two activation signal motifs, including CD3ζ (provides co-stimulatory signal 1), CD28 (provides co-stimulatory signal 2), 4-1BB (provides co-stimulatory signal 2), or OX40 (provides co-stimulatory signal 2). The transmembrane domain derives from the same part of T-cell receptor (TCR) or the corresponding domain of CD28/CD8. The extracellular domain is formed by one or more single chain variable fragments (scFv) to aim at the specific target on neoplastic cells. As such, CAR molecules provide the essential stimulatory signal, which can antagonize the immune suppression effect in the tumor microenvironment (TME) and trigger the immune response of CAR-armed cells.

CAR-T

Development of CAR-T has achieved impressive outcomes in patients with B-cell malignancies. Anti-CD19 CAR-T cell is the first engineered CAR-armed cell, and it has already been approved by the Food and Drug Administration (FDA) for use in the market. Building on the inspiring clinical success, it is expected that CAR-T therapy would also work well in solid tumors, especially in glioma. In fact, tentative explorations into CAR-T therapy for glioma have been recently performed. Some of the cutting-edge CAR-T therapeutic strategies or the ones that can be utilized against glioma are summarized below.

Anti-IL13Rα CAR-T

A 50-year-old man with recurrent multifocal IDH1-wt and MGMT non-methylated GBM was enrolled into a clinical trial [7]. The 2nd generation CAR was constructed with the IL13R-specific extracellular domain, 4-1BB, and CD3ζ. Autologous CD8^{+}T cells were armed with the CAR molecule. Six sessions of intracranial injections of CAR-T cells, followed by ten sessions of intraventricular injections of CAR-T cells were administered weekly. Tumor regression in all lesions was detected after the 3rd session. Pro-inflammatory cytokines were detected in the cerebrospinal fluid after each infusion. No cytokine storm-associated symptoms developed during treatment. A dramatic response was sustained for 7.5 months with no initial focal recurrence. Unfortunately, four new anatomic sites were noted after the 16th session. This might be due to antigen escape in the tumor.

Similarly, Khun et al. constructed IL-13 zetakine CAR-T targeting IL13Rα^{+} GBM [8]. Mutant IL-13 with higher affinity for IL-13Rα was linked with the CD3ζ domain. CAR-T cells were also equipped with herpes simplex virus type-1 thymidine kinase gene (HSV-1 tk), a suicide and reporter gene in frame with hygromycin phosphotransferase (HPH) as the selection marker. Seven patients with high-grade glioma were treated intracranially with the administration of IL-2. No side effect or normal tissue damage was observed after CAR-T infusion. CAR-T cell infusion and an increase in the activity was detected on positron emission tomography (PET) imaging. But neither tumor shrinkage nor prolonged survival was detected.

Anti-EGFRvIII CAR-T

Epidermal growth factor receptor (EGFR) variant III (EGFRvIII) is a traditional target for GBM. A total of 10 patients with recurrent GBM were treated with anti-EGFRvIII CAR-T cells [9]. 9 of these 10 patients had multifocal disease. A single dose of 1.75-5 × 10^{8} cells was injected intravenously. Infiltration of CAR-T cells in lesions only appeared after two weeks. EGFRvIII^{+} tumor cells were thoroughly decreased in the post-resection samples. Two patients underwent surgical intervention after 2 weeks of CAR-T injection. CAR-T was detected at a higher concentration in surgical samples than in the corresponding blood samples, suggesting CAR-T trafficking. But the persistence of CAR-T cells remained limited. Cytokine release syndrome was not observed in these patients.

Anti-HER2 CAR VST

Seventeen patients with recurrent or progressive HER-2^{+} GBM were enrolled in a phase I clinical trial [10]. 16 of these 17 patients were seropositive for cytomegalovirus (CMV). Considering that HER2-specific 3rd generation CAR-T cells were reported to trigger a lethal cardiopulmonary effect after injection into a

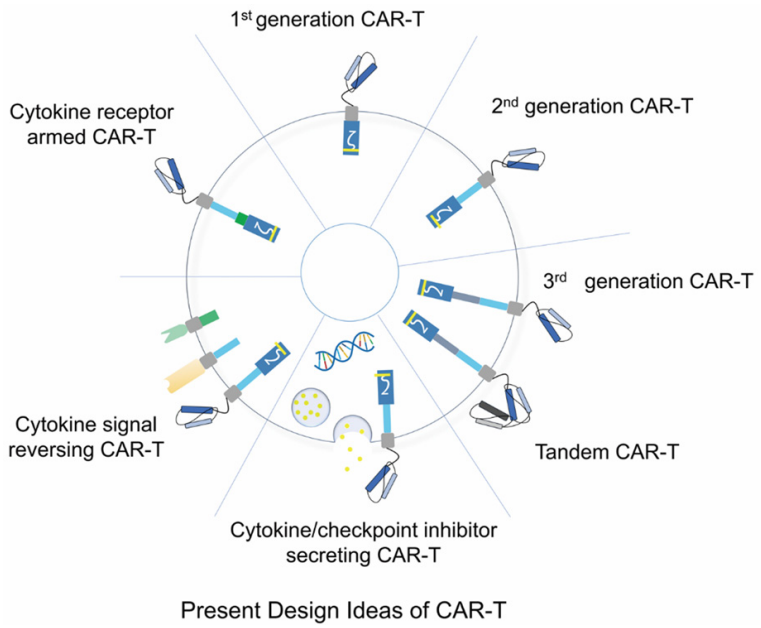

Figure 1. The main design of CAR-T has been developed rapidly. The 1st generation CAR molecule was constructed with scFv, CD8 transmembrane domain, and CD3ζ domain. The 2nd generation CAR molecule was developed on the basis of the previous molecule by introducing the CD28/4-1BB co-stimulatory domain between CD3ζ and the transmembrane domain. The 3rd generation CAR molecule was constructed by adding another co-stimulatory domain in order to achieve stronger activation, whereas linking two different scFvs in a series was performed for targeting improvement. Recent designs of the CAR molecule vary, which can be concluded by the three sketch maps on the left. By utilizing T2A (figure not shown), cytokines/checkpoint inhibitors can be expressed in a 1:1 relationship with the CAR molecule. However, introducing the NFAT sequence can provide inducible cytokines/checkpoint inhibitor expression. Reversing the signal of immune-suppression cytokines is another strategy to confront TME and prolong the lifespan of CAR-T. The chimeric cytokine receptor (e.g. TGFβ receptor extracellular domain+IL-7 transmembrane and intracellular domain) can perfectly reverse the immune-suppression signal to the pro-inflammatory signal. Otherwise, adding the intracellular domain of the type I cytokine receptor family (e.g. truncated IL-2 receptor β) between the co-stimulatory domain and CD3ζ provides another strategy for promoting CAR-T activation.

patient with refractory colon cancer [11], the investigators utilized 2nd generation CAR-T cells. FPR5 (HER2-specific murine scFv [12]), CD28, and CD3ζ were integrated into virus-specific T cells (VSTs). Adenovirus, cytomegalovirus, and Epstein-Barr VSTs have been previously established. CAR-T cells were injected intravenously, at a dose ranging from $1 \times 10^6/m^2$ to $100 \times 10^6/m^2$. Patients received different doses and sessions during treatment. No side effect was reported during treatment. HER2-CAR VSTs had been in the peripheral blood for up to 1 year after injection but without proliferation. About 6 weeks after infusion, MRI showed an increase in peritumoral edema. In total, the median overall survival (OS) was 11.1 months. Three patients were alive at the last follow-up with no disease progression, and 5 patients had durable and stabilized disease for over 24 months. The main theme of this clinical trial was a large dose span and an expected antitumor effect in FPR5 CAR-VST treatment. But it also highlighted the fact that the virus antigen presented by antigen-presenting cells might promote CAR-VST activation, but it does not trigger its proliferation.

In summary, the clinical trials of CAR-T therapy against glioma featured single-target 2nd generation CAR-T cells. A more inspiring design has already been tested in xenograft models. The main strategy of constructing CAR-T cells was to enhance the accuracy and prolong the persistence. However, to date, CAR-armed leukocyte therapy in the field of glioma lags behind that for other malignant tumors. Novel designs of CAR-T mentioned below are not precisely against GBM, but these experiments might be a future direction for CAR-T therapy in GBM (**Figure 1**).

Cytokine secreting single-target CAR-T

Cytokine-delivering CAR-T cells were also named as TRUCKs or 4th generation CAR-T cells [13]. The strategy of secreting recombinant pro-inflammatory cytokines in the TME has produced promising outcomes in eliminating tumor cells and facilitating the proliferation and persistence of engineered T cells. The 4th generation CAR-T cells can release cytokines after activation, while they remain silent beyond the tumor tissue. So far, several cytokine structural genes have been fused into T cells, and they mainly include IL-7, CCL-19 [14], IL-15 [15], IL-18 [16], and IL-12 [17, 18]. These pro-inflammatory cytokines are able to antagonize immune suppression in the TME. By using the 2A peptide

technique, the CAR molecule could be designed such that it was linked with cytokine genes to transcript continuously and translate separately [19, 20]. Alternatively, introduction of the nuclear factor of activated T cells (NFAT) response elements to construct activation-associated cytokine expression is another method. Here, we exemplified these two kinds of outstanding cytokine-secreting CAR-T against a solid tumor.

Eg.1: IL-7 is a cytokine that reduces tumorigenesis and prolongs T cell life span [21]. CCL19 has been proved to attract T cells as well as APCs. Keishi Adachi, et al. constructed CD20-target CAR-T cells that express IL-7 and CCL19 against mastocytoma [14] (**Table 1**). An anti-human CD20 2nd generation CAR molecule was linked with IL-7 and CCL19 (7 × 19 CAR-T). In vitro experiments proved its ability to prolong and attract T cells of IL-7 and CCL19. The 100% long-term survival of the mouse model indicated the advantage of 7 × 19 CAR-T compared with the 30% survival rate of conventional CD20-targeted CAR-T. Also, 7 × 19 CAR-T could effectively restrict the tumor burden.

Taking a further step, an anti-PD-1 scFv-expressing CAR-T was generated by using the same process and it provided an inspiring result in leukemia treatment as expected [22].

Eg.2: IL-18 is a stimulatory cytokine of NK cells and T cells. It is a key cytokine for IFN-γ releasing. Markus Chmielewski, et al. compared the killing effect of 4th generation CAR-T with that of 2nd generation CAR-T (targeting CEA) in treating pancreatic adenocarcinoma [23] (**Table 1**). They firstly rejected the effectiveness of 2nd generation CAR-T in advanced tumors. Then they took a further step by constructing activation-associated IL-18/IL-12 expression. The DNA of NFAT and IL-2 promoter followed by IL-18/IL-12 was linked with 2nd generation CAR-T. In mouse models, inducible IL-18 expression showed great advantages in increasing the number of NK cells and reducing regulatory T cells (Tregs) and FoxO1 expression. Furthermore, release of IL-18 effectively reduced the activation and expression of PD-1 and other immune checkpoints. In brief, the newly engineered T cells with inducible IL-18 expression showed prolonged tumor-specific rejection and it was proved to be safe in mouse models.

The main theme of such type of CAR-T cells is to combine the 2nd generation CAR molecule with cytokines or other TME modifiers. As mentioned above, these secreted modifiers might normalize the metabolism chain of tumor-infiltrating leukocytes (TILs) and reactivate them directly. These studies also demonstrated their effectiveness and safety. Definitely, the 4th generation CAR is a future direction for immune therapy.

Cytokine receptor-armed CAR-T

Another method to introduce a pro-inflammatory cytokine signal into CAR-T cells is to provide the cytokine receptor intracellular domain. This strategy may avoid the potential toxicities (such as liver dysfunction, high fever, and hemodynamic instability), which are likely attributed to the secreted cytokine as mentioned in a clinical trial [24]. Type I cytokine receptor (e.g. IL-2, IL-7, and IL-15 receptors) shared common γ chains to trigger the JAK-STAT pathway [25]. STAT3/5 activation was triggered by the tyrosine-containing motif within the γ chain [26]. Two methods were introduced, and they achieved inspiring outcomes.

(i). Yuki, et al. constructed a novel 2nd generation anti-CD19 CAR molecule by infusing the cytoplasmic domain of IL-2 receptor β between the CD28 and CD3ζ domain. The CD3ζ domain was linked with the YXXQ motif to trigger the STAT3 pathway. The 28-ΔIL2RB-z (YXXQ) CAR-T cells showed impressive persistence, proliferation, and cytokine releasing ability in vitro. STAT-3 and IL-21 induced gene expression was magnified after CAR-T cells were cultured with the target cells. Transduced $CD19^+$ melanoma models and $CD19^+$ leukemic models were constructed for the in vivo experiments. In summary, the 28-ΔIL2RB-z (YXXQ) CAR-T cells did not affect CAR-T mediated toxicity, and they possessed outstanding tumor-controlling ability and persistence.

(ii). Thomas, et al. constructed an additional constitutive IL-7 signaling molecule [26] (**Table 1**) on the 2nd generation anti-GD2 CAR-T cells [27] (GD2-CAR.C7R). GD2-CAR.C7R showed outstanding persistence when cultured without the target cells or interleukins. When co-cultured with the target cells, GD2-CAR.C7R showed stronger proliferation and cytokine secreting ability. Results of the in vivo experi-

Table 1. Summary of current studies on CAR-armed lymphocyte therapy for human solid tumors

Study name	Reference	Target Cell	CAR structure	Summary of the effect	Innovation	Institution
7x19 CAR-T	[12]	mastocytoma	Anti-human CD20 scFv-CD28/4-1BB intracellular domain-CD3ζ-P2A-human IL-7-2A-human CXCL19	Long-term survival with additional CPA treatment. Low side effects.	Prolong CAR-T survival Recruiting of macrophages	Department of Immunology, Yamaguchi University Graduate School of Medicine, Ube, Japan
IL-18 TRUCKs	[21]	Pancreatic carcinoma	Anti-CEA scFv-CD28 intracellular domain-CD3-CD3ζ & 6x NFAT-IL-2minimal promoter-IL-18	Convert tumor infiltrate T cell into T-bet^{high} FoxO1^{low}	Inducible secreting IL-18	Center for Molecular Medicine Cologne, University of Cologne, Cologne, Germany
C7R	[24]	neuroblastoma	Anti-GD2 scFv-4-1BB intracellular domain-CD3ζ & Ectodomain of CD34-IL7Rα transmembrane-CD34-IL7Rα intracellular domain	Long-term survival and proliferation of CAR-T. Advanced effect in re-challenge	Constitutive IL-7 signal that is related to the size of the ectodomain.	Center for Cell and Gene Therapy, Texas Children's Hospital, Houston Methodist Hospital, and Baylor College of Medicine, Houston, Texas
Tandem CAR-T	[28]	GBM	IL-13 mutein-linker-anti-HER2 scFv-CD28 intracellular domain-CD3ζ	Advanced killing effect compared with pool CAR and bi-CAR	Tandem CAR-T utilized in GBM	Center for Cell and Gene Therapy, Texas Children's Hospital, Houston Methodist Hospital, Baylor College of Medicine, Houston, Texas, USA
U-CAR-T	[29]	GBM	2nd generation of HER2 CAR-P2A-2nd generation of IL13Rα CAR-P2A-2nd generation of EphA2 CAR	Advanced killing effect compared with classical 2nd generation CAR-T in the PDX model	Trivalent CAR-T utilized in the PDX model	Center for Cell and Gene Therapy, Texas Children's Hospital, Houston Methodist Hospital, Baylor College of Medicine, Houston, Texas, USA
CD70 CAR-T	[30]	GBM	Anti-CD70 scFv-4-1BB intracellular domain-CD3ζ	Eliminating the target cells	Novel target that is negative corelated with prognosis	UF Brain Tumor Immunotherapy Program, Preston A Wells Center for Brain Tumor Therapy, Lillian S. Wells Department of Neurosurgery, University of Florida, Gainesville, Florida, USA
SmarT	[31]	pancreatic tumor cells	Anti-PSCA scFv-CD3ζ & TGFβRII-4-1BB & IL4Rα ectodomain-IL7Rα intracellular domain	Conditional activation Advanced proliferation and killing ability	Reverse the negative effect of IL-4 & TGFβ	Center for Cell and Gene Therapy, Baylor College of Medicine, Texas Children's Hospital and Houston Methodist Hospital, Houston, Texas.
BiTE-CAR-T	[28]	GBM	Anti-EGFRvIII scFv-CD28 intracellular domain-CD3ζ-T2A-BiTE	BiTE induces untransduced T cell contact and kill tumors	Delivering a novel structure of a small molecule protein.	Department of Dermatology, Massachusetts General Hospital and Harvard Medical School, Boston, MA, USA
CAR-NK-92	[41]	GBM	Anti-ErbB2 scFv-CD28 intracellular domain-CD3ζ	Advanced tumor suppression Inspiring effect in re-challenge	CAR-armed NK-92 cells	Institute for Tumor Biology and Experimental Therapy, Frankfurt am Main, Germany
CAR-iNKT	[54]	neuroblastoma	Anti-GD2 scFv-CD28 intracellular domain-4-1BB intracellular domain-CD3ζ	Advanced tumor suppression Less GVHD compared with corresponding CAR-T	iNKT armed with the 3rd generation CAR molecule	Texas Children's Cancer Center, Department of Pediatrics

Designs of CAR-armed cells mentioned in the article are displayed in the table. Several representative trails indicated the need for improving the effectiveness of immune modulation in CAR-T treatment. Proinflammatory and anti-immunosuppressive strategies should be generalized in CAR therapy for glioblastoma (e.g. IL-7, IL-18). Furthermore, since markers essential for tumors also tend to be important for normal tissue (e.g. CD70), target selection should not only focus on searching the absolute specific target, but also on taking the ameliorating on-target-off-tumor effect into consideration. Traditional markers, such as IL13Rα and EGFRvIII, are specific but not stable and full-scale targets for CAT-armed cell therapy. Filtrating full-scale but relatively nonspecific target while remitting the on-target-off-tumor effect might be a new strategy for the CAR-T design.

ment showed the same anti-tumor effect. Furthermore, the GD2-CAR.C7R cells could be eliminated by AP20187 after the transduction of iCaspase9.

The main theme of the strategy is to induce the 3rd signal of proliferation and persistence of T cells by means of introducing the STAT pathway.

Small molecule protein delivering CAR-T

Bryan D. Choi, et al. developed a novel EGFRvIII targeted CAR-T, which can actively deliver BiTE [28]. BiTE is a newly designed scFv, which was constructed as the "anti-CD3 scFv A-linker-anti-EGFR-scFv". Tumor-infiltrating $CD3^+$ cells were expected to be linked with $EFGR^+$ tumor cells and they exerted an extra killing effect. However, the target selection does not seem to be ideal, as not all patient-derived cell lines highly expressed either EGFRvIII or EGFR.

Multi/novel-target CAR-T

Antigen escape has long been a critical problem in CAR designing. In spite of classical targets, such as EGFRvIII [29], EphA2 [30], HER2 [10], and IL13Rα [7], several novel and more stably expressed markers have been identified.

Multi-target CAR is a simple strategy for mitigating antigen escape. Tandem CAR-T and even trivalent CAR-T have been discovered. Tandem CAR [31] (**Table 1**) molecule contains two scFv chains with a linker between them. The remaining part of Tandem CAR molecule is the same as the classical 2nd generation. Tandem CAR-T showed an outstanding anti-tumor effect in a mouse model, compared with pool CAR-T (a mixture of anti-HER2 and anti-IL13Rα CAR-T cells) and bi-CAR-T (anti-HER2 and anti-IL13Rα scFv are linked with CD28 or CD3ζ, respectively) counterparts. Recently, Kevin Bielamowicz, et al. took a further step by constructing trivalent CAR-T cells (patient-derived T cells armed with HER2, IL13Rα2, and EphA2 2nd generation CAR molecule simultaneously, UCAR-T) [32]. UCAR-T (**Table 1**) treatment produced long-term survival and impressive tumor shrinkage in PDX mouse models, while other types of CAR-T (biCAR and single CAR) did not show a promising outcome.

During the search of a new target, CD70 was proved to be a potential choice [33] (**Table 1**), as CD70 expression was not detected in the normal brain tissue or tumor-infiltrating cells. But so far, no inspiring new cell surface marker has actually been identified.

SmarT (**Table 1**) cells is a novel concept that has been proposed recently [34]. Engineered T cells were transduced to express the following three molecules: (i) CAR molecule, which consists of scFv, transmembrane domain, and CD3ζ domain. (ii) TBBR molecule, which consists of TGFβ receptor type II extracellular and its transmembrane domain, and the 4-1BB domain. (iii) 4/7 molecule, which consists of IL-4 receptor α chain extracellular and its transmembrane domain, and the IL-7 receptor α chain intracellular domain. These three independently expressed molecules provided the essential signal for the activation, proliferation, and development of T cells. In every respect, SmarT cells showed an outstanding tumor rejection effect against prostate stem cells. In vitro experiments showed huge upregulation of granzyme and IFNγ and lower expression of immune checkpoints. The fold expansion in the $PSCA^+$ $IL4^+$ $TGFβ^+$ environment was significantly higher compared with that in controls. In vivo experiments showed inspiring outcomes suggesting that SmarT cells eliminated tumor cells thoroughly and presented considerable memory rejection during the re-challenge experiment. The main theme of the design was to introduce a chimeric cytokine receptor to reverse the suppression effect in the TME.

CAR-NK

Due to some special advantages of NK cells over T cells, the CAR-NK cell technique has also been developed in recent years.

NK cells do not need HLA matching to become activated [35, 36], but they need the HLA molecule to be inhibited. In fact, downregulation of HLA molecules makes abnormal cells susceptible to attack by NK cells, although this is a strategy for tumor cells to escape T cell immune surveillance [37]. Thus, there is no need to autograft engineered NK cells. This feature makes it possible to transplant a subset of NK cells, NK-92 cells [38]. The unique advantages of NK cells are as follows: (i) according to allogeneic adoptive NK cell infusion in a clinical

trial [39], NK cells did not trigger Graft-Versus-Host Disease (GVHD) [40], while allogeneic T cells were reported to cause GVHD [41]. (ii) CAR-NK cells retained their innate function to recognize tumor cells directly, thus theoretically they possessed a wider target spectrum [42].

The method of engineering NK cells with the CAR molecule is similar to that for CAR-T; however, the CAR-NK technique is in a preliminary stage compared with the CAR-T technique. Several clinical trials of leukemia have been performed (NCT02944162, NCT02839954, and NCT03065339). However, the development of CAR-NK was relatively slower in solid tumors. Michael C, et al. firstly engineered HER2 targeting CAR-NK cells for GBM [43] (**Table 1**). NK-92 cells were armed with the classical 2nd generation CAR molecule. As expected, CAR-NK cells produced a longer survival and protected against GBM recurrence. Taking a further step, infusion of IL-15 into the CAR molecule was a novel strategy for long-term persistence in leukemia [44]. So far, no study comparing the therapeutic effects of CAR-T and CAR-NK has been published.

CAR-NKT

NKT (invariant NKT) cells have recently been discovered, and they have been proved to play an important role in the anti-cancer immune response [45]. NKT cells contain conventional T cells as well as NK cells [46]. NKT cells do not have the shortcomings of T cells and NK cells, such as GvHD [47, 48] and NKG2D mediated immune suppression. Their killing effect and the antigen recognition mechanism are similar to those of NK cells and T cells, respectively. NKT cells are mainly directly activated by the interaction of iNKT TCRs and CD1d-lipid agonists (presented by APCs or tumor cells). But, they can also be activated via Toll-like receptor and inflammasome components, NOD1 and NOD2 [49, 50], or they can be indirectly activated by IL-18, IL-12, and IFN [51, 52]. Glioma was found to express CD1d, which was a rare phenomenon among cancers [53]. It has been reported that patient-derived NKT cells preserved the CD-1d mediated killing effect and the ability of expansion [54, 55]. As such, NKT cells can be regarded as innate glioma-eliminating cells. NKT cells have been identified as potential vectors for the CAR molecule [56].

However, currently, the biology and function of NKT cells have not been completely described, and their roles in tumorigenesis and tumor development remain unclear. The development of the CAR-NKT technique is limited. In fact, anti-GD2 CAR-iNKT was the only experiment for solid tumor hitherto [57] (**Table 1**). The anti-GD2 2nd generation CAR molecule was armed with the iNKT cells. Engineered NKT cells showed bi-target effects in vitro, indicating that the innate CD1d-targeting ability was preserved. Compared with the CAR-T technique, the CAR-iNKT technique presented better persistence and anti-tumor effect with mild tissue (liver and lung) injury. The experiments showed promising prospect for CAR-NKT.

Obstacles to CAR-armed cell working

Non-cellular immune suppression

Many studies have attempted to reveal the mechanism of immune suppression in TME. Metabolic regulation between the tumor and immune cells is one of the research hotspots [58]. (i) Hypoxia, which reduces the survival and proliferation of T cells [59] and recruits Tregs by inducing the expression of CCL28 [60]. (ii) Glycolysis, which is influenced by hypoxia. Infiltrating lymphocytes need more time for adaptation. The acidification of the TME can inhibit the expression of pro-inflammatory cytokines [61]. (iii) Amino acid metabolism. The upregulation of indoleamine-2,3-dioxygenase (IDO) induces tryptophan deprivation in T cells and directly downregulates CD3ζ expression. In addition, numerous tumor-secreting cytokines (IL-4 and IL-13) induce tumor-associated microglia/macrophages (TAM) to express arginase, which cause a great obstacle to oxidative phosphorylation in T cells [62, 63].

The expression of immune system signaling inhibitors is another hot research topic. Except for the secretion of specific cytokines, the regulatory effects of checkpoints (PD-1, TIM-3, LAG-3, and CTLA-4) have received increasing attention. These researches have encouraged us to think whether there is an interaction between the marker expression and the regulation of TME. Studies have shown that specific markers, such as CD44, SOX2, and SOX4, are correlated with the up-regulation of cytokines or pathways (like TGF-β and hyaluronic acid).

These findings provide a remarkable target for immune therapy.

Cellular immune suppression

It is well-known that there is no shortage of leukocytes, but there is a lack of immune response in gliomas [64]. Among all types of TILs, tumor-infiltrating T cells are the most thoroughly studied. Tumor infiltrated T cells are not only the victim of immune suppression, but also the participant. (i) The accumulation of Tregs ($CD4^+CD25^+FOXP3^+$) has been considered as one of the core issues in immune dysfunction [65]. (ii) In spite of multiple metabolism and molecule inhibitors as mentioned above, tumor-infiltrating T cells show self-repression by upregulating different types of checkpoints and downregulating the sensitivity for IFNγ and IL-2 [66]. (iii) However, tumor-infiltrating T cells have been proved to preserve their anti-tumor effect. Central memory T cells ($CD45RA^-$ $CCR7^+$) were found to be the main subset of TILs in GBM samples. Almost all infiltrating T cells could be reactivated in vitro [67]. These facts underlined the importance of management rather than elimination of TILs during the intervention of adoptive immune therapy.

Beyond Tregs, TAMs are also common in GBM. TAMs remain in a dynamic equilibrium between the M2 and M1 phenotypes. In this situation, a considerable ratio was aligned with the M0 non-polarized phenotype [68]. This conclusion opened up the possibility of inducing a differentiation strategy to elevate the M1 phenotype. Inhibiting the colony stimulating factor-1 receptor was one of the most inspiring examples. By means of blocking CSF-1 with PLX3397, M2 phenotype TAMs were induced to the M1 phenotype, which might trigger a pro-inflammatory response and restrict the tumor growth [69].

Concluding remarks and future perspectives

Cell-based therapy is one of the most attractive treatment options for human cancers. Armed with the CAR molecule, leukocytes can recognize tumor cells and be activated directly. The core issue for CAR therapy is the sporadic tumor special antigen discovery. Several strategies for balancing the killing and protecting effects have been discovered. They include the following: (i) Modulating the co-stimulatory domain (CD28 or 4-1BB) [70]. (ii) Alternating the specific amino acid residue in the simulation domains of the CAR molecule [71]. (iii) Establishing the "switch" or "brake" mechanism, e.g. glucocorticoid, antagonists of IL-1 and IL-6, suicide genes, or simply designing a CAR molecule that targets the marker on the anti-tumor Fab fragment [72].

Targeting GSC is a new way to avoid immune escape caused by heterogeneity. Given the immune suppression and hypoxia environment in the niche [73], engineered cells should be designed to overcome these obstacles, and more questions need to be answered. (i) Immune suppression should be first taken into consideration. Given that there is no lack of TILs in GBM, it is essential to mobilize the potential immune reaction [74]. Several strategies have been described in the main text. However, in our daily researches, we found that introducing new structures (including Bi-specific CAR-T, scFv delivery) would have an all-round impact on the biological function of CAR-T, as well as peripheral blood T cells cultured in the same system. Such a novel design may change the molecular exchange between the tumor and CAR-T [75]. The corresponding function of a novel design seems to be full of surprises and unpredictable. New immunosuppressive mechanisms and new solutions should be explored. (ii) For hypoxic and metabolism inhibiting the environment, T cells, on the contrary, become the first choice, since deactivation of NK cells in hypoxic tumors has been described repeatedly [76, 77].

In summary, CAR-armed cells are becoming powerful and multifunctional. The future design should not only be limited to the killing effect, but it should also be devoted to regulating the TME and activating the TILs or TAMs.

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