Review

Strategies to improve chimeric antigen receptors therapies for neuroblastoma

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Abstract: Chimeric antigen receptors (CARs) is one of the curative immunotherapeutic approaches that exploit the antigen specificity and cytotoxicity function of potent immune cells against cancers. Neuroblastoma, the most common extracranial pediatric solid tumors with diverse compartment, could be a promising candidate for using CARs therapies. Several methods harness CARs modified cells in neuroblastoma to increase therapeutic efficiency, albeit the assessment has still been less successful. Regarding the improvement of CARs, various trials have been launched to overcome insufficient capacity. However, the reason behind the inadequate response against neuroblastoma of CARs modified cells are still not well understood. It is essential to update the present reveal of comprehension of CARs to improve the efficiency of CARs therapies. This review summarizes the crucial features of CARs and its design for neuroblastoma, discusses challenges that impact the outcomes of the immunotherapeutic competence, and focuses on devising strategies currently investigated to improve the efficacy of CARs for neuroblastoma immunotherapy.

Keywords: CAR T cells; immunotherapy; pediatric neuroblastoma; strategy

1. Introduction

Neuroblastoma, an extracranial solid tumor that initiates from the sympathetic nervous system’s neuroendocrine tissue, is one of the most common causes of death in pediatric cancers [1,2]. It is often diagnosed during the perinatal period, which accounts for 8% in patients under 15 years. This childhood neoplasm appears each year in more than 600 cases in the United States and 200 in Japan [3-5]. According to clinical presentation, neuroblastoma is an extremely variant characteristic tumor. It ranges from an adrenal mass tumor that regresses without treatment to a metastatic tumor that causes critical illness [6]. At present, while intensive therapies can be beneficial for patients with localized disease, these therapies have frequently not been useful against patients with high-risk disease (approximately 40% of cases associated with the extent of metastases and genetic factors) nor patients with relapsed [7,8]. Hence, novel therapies based on immunotherapy are subsequently developed to improve survival for high-risk patients.

One such approach is treatment with anti-GD2 monoclonal antibody, which has already been assessed in the nodal phase III clinical trial. The use of this antibody-based therapy was compiled into a therapeutic protocol for high-risk neuroblastoma patients and disclosed the promising results [9,10]. This effectiveness has led other immunotherapeutic approaches, even though their integration into conventional multimodality therapies requires further investigation.
After discovering that GD2, a disialoganglioside highly expressed in most neuroblastomas, is also targeted by T cells, cellular immunotherapies including genetic engineering of T lymphocytes to express anti-GD2 chimeric antigen receptor (CAR) have emerged and now being studied. With the combination of antigen specificity and cytolytic capacity, anti-GD2 CAR T cells have demonstrated safety and antitumor efficacy in relapsed neuroblastoma patients [11,12]. Various preclinical studies have improved antitumor effects, proliferation, and cytokine release of CAR T cells, and some approaches have reached clinical trials (Figure 1). Currently, anti-GD2 CAR T cells approach might represent the potential therapeutic for pediatric neuroblastoma.

![Figure 1](image_url)

Figure 1. Cumulative publications of CAR immunotherapy in clinical trials.

However, unlike the success of using CAR T cells in hematological malignancies [13-15], the efficacy of CAR T cells in neuroblastomas has been limited by several factors, including tumor microenvironment, T cells exhaustion, and T cells persistence and potency, which may lead to therapeutic resistance [16-18]. Therefore, CAR T cell improvement as a feasible alternative to conventional therapies for patients with neuroblastoma is still a significant challenge to immunotherapy achievement.

In this review, we provide an updated summary of preclinical and clinical experience of CAR-based neuroblastoma therapies and discuss the improvements of CAR in different ways that could overcome clinical problems of applying this approach for treating neuroblastoma. Defining these strategies would suggest an attractive route of improving the potency of CAR immunotherapy.

2. CARs in neuroblastoma

CARs have been developed to fulfill the applicability of adoptive cellular immunotherapy for neuroblastoma in a major histocompatibility complex (MHC)-unrestricted manner of effector T cells. Effector immune cells, commonly T lymphocytes, have been genetically engineered to express an extracellular antigen-binding domain that is mostly single-chain variable fragment (scFv) joined with a transmembrane domain and intracellular signaling domain. The 1st generation CARs were designed to have a single CD3-ζ intracellular signaling domain. The 2nd and 3rd generation CARs products were improved by adding one or two costimulatory endodomains to the CD3-ζ motif to achieve the optimal activation and survival of CAR cells. Current intracellular endodomain based on the costimulatory receptors include CD27, CD28, 41BB, ICOS, and OX40 [17,19]. Each of the CAR design components reflects the variations of therapeutics achievement, and novel CAR engineering has been developed for decades to broaden CARs therapeutics in solid tumors like neuroblastoma.
2.1 Summary of CARs experience

Several CAR approaches in neuroblastoma have been developed according to discovered putative cancer antigens. There are some novel target antigens for CAR T cell therapy in neuroblastoma that have been investigated in the preclinical phase (Figure 2).

![Figure 2](image)

**Figure 2.** Target antigens conducted on the safety and efficacy of CAR therapy for neuroblastoma. Six surface antigens of neuroblastoma, including L1-CAM, GPC2, NCAM, GD2, ALK, and B7H3, are under development and investigation. L1-CAM and GD2 are only two target antigens currently in completed clinical trials for neuroblastoma (labeled with star).

Anaplastic lymphoma kinase (ALK), an oncogene expressed on neuroblastoma cells, is associated with familial neuroblastoma cases [20,21]. Anti-ALK CAR has demonstrated its effectiveness against this neuroblastoma subtype in vitro and in vivo [22,23]. This line of research also suggested that antigen density must be concerned to achieve CAR T cell potential. Another tyrosine kinase receptor that may be rendered an ideal target for CAR therapies is glypican 2 (GPC2). The high expression of GPC2 on neuroblastoma cell surface brought promising clearance of disseminated neuroblastoma in the mouse model by anti-GPC2 CAR T cells [24]. B7H3 (CD276), a checkpoint molecule expressed on neuroblastoma, is another candidate for CAR therapies of neuroblastoma [25,26]. This attractive target brought useful immunotherapeutic strategies, including monoclonal antibodies and CAR targeting B7H3. Recently, the efficacy of anti-B7H3 CAR has been being demonstrated in vivo [27,28]. Also, many target antigens that are specific to neuroblastoma cells have been more characterized. Such antigens, including NCAM (CD56), NY-ESO1, and PRAME, were investigated both in vitro and in vivo for safety and efficacy, which gained attention for further development as CAR features [29-32].

To date, only CAR T cells targeting L1-CAM (CD171) and GD2 have been reached the early phase of clinical trials [33] (Table 1). L1-CAM, an adhesion molecule in the immunoglobulin superfamily, is another suitable target in neuroblastoma [34]. Because of the specificity of CE7, the monoclonal antibody that can bind to the L1-CAM epitope, the anti-L1-CAM CAR with the scFv from CE7 was generated. This 1st generation anti-L1-CAM CAR efficacy and safety was investigated in
patients with relapsed/refractory neuroblastoma in phase 1 clinical trial [12]. To augment the persistence of anti-L1-CAM CAR, 2nd generation CAR was generated using a 41BB costimulation domain following 3rd generation CAR, including CD28 costimulation addition, which is currently being investigated in phase 1 clinical trials [35,36]. Until now, the most critical target antigen in neuroblastoma is GD2, a disialoganglioside highly expressed on neuroblastoma tissue [37]. Owing to the presence of this antigen during chemotherapy and the success of anti-GD2 monoclonal antibody therapy, this antigen has been the most studied targeted for CAR T cell therapy in neuroblastoma [38]. Many approaches of 1st generation of anti-GD2 CAR have been reports, including anti-GD2 CAR containing single-chain variable fragment (scFv) derived from 14g2a monoclonal antibody or Epstein-Barr virus-specific cytotoxic T cells transduced CAR (so-called GD2 CAR-CTL) with the fact that the prolonged persistence in vivo was associated with costimulation domain of CAR [39-42]. Anti-GD2 CAR constructs are now considered on costimulatory endodomains. The 2nd and 3rd generations of CAR were then generated for in vitro and in vivo assessments on CAR T cell survival [43,44]. The 3rd generation anti-GD2 CAR, containing an inducible caspase 9 (iC9) safety switch, has been tested in clinical trials for its safety (clinicaltrials.gov identifier NCT01953900 and NCT01822652). Various clinical trials based on CAR therapy are underway to augment the reliable therapeutic outcomes. However, improving the efficacy and persistence of CAR is still a significant issue.

Table 1. An outline of clinical trials for CAR immunotherapy in neuroblastoma.

| Clinicaltrials.gov identifier | Type of study | Target/ScFv/signaling domain | Key results | Reference |
|-----------------------------|---------------|-----------------------------|-------------|-----------|
| N/A                         | Phase I       | L1-CAM/CE7R/CD3ζ            | Partial response in one patient | [12]       |
| NCT02761915                 | Phase I       | GD2/KM8138/CD28.CD3ζ        | On target activity in bone and bone marrow were detected | [42]       |
| NCT01822652                 | Phase I       | GD2/14g2a/CD28.OX40.CD3ζ   | The modest early antitumor responses were detected | [44]       |
| NCT00085930                 | Phase I       | GD2/14g2a/CD3ζ             | Complete response in one patient | [18,45]   |
| NCT03635632                 | Phase I       | GD2/14g2a/CD28.OX40.CD3ζ   | N/A         | Unpublished |
| NCT01953900                 | Phase I       | GD2/14g2a/CD28.OX40.CD3ζ   | N/A         | Unpublished |
| NCT02107963                 | Phase I       | GD2/14g2a/CD28.OX40.CD3ζ   | N/A         | Unpublished |
| NCT03373097                 | Phase I/II    | GD2/14g2a/CD28.4-1BB.CD3ζ  | N/A         | Unpublished |

2.2 Obstacles of using CARs in neuroblastoma

2.2.1 CAR T cells persistence and exhaustion

Restrictive CAR T cell persistence has occurred as a major problem in neuroblastoma. Evidence from 1st generation of CAR studies in vivo and clinical trials suggested that the limited persistence of CAR T cells from low activation and proliferation of cells also affected the antitumor efficacy [18,43,45,46]. One clinical study demonstrated that the infused, 1st generation, anti-L1-CAM CAR cells were detectable in the peripheral blood up to 1-7 days after adoptive transfer in most patients with bulk disease but significantly longer (42 days) in a patient with limited disease burden [12]. T cell exhaustion might be a significant cause of shortening persistence. This is confirmed by
discovering the exhausted CAR T cell phenotype in GD2 CAR T cells with low-level tonic signaling [47]. The persistence of infused CAR T cells might be prolonged if the exhaustion was reduced. Thus, several methods have been proposed to increase the persistence of CAR T cells. One such way is the utilization of 2nd and 3rd generation CAR, which improves costimulation after antigen binding (e.g., 4-1BB costimulatory domain) to protect shorten persistence. This development is under investigation for feasibility [13,15,47].

2.2.2. Target selection and on-target, off-tumor effect

Ideally, the target antigen for CAR T cells should have a high expression on cancer cells, a low expression in normal cells, and not associated with oncogenesis [48]. It is known that there are challenges in the way of choosing an optimal CAR T cell target antigen in neuroblastoma is challenging since many target antigens are related to normal peripheral nerves or neural tissue expression. Toxicities caused by particular interactions between the CAR and its target antigen expressed by normal cells termed on-target, off-tumor effects have been reported in previous CAR studies in solid tumors [49-52]. One clinical trial in metastatic colon cancer reported the pulmonary infiltration by CAR T cells that cause a systemic cytokine storm in patients who received HER2-targeted CAR T cell therapy, demonstrating strong evidence of on-target, off-tumor toxicities [51]. On the other hand, there is no such effect in pediatric sarcomas study using anti-HER2 CAR T cells and anti-GD2 CAR T cells in neuroblastoma studying [42,53,54]. This evidence suggested that the variation of antigen density on the different types of cancer is an additional factor to concern during target selection to avoid on-target, off-tumor effects.

2.2.3. Tumor microenvironment

Unlike the remarkable success of CAR T cells in the treatment of hematological malignancies, the efficacy of CAR T cells in neuroblastoma can be obstructed by the immunosuppressive tumor microenvironment (TME), which is a manifest barrier to achieve full effective CAR T cell therapy for solid tumors [55]. Significant factors derived from TME in neuroblastoma that include immunosuppressive cells like tumor-associated macrophages (TAMs), type 2, regulatory T cells (Tregs) and myeloid-derived suppressor cells (MDSCs) which are attributed to poor result of CAR therapy [16]. Another factor is inhibitory ligand present in the TME, such as PD-L1, the ligand for an inhibitory receptor expressed on activated T cells, named PD-1 [56,57]. Remarkably, this habitual expression of the inhibitory ligand in neuroblastoma can cause the loss of CAR T cells [58]. In addition to inhibitory signals, the availability of soluble factors in TME including galactin 1 and 3, TGF-β, and IL-10 can trigger T cell inhibitory pathways or inhibit T cell function [57,59-62], while secretory HMGB1 may be responsible for Treg differentiation in neuroblastoma TME [63]. Furthermore, there are physical barriers that prevent tumor access of T cells, such as protease fibroblast activation protein (FAP) expressed by tumor-associated stromal fibroblasts, extracellular matrix (ECM), and immunosuppressive tumor vasculature like vascular endothelial growth factor (VEGF) [64,65].

2.3.4. CAR trafficking

Trafficking of CAR cells into solid tumor sites to exert antitumor activity needs to be improved, especially in neuroblastoma. Several chemokines that can mediate immune cell trafficking are generally excreted by tumor or stromal cells like CC-chemokine ligand 17 (CCL17), CCL22, and CCL2 to enhance localization of immune cells [66]. Moreover, suitable trafficking of immune cells, like T cells, can occur when there is an upregulation of a chemokine receptor that is matched to chemokine related trafficking on T cells. However, in a previous study using CAR T cells derived from neuroblastoma patients, low expression of CCR2, a chemokine receptor, was detected [67-69]. Thus, various approaches to generate CAR T cells with an ability to traffic to neuroblastoma sites are underway.

3. Strategies to improve CARs in neuroblastoma
Remarkable the engineering of T lymphocytes to target tumor-associated antigens by forced expression of CARs has been successful against CD19+ leukemia [70-72]. Similar results have not yet been achieved against neuroblastoma [73-75]. The key factors that challenge the success of CARs immunotherapies are the immune effector cells, the design of CARs construct, and the intrinsic tumor factors. The proposed strategies for improving CARs efficacy in neuroblastoma therapy are illustrated in Figure 3.

Figure 3. Proposed strategies to improve CARs efficacy against neuroblastoma. To enhance the anti-tumor toxicity of chimeric antigen receptor (CAR) T cells, several strategies can be applied; (a) The use of extracellular vesicles (EVs) inhibitor. EVs are cargo ships containing functional molecules that enhance tumor growth and metastasis, and may decoy CAR immunotherapy. Inhibiting secretion of EVs from tumor cells may improve killing efficiency CAR T treatment; (b) Engineering CAR T cell with suicide gene. To improve CARs, and any side effects of CARs, fusing iCaspase-9 gene in CARs structure can break T cell activations via induction of apoptosis in CAR T cells; (c) Engineering CAR T cells secreting cytokine to support function. IL-15 cytokines showed ability to increase CARs anti-tumor toxicity in both T and NKT cells; (d) Use of T cell subsets with greater proliferative ability. Naïve and central memory effector cells have potential to both enhance CARs efficacy and persistency of immune effector cells; (e) Use of different effector cells with greater function. For various solid tumors, CARs-derived from NK, NKT or γδT cells showed more penetration into tumor sites and more potent toxicity than CAR T cells; (f) Engineering CAR T cells to overcome TME and increase penetration. Supplementation with tumor-enriched chemokine inhibitor or using extracellular matrix remodeling enzyme during treatment might be improve the trafficking and infiltration of CARs T cells; (g) Finally, engineering CAR NK cells to support function of CAR T cells.
Inhibition of immunosuppressive cells function in TME via CAR NK cells can enhance homing and efficacy of neuroblastoma-targeted CAR T cell.

3.1 Improving effector immune cells

Solving the CARs T cell obstruction or use of other effector cells might increase CAR’s tumor-killing activity on neuroblastoma. Regarding the killing effect against tumor cells, natural killer T (NKT) [76], gamma-delta T (γδT) [77], and natural killer (NK) [78] are the valuable candidates for generating CAR-derived cells. These immune cells have different pros and cons. Thus, choose the proper effector cell for each tumor targets is extremely important. This part will assemble information about these CAR-derived effector cells, some of which are summarized in Table 2.
Table 2. Comparison of CAR-derived effector cells for neuroblastoma.

| Effector cell | Ex vivo expansion | Type of target cells | CAR design | Combination |
|---------------|-------------------|----------------------|------------|-------------|
|               |                   | Localized            | CTC        | Cytokine    | Effector cell | Signaling inhibitor |
| T             | ***               | *                    | ***        | Dim expression | IL-2, IL-7, IL-15 | NKG2D.CAR NK | anti-PD-1/PD-L1, anti-IL-10, IL-6 inhibitor |
|               |                   |                      |            | High expression |                 |                  |
|               |                   |                      |            | target: CD28 |                 |                  |
| NK            | **                | ***                  | ***        | CD28, 4-1BB, CD3ζ and DAP12 | IL-2, IL-7, IL-21 | NB-targeted CAR T | anti-PD-1/PD-L1, anti-TGFβ, anti-IL-10, IL-6 inhibitor |
| NKT           | ***               | ***                  | ***        | CD28 | IL-2, IL-7, IL-15 | N/A | anti-PD-1/PD-L1, anti-IL-10, IL-6 inhibitor |
| γδT           | ***               | ***                  | ***        | Both CD28 and 4-1BB, CD3ζ | IL-2, IL-7, IL-15 | N/A | anti-PD-1/PD-L1, anti-IL-10, IL-6 inhibitor |

Score: *** = high, ** = moderate, * = low; CAR, chimeric antigen receptor; CTC, circulating tumor cells; DAP12; DNAM1, DNAX activating protein 12; ECM, extracellular matrix; IL, interleukin; NA, not applicable; NB, neuroblastoma; NK, natural killer cell; NKG2D, natural killer cell receptor D; NKT, natural killer T lymphocyte cell; PD-1, program cell death-1; PD-L1, program cell death-ligand 1; T, T lymphocyte; γδT, gamma delta T lymphocyte.
3.1.1. T lymphocyte

To date, the immunophenotype of T cells is well understood as an essential parameter in safety and efficacy features in CAR T products [74,79]. The ratio of subtype composition, available of naïve and central memory T (Tcm) cell population, is essential to increase the therapeutic efficacy of CAR T cells. Shah NN. et al., reported that enriching the CD4+ and CD8+ in starting T cell population before CAR-lentiviral transfection can enhance the efficacy of CAR T cells on neuroblastoma [80]. Also, the 1:1 ratio of subtype composition between CD4+ and CD8+ possibly promotes high efficiency with more safety in patients [75]. Furthermore, another study exhibited an increased percentage of CD4+ T cell and CD45RO+CD62L− Tcm in CARs treatment of neuroblastoma shown to prolong the persistence of CAR T cells in clinical trials [74]. Interestingly, CAR T cells derived from naïve T cells and Tcm cells strongly proliferated ex vivo resulting in 89% of CAR T cell population [81]. Likewise, the procession of CD62L+, a standard marker of memory cells, in CARs population is more effective against both hematological malignancy and solid tumors, and promoted longer persistence of peripheral NKT and T cells [82-84]. Because of timing in infiltration into the tumor site, recognizing the tumor antigen, and performing their function, the memory subtype of CAR T cells is exigency [85]. The selection of T cell subtype population during CAR production or before administration to patients is recommended for improving the efficacy of CARs T cell against neuroblastoma. Nonetheless, CAR T cells are mostly detected in peripheral blood but have lower tumor infiltration than other effector cells such as NKT and NK cells. Thus, a combination of other infiltrated-effector cells or ECM remodeling enzymes may enhance tumor infiltration of T cells. In contrast, neuroblastoma-targeted CARs T cells may be more appropriate for eradicating the circulating neuroblastoma cells in patients’ blood and lymphatic system.

3.1.2. Natural killer cells

Natural killer (NK) cell, a lymphoid component of the innate immune system, was also applied in CARs immunotherapy against both hematological malignancy and solid tumors [86-88]. NK cells can localize, migrate, and infiltrate into tumor sites to elicit MHC-unrestricted cytotoxicity and proinflammatory cytokines and chemokines against solid tumors [89]. In addition, NK cell showed the antitumor responses via NKG2D cytotoxic adapter molecule DAP10 to eliminate MDSCs, the immunosuppressor cells in neuroblastoma TME. These leading other lymphocytes, such as T cell infiltration into tumor [90], NK cells might requisite proper CARs constructs such as costimulator and/or signaling molecule, which are discussed in the next topic. Several researchers informed that antitumor properties of CARs NK cells were downregulated by suppressive molecules of the TME, such as TGFβ, thereby limiting the tumor-killing capability of NK cells [90,91]. In this direction, a combination with the inhibitor of immunosuppressive molecule (e.g., anti-TGFβ) might increase the antitumor activities of neuroblastoma-targeted CARs NK.

3.1.3. NKT cells

Vα24-invariant natural killer T cells (NKTs) are a subset of innate lymphocytes that share T and NK cells properties [92,93]. The first use of CARs NKT cells against neuroblastoma had been reported since 2014 [76]. Both 2nd and 3rd generation of anti-GD2 CAR NKT cells exhibited potent antitumor activity and significantly presented prolong survival without any graft-versus-host disease in a mouse xenograft model [76]. Unlike T cells, NKT effectively traffic and infiltrate into the tumor site [83] to mediate antitumor activities, inhibit tumor-supportive macrophage, and transactivate the localized NK and CD8+ T cells [94,95]. Additionally, a high percentage of NKT cells in tumor-infiltrating lymphocytes have been detected in neuroblastoma than in patients’ peripheral blood [96]. Likewise, Xu X. et al. reported that CARs NKT cells enhanced CARs treatment efficacy through their persistence property and provided the high localization onto the tumor site without significant histopathological toxicity [97]. These suggested that NKT cells are easily infiltrated into neuroblastoma than T lymphocyte. Moreover, the first CAR NKT cell trial is now recruiting
(NCT03294954) [97]. Neuroblastoma-targeted CARs NKT cells are noticeable as the potential effector cells against neuroblastoma and other solid tumors. Of note, an exhausting phenotype of NKTs upon in vitro stimulation is associated with the upregulation expression of PD-1 and TIM-3 [83]. Thus, CAR NKT cells should be used in combination with anti-PD-1 or anti-TIM-3 to generate an intense tumor-killing effect.

3.1.4. γδT cells

γδT cells are innate T lymphocytes that independently recognize MHC targets, have a natural killing activity against pathogens, and respond to the cells [98-100]. Normally, neuroblastoma mostly escapes from the immune system via the downregulation of MHC class I molecule [96]. Thus γδT cells have the potential to solve this problem. Additionally, γδT cells have been found in neuroblastoma tumors [96] and a wide variety of tissues [101]. These cells can differentiate into professional antigen-presenting cells (pAPCs), which present the antigenic fragments for CD4+ and CD8+ αβT cells [102]. γδT cells expressing the Vγ9Vδ2 TCR is a common subset that can be activated and proliferated ex vivo for more than 1000 fold expansion [103]. Although γδT cells exhaustion is associated with PD-1, an immune checkpoints molecule [104], γδT cells have lower expression of PD-1 compared to αβT cells, thereby recognized as a promising effector cell for CAR immunotherapy. Like T cells, the naïve and memory phenotype of γδT cells sufficiently survive to promote persistence of CAR γδT cells in the recipients without decreasing the killing activity against tumor cells [98]. From these properties, γδT cell is a potential candidate for using as effector cells in adoptive CAR immunotherapy against neuroblastoma.

3.2 Modification of CARs constructs

The CAR construct design is one of the key features that affect the kinetics of expansion and the duration of persistence. CARs are recombinant receptors responsible for both antigen-binding and activation functions for immune cells [105]. The 2nd generation CAR offers a clinical benefit via both TCR stimulatory domain (CD3z) and a single-co-stimulatory domain [80]. Recently, FDA-approved products contain either CD28 or 4-1BB (CD137) costimulatory domain [70]. Choosing the appropriate combination of costimulatory ligands, chimeric costimulatory receptors, or cytokines in CARs structure to each objective immune cell may differently enhance the effector cell function, proliferation, and survival [105]. The well-design of CAR structures can overcome the significant barrier of CARs in cancer immunotherapy.

3.2.1. Hinge and transmembrane domain

The access of CAR to the target antigen is depended on their flexibility and length of hinge and transmembrane domain [106]. In the case of anti-CD19 CAR T cells, human CD8α hinge and transmembrane molecule promoted efficacies indifferently against tumor xenograft but induce lower cytokine levels compared to CD28 hinge and transmembrane [107,108]. These confirmed that the length and composition of hinge provided antigen binding and signaling cascade through the CAR T cells. Accordingly, the CD8α hinge and transmembrane domain may be an excellent choice to generate neuroblastoma-targeted CARs. However, further studies on this topic are required to pursue a suitable composition that offers the highest tumor-killing efficacy but bestows the patients' low or no side effect.

3.2.2. Costimulatory domains

Costimulator molecules play a critical role in developing, activation, and functional response of effector immune cells [83]. A set of costimulatory receptors that noticeable in human T cells are including; CD28, 4-1BB, and OX40 [83], while the costimulators for activating NK cells are composed of CD28, 4-1BB, 2B4, and NKG2D [109,110]. For creating CAR T cells, CD28 has more potent signaling through T cell activation with a short persistence, while 4-1BB provides a less potent signal but longer persisted [111]. To address cytokine releasing syndrome (CRS), CD28 co-stimulator is worthy of the
dim target antigen, whereas 4-1BB is suitable for the high density of target [108]. Unlike T cells, CD28 costimulator is suitable for enhancing proliferation, cancer-killing function, and persistence of NKT cells. Even though CD28 and 4-1BB prevent the loss of CD62L expression that is important to prolong the survival of NKT cells [83], the CD28 costimulator also forceful the CAR NKT cell to massive expansion while 4-1BB induce excessive activation leading to cell death [97]. For CAR γδT cell, both CD28 and 4-1BB [112] were applied as the costimulator [113,114]. However, data on the comparison and optimization of costimulators for CAR γδT cells are scant. For NK cells, CD28 is mostly used as a costimulator. Oelsner S et al. suggested that CD19-CAR NK with CD28 costimulator showed better performance against B-cell malignancy than 4-1BB [115]. Also, fusing DNAM1 and 2B4 costimulatory domains in CAR NK design can enhance cytotoxicity against hepatocellular cancer cells in vitro [116].

3.2.3. Signaling-transducing domain

The CD3ζ is commonly used as the signaling molecule in CAR constructs to activate T and NK cells functions for both hematologic malignancy and solid tumors [71,80,107,117]. Immunotherapy resistance and CRS are associated with highly active CD3ζ-activated CAR T cells [118,119]. Implementing other activation domains in CAR design should be considered an alternative to provide a robust clinical advantage in relieving the risk of CRS and overcoming these barriers.

DNAX activation protein of 12 kDa (DAP12), an immunotyrosine-based activation motifs-containing adaptor, is constitutively expressed in NK cells while expressed in a subset of human T cells [120]. The first use of DAP12 as the alternative cytoplasmic domain in CAR design was emerged in 2015 [121]. The chimeric target receptor fusing with the transmembrane and cytoplasmic domains of KIR2DS2, a stimulatory killer immunoglobulin-like receptor, and DAP12 (KIR-CAR DAP12) triggered robust antigen-specific proliferation, effector function, and enhanced antitumor activity in leukemia cell lines [121]. Although only a few studies used the DAP12 as the newly activation domain in CAR-modified T cells, the low cytokine markers of CRS could be observed in DAP12-based CARs preclinical studies [118]. Comparing to CD3ζ-based 2nd generation CARs, T cells modified with the natural killer group 2D (NKG2D) ectodomain combining with 4-1BB and the DAP12 signaling domain release lower levels of interferon-gamma (IFN-γ), Tumor necrosis factor-alpha (TNF-α), and interleukin-2 (IL-2) during tumor cell lysis without significant difference of tumor-killing effect both in vitro and in vivo [118]. DAP12 is also involved in signal transduction of activating NK cells [122]. DAP12-based CARs NK cells could eradicate neuroblastoma in the mouse xenograft model [122]. This DAP12 might be the new brilliant signaling molecule for high efficacy with more safety in CARs development

3.2.4. Others generation of CARs structures

Although the 2nd generation of CARs is mostly used in neuroblastoma clinical trials, the 4th generation CAR has been developed. With neuroblastoma-targeted antigen incorporated multiple costimulatory molecules and a suicide gene inducible caspase9 (iCasp9), the success of 4th CAR T cells against MYCN amplification neuroblastoma has been reported [123]. This 4th generation CAR exhibited higher tumor-killing activity than the 3rd generation CAR with a safety profile, leading to 4 years survival of a neuroblastoma patient after 4th generation CAR treatment [123]. Successful of this 4th generation CAR T cells is explored to the new era treatment for neuroblastoma and other solid tumors.

3.3 Overcoming TME

The intrinsic tumor factors, including extracellular matrix (ECM) and the TME, are the critical challenges for CAR immunotherapy against neuroblastoma [124]. The counteracts for these tumor escape mechanisms are showed in this topic.

3.3.1. ECM: trafficking and infiltration of effector cells
The low T cell infiltration has been observed in neuroblastoma so-called immunologically “cold” tumors [125]. Various studies demonstrated that poor trafficking and limited CAR T cell persistence to solid tumors are significant burdens in CAR immunotherapy [126]. Tumor chemokines and their receptor play an essential role in TME [127]. Chemokines also mediate proliferation, infiltration, and persistence of leukocytes [128], which affected tumor control [129]. Aberrant expression of tumor chemokine-chemokine receptor network affected several tumor biological functions, i.e., stimulating tumor cell proliferation, inducing tumor angiogenesis [130], enrolling inhibitory immune cells [131], preventing recruitment of effector lymphocytes [132], and resisting their antitumor response [133], leading to cancer immune escape [134]. Increased trafficking ability of CAR T cells via presenting the matched chemokine receptors or adding therapeutic agents against tumor-enriched chemokines may be a practical strategy for neuroblastoma and other solid tumor treatments [135]. For example, in hepatocellular carcinoma in which the high expression level of CXCR2 ligand was observed, Liu G, et al., confirmed that using CXCR2 CAR T cells could enhance in vivo trafficking and tumor cytotoxicity [127]. Additionally, the fusion of IL-7 and CCL19 on CAR structure showed increased infiltration and exhibited high antitumor activity of CAR T cells [136]. Moreover, given the prevalence of IL-8 production in human cancer [137], this approach may find broad applicability in the potentiation of CAR T cell immunotherapy for solid tumors. Accordingly, the tumor chemokine-chemokine receptor network in neuroblastoma should be identified. Studies showed that VEGF, IL-6, and IL-10 receptor (IL-10R) in neuroblastoma were associated with poor outcomes [138-140]. Thus, the fusion of anti-VEGF, anti-IL-6, or anti-IL-10R into CAR structure may also be effective against neuroblastoma.

3.3.2. Myeloid-derived suppressor cells (MDSCs)

MDSCs are the immunosuppressive immune cells found in TME [141,142]. These MDSCs enhance tumor growth and suppress the infiltration, proliferation, and tumor-killing activity of CAR T cells [143]. These cells also express PD-L1 themselves, which contribute to immune escape in various solid tumors [144]. Researchers found that NKG2D ligand, a cytotoxicity receptor activated by non-classical MHC molecules, is overexpressed on these tumor-infiltrating MDSCs [145,146]. Combination with NKG2D-CAR NK cells enhanced tumor infiltration and expansion of CAR T cells at tumor sites and prolonged CAR T cell survival compared to single treatment [90,147]. The mixed NKG2D-CAR NK and neuroblastoma-targeted CAR T cell treatment may safely enhance antitumor activity against neuroblastoma that are supported and protected by MDSCs.

3.3.3. Tumor extracellular vesicles

Extracellular vesicles (EVs) are the lipid bilayer vesicle released from the cells [148], which can be classified into three major groups; exosome, microvesicles, and apoptotic bodies, based on their size [149]. Like cargo, EVs containing soluble proteins such as growth factors, cytokines, and chemokines [150], lipids, metabolites, nucleic acids, including regulatory microRNAs (miRs) [149,151]. Several studies reported that EVs play a crucial role in tumor-tumor [152,153] and tumor-immune cell communications [154] and affect tumor cell phenotype and metastatic potential [155-157]. Neuroblastoma cells release EVs in their extracellular space [150]. Previous studies showed that neuroblastoma-EVs induced the production of pro-tumorigenic cytokines and chemokines such as IL-6, IL-8, VEGF, and CCL2 by MSCs [158]. Additionally, neuroblastoma-EVs also trigger a proinflammatory response in monocytes and promote neuroblastoma chemoresistance [159]. Future studies aim to reveal the exact roles of EVs in the communication between neuroblastoma and TME is required to decipher a novel treatment strategy against neuroblastoma TME.

3.4 Combination of CARs

3.4.1. PD-1/PDL-1

Program cell death 1 (PD-1 or PDCD1) is a coinhibitory receptor expressed by all CAR effector cells, including αβT [160], γδT [113], NKT [97], and NK cells [104]. PD-1 binding to its ligand, PD-L1
[or CD274], leading to a decrease in effector cell receptor downstream signaling and diminishing T cell activation [161]. Combining with PD-1/PD-L1 inhibitor is recommended for turning neuroblastoma microenvironment to response to immunological treatment. These might enhance the efficacy of CAR therapy against neuroblastoma as well [162,163].

3.4.2. Cytokines

Another approach to improve the potency of CARs is to genetically modify the effector cells secreting pro-inflammatory or pro-proliferative cytokines, aiming to strengthen effector function, proliferation, persistence, and to alter the TME [105,164]. The cytokines IFN-γ, IL-2, IL-7, IL-12, IL-15, IL-18, IL-21, and IL-27, promoted neuroblastoma regression via enhancing the efficacy of effector cells, whereas VEGF, IL-6, IL-10 induced neuroblastoma progression through their immunosuppressive activities. Several studies reported that increasing the proinflammatory chemokine IL-8 levels in patients was related to poor prognosis in neuroblastoma and other solid tumors [165,166]. This information is useful for the new design of CAR construct or combination therapy to enhance the efficacy of CARs against neuroblastoma.

To date, IL-15 cytokine has been studied for its ability to enhance CAR antitumor. IL-15 plays a crucial role in T and NKT cells [97,167] via enhanced CAR antitumor activity and survival in peripheral blood and tumor tissue [167]. This study suggested that IL-15 conjugated CD28-CAR structure reduced the exhausting marker, improved the persistence and the tumor-killing activity of NKT-established CARs. Recently, Xin Xu, et al., utilized mice bearing neuroblastoma xenografts to demonstrate that GD2-CAR IL-15 NKT cells enhanced in vivo persistence, increased localization to tumor sites, and improved tumor control as compared with GD2-CAR NKT cells [97]. Interestingly, IL-15 conjugated GD2 CAR T cells reduced the expression of the PD-1 receptor [167]. These characteristics have made IL-15 a promising candidate for CAR T (or NKT) design for neuroblastoma immunotherapy.

4. Future perspective

The strategies to improve CAR T cells using in neuroblastoma are mostly concerned with increasing the antitumor activity and persistence of the infused CAR T cells. Therefore, discovering new tumor antigens that are more restricted to neuroblastoma with less neuronal toxicity is very necessary. The evaluation of new target antigens such as B7H3 or o-acetyl-GD2 (oaGD2) is on-going [168,169]. Researchers can also modify the construct of CAR T cells to be more effective by adding new costimulatory endodomains such as 4-1BB or ICOS, which can be seen in the 4th generation of CAR therapy [170,171]. Seeking a way to overcome the immunosuppressive TME in neuroblastoma is a critical challenge in CAR studies. Various trials of CAR T cell therapy in neuroblastoma, which were relied on the strategies mentioned above, are underway validating to improve outcomes. Alternatively, administration of antitumor related cytokine might enhance immune response and amplify the direct neuroblastoma cell killing of CAR cells in complex TME. For example, IL-15 has been used to improve in vivo persistence and antitumor activity against neuroblastoma. Hence, another area of interest in CAR structure design is the induction of cytokine expression.

Given all these findings, understanding the mechanism of TME affects the resistance of CAR cells and inhibits the in vivo antitumor function is vastly relevant. The exploration of exosomal miRs or exosome decoy released within neuroblastoma TME may be essential alternatives to embrace the barriers posed by the TME. Furthermore, by understanding molecular mechanisms within the TME, researchers can efficiently engineer molecular targeting CAR T cells to restore TME resistance sensitivity.

Lastly, a more practical approach for CAR T cell manufacturing is still in need. The ability to manufacture prompt quantities of an efficacious cell product is required for neuroblastoma patients with varied conditions. In the end, good manufacturing might help to create highly active CAR T cells for patients with relapsed disease in time.

5. Conclusions
In summary, immunotherapy using CARs in neuroblastoma treatment has shown promising efficacy and safety. To utilize the therapeutic benefits of CARs as the first line of immunotherapeutic approach in patients with high risk or relapsed neuroblastoma, several approaches have been raised further to augment the antitumor function of CAR-modified cells in vivo. However, unlike the remarkable success in hematological malignancies, various barriers restrict the effective use of this CAR treatment in clinical trials, as indicated earlier. The strategies provided in this review may offer suitable approaches to address the benefits of CAR therapy in neuroblastoma.

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