



Anti-Tumour Treatment

Chimeric antigen receptor T (CAR-T) cell immunotherapy for sarcomas: From mechanisms to potential clinical applications

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ABSTRACT

Survival rates for sarcoma patients have plateaued in the past few decades and remain especially grim for those with recurrent or metastatic disease. This has prompted investigation into novel immunotherapies for sarcomas, especially after their recent and well-recognized successes in other cancers. One such modality, the Chimeric Antigen Receptor (CAR) T Cell therapy, has shown promising results in treating B-cell lymphoma and acute lymphoblastic leukemia. This novel therapy functions by fusing a specific antibody derived single-chain variable fragment (scFv) with a T-cell which recognizes a specific tumor-associated antigen (TAA). Several sarcoma-associated antigens (SAA) amenable to CAR-T cell treatment have recently emerged with encouraging results. These include human epidermal growth factor receptor 2 (HER2), disialoganglioside (GD2), interleukin 11 Receptor Subunit Alpha (IL-11RA), fibroblast activation protein (FAP), B7-H3, CD44v6, insulin-like growth factor 1 receptor (IGF-1R), and tyrosine kinase orphan-like receptor 1 (ROR1). Given the limitations of current medical therapies, novel treatment strategies are urgently needed. As a sarcoma treatment modality, CAR-T cell therapy is highly promising and continues to draw interest especially as new clinical trials emerge. Here we review recent breakthrough CAR-T cell studies in sarcoma, the targets which define them, and approaches to minimizing host cytotoxicity.

Introduction

Sarcomas are a group of mesenchyme-derived solid tumors with more than 50 histologic classes. They are broadly divided into bone or soft tissue subtypes. Approximately 16,250 patients are expected to receive a sarcoma diagnosis in the United States for 2019, with 3500 having the bone subtype and 12,750 having the soft tissue subtype [1]. Total deaths are estimated at 6930 annually, at a rate of 0.4 and 1.3 deaths per 100,000 people in bone and soft tissue sarcomas, respectively [1,2]. The conventional treatment strategy for localized sarcoma is complete surgical resection with adjuvant or neoadjuvant radiation [1]. Perioperative chemotherapy has been increasingly demonstrated to improve overall survival and disease-free survival in bone and high-risk soft tissue sarcoma treatment [3–6]. However, the prognosis for sarcoma patients has not improved significantly over the past few decades. According to data from the Surveillance, Epidemiology, and End Results (SEER) Program from 2008 to 2014, the five-year overall survival for

patients with bone or soft tissue sarcoma are 66.9% and 64.9%, respectively [2]. Additionally, the sarcoma recurrence rate remains high at approximately 35%. Sarcoma patients with metastasis have even worse outcomes, with a 10 – 25% five-year overall survival rate and a median overall survival of 1.5 – 2 years [1,2]. Despite increasing efforts on targeted therapies, including tyrosine kinase inhibitors (e.g., pazopanib, sorafenib, regorafenib) for soft tissue and bone sarcomas, clinical results have not significantly improved in these patients [7–12]. Given the limitations of current medical therapies, novel treatment strategies are urgently needed to improve sarcoma patient outcomes.

Cancer immunotherapy is rapidly expanding and divided into three main categories: tumor vaccinations, adoptive T cell transfer (ACT), and immune checkpoint blockade. Recently, advanced *ex vivo* cellular engineering has shown ACT to have durable clinical responses in various cancers. In brief, the ACT method modifies patient-specific T cells for enhanced targeting of tumor-specific antigen. The three ACT types used for cancer immunotherapy include tumor-infiltrating lymphocytes

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(TILs), T cell receptor (TCR) T cells, and CAR-T cells [13]. Of note, CAR-T cells have shown great success in treating CD19 + B-cell lymphoma and acute lymphoblastic leukemia, leading to their recent approval by the FDA as a new cancer treatment modality. Subsequent interest has been generated for CAR-T cell-based technology for sarcoma therapy [13]. Here we review the basis of CAR-T cell immunotherapy and evidence for clinical application in sarcoma treatment.

Mechanism of CAR-T cell therapy

CAR T cell therapy relies on the manipulation of autologous T cells *ex vivo*. In short, a specific antibody derived single-chain variable fragment (scFv), acting as the specific receptor, is fused with a T-cell signaling domain. When reintroduced *in vivo*, the T-cell can be activated after binding of the specifically engineered antigen binding site without the need of a major histocompatibility complex (MHC). The current CARs are categorized into three generations according to their signaling compartments and have three major components [13]. First, the extracellular antigen identification component (scFv) recognizes tumor-associated antigen (TAA). Second, the transmembrane component (CD3, CD8, CD28, or FcεRI) fixes scFv to the surface of T cells and transduces the signal. The final component is the intracellular signal transduction segment (CD8, CD28, or CD137 intracellular area and CD3ζ), which contains the immune-receptor tyrosine-based activation motif (ITAM) (Fig. 1). While the first-generation CARs only contain an intracellular CD3ζ domain, the second- and third-generation CARs have

additional co-stimulatory domains [13] (Fig. 1). Various co-stimulatory molecules such as CD28, 4-1BB (CD137), and OX40 have been used to enhance CAR-T cell proliferation and survival. Recently, newly designed so-called “fourth-generation CAR” constructs have been equipped with nuclear factor of activated T cell-responsive expression elements, allowing for inducible transgenic products (Fig. 1). Currently available expressible components of these fourth-generation CARs include pro-inflammatory cytokines such as IL-12, IL-8, IL-9, IL-15, and IL-18. These T cells redirected for universal cytokine killing (TRUCK engineered T cells) are advantageous in that they can release tailored cytokines upon CAR-T cell activation. When a cytokine such as IL-12 is released, it increases CAR-T cell activation. Additionally, TRUCKs activate innate immune cells such as NK cells and macrophages, which in turn support the antitumor response by destroying the antigen-negative cancer cells which CAR-T cells are unable to recognize as they lack a CAR-recognized target [14,15]. Another advantage of fourth-generation CARs is that the cellular and potentially toxic cytokine milieu is more precisely controlled by the CAR-T, as there is an additional level of control within their highly engineered transcription mechanism.

The differences between TCR-T cell therapy and CAR-T cell therapy are summarized in Table 1 [13]. Briefly, TCRs consist of α and β-subunits associated with a surface CD3 signaling complex, whereas CARs contain scFv derived from the variable domains of tumor targeting antibodies and a CD3ζ chain as well as other less specific costimulatory elements [13] (Fig. 1). Hence, TCR T cells require the TCR to interact with TAA bound to MHC on the tumor cells or antigen-presenting cells

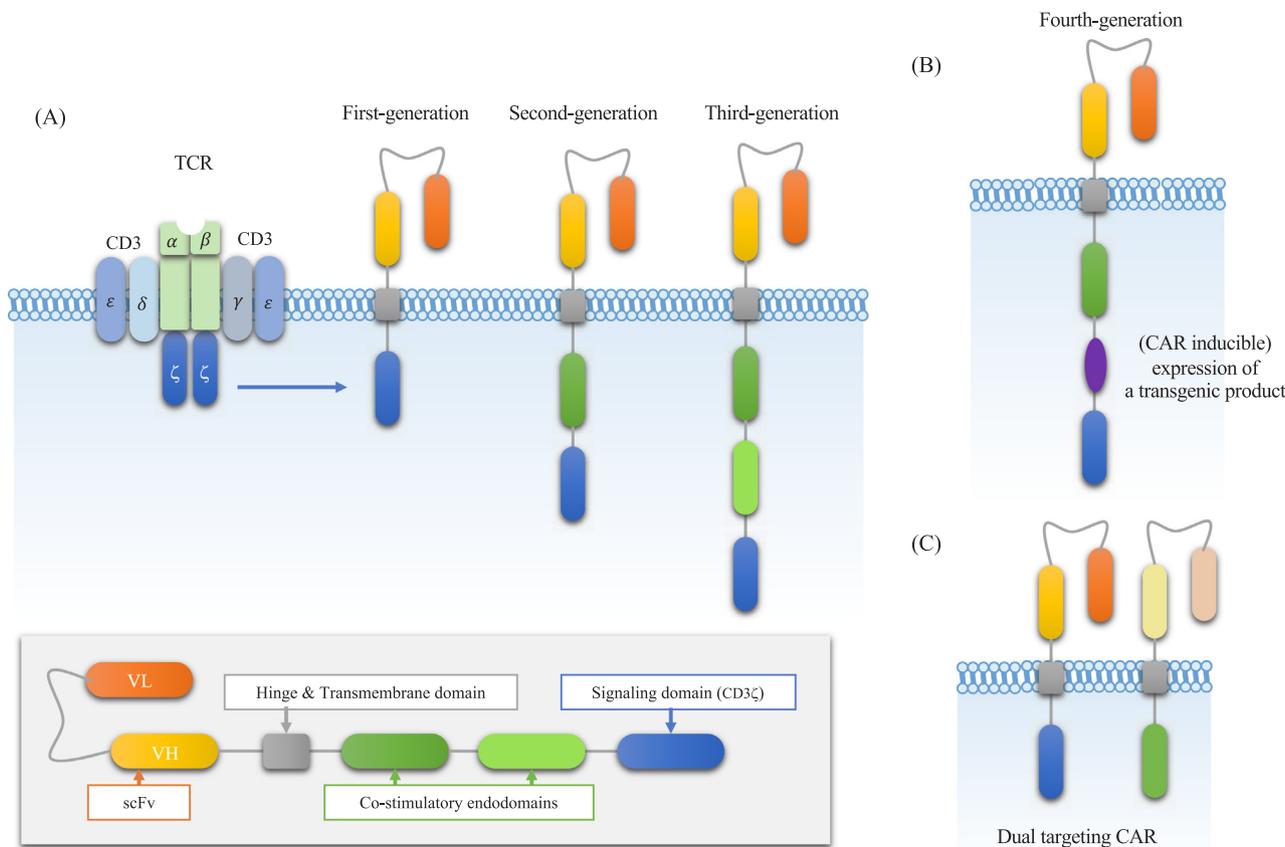


Fig. 1. Structural features of chimeric antigen receptors. An antigen-binding domain typically consists of variable heavy (VH) and light (VL) chains from a monoclonal antibody assembled through a linker sequence to form a single chain variable fragment (scFv). (A) In first-generation CAR, the scFv is coupled via a hinge and transmembrane domain to an intracellular T cell signaling domain, typically the CD3ζ of the T cell receptor. In the second and third generation CARs, the structure contains an additional one or two co-stimulatory domains, respectively. (B) the novel fourth generation CAR is equipped with a nuclear factor of activated T cell responsive expression element, for an inducible transgenic product such as interleukin-12. (C) A dual-targeting CAR with two CARs expressed by the same T cell, each targeting an independent antigen. T cell signaling domains are split between the two CARs to enable optimal T cell activation only upon simultaneous engagement with both antigens.

Table 1
Comparison between CAR- and TCR-T cell therapies [13].

CAR-T cells therapy	TCR-T cells therapy
T-cell signaling amplification from synthetic biology	T-cell signaling amplification derived by evolution of the TCR
Avidity-controllable	Low-avidity, unless engineered
CAR targets tumor surface structure such as proteins, glycans	TCR targets intracellular proteome of the tumor cell
MHC-independent recognition of tumor targets	MHC class I expression and HLA matching requisition
Cytokine release syndrome, which could be more severe than with TCR T cell therapy	Off-tumor target toxicity, which is difficult to predict

Abbreviations: chimeric antigen receptor (CAR), human lymphocyte antigen (HLA), major histocompatibility complex (MHC), T cell receptor (TCR).

(APCs), whereas CAR-T cell activation is MHC independent. Although the TCR-MHC interaction is normally important for reducing host autoimmunity, it is also a barrier for designing precision immunotherapy. Moreover, in the case of relatively low affinity TCR binding, there is potential for cross-reactivity and off-target neurotoxicity and cardiotoxicity [13,16]. In contrast, the absence of MHC restriction in CAR-T cell therapy offers several advantages. It circumvents immune-evasion if HLA expression is modified while also maintaining its TCR binding affinity and antigenic intracellular processing [13,17].

The general workflow for CAR-T cell therapy involves collecting T cells from the patient with subsequent *ex vivo* activation. The CAR molecules are then introduced to the T cells by retroviral or lentiviral transduction. Those CAR-T cells with preferred expression patterns are then selected and expanded, and eventually infused back into the patient (Fig. 2). Recent works have identified several novel candidate TAAs amenable to CAR-T cell therapy in various sarcomas with promising results at the pre-clinical and clinical level.

Application of CAR-T cells in sarcomas

Human epidermal growth factor receptor 2 (HER2)

HER2 (also known as Erb-b2 receptor tyrosine kinase) is a member of the human epidermal growth factor receptor (HER) family, which includes EGFR (HER1), ERBB2 (HER2), ERBB3 (HER3), and ERBB4 (HER4). A major HER ligand is epidermal growth factor (EGF) and transforming growth factor- α (TGF- α), which promotes downstream signaling of pathways such as Ras/Raf/MEK/ERK1/2 as well as the phospholipase pathway [18]. HER2 can exhibit both ligand-dependent and ligand-independent activity [18]. In breast cancer, HER2 is routinely tested for as it informs prognosis, metastasis, and treatment [19]. Aside from acting as a biomarker, HER2 induces tumor cell proliferation, angiogenesis, migration, and survival. In an effort to mitigate its role in oncogenesis, targeted HER2 monoclonal antibodies such as trastuzumab have been developed and significantly improved outcomes in HER2-positive breast cancer patients. HER2 overexpression has also been reported in other solid tumors such as medulloblastoma, gastric, endometrial, esophageal, and lung cancers [20]. Importantly, overexpression or amplification of the HER2 gene has also been observed in

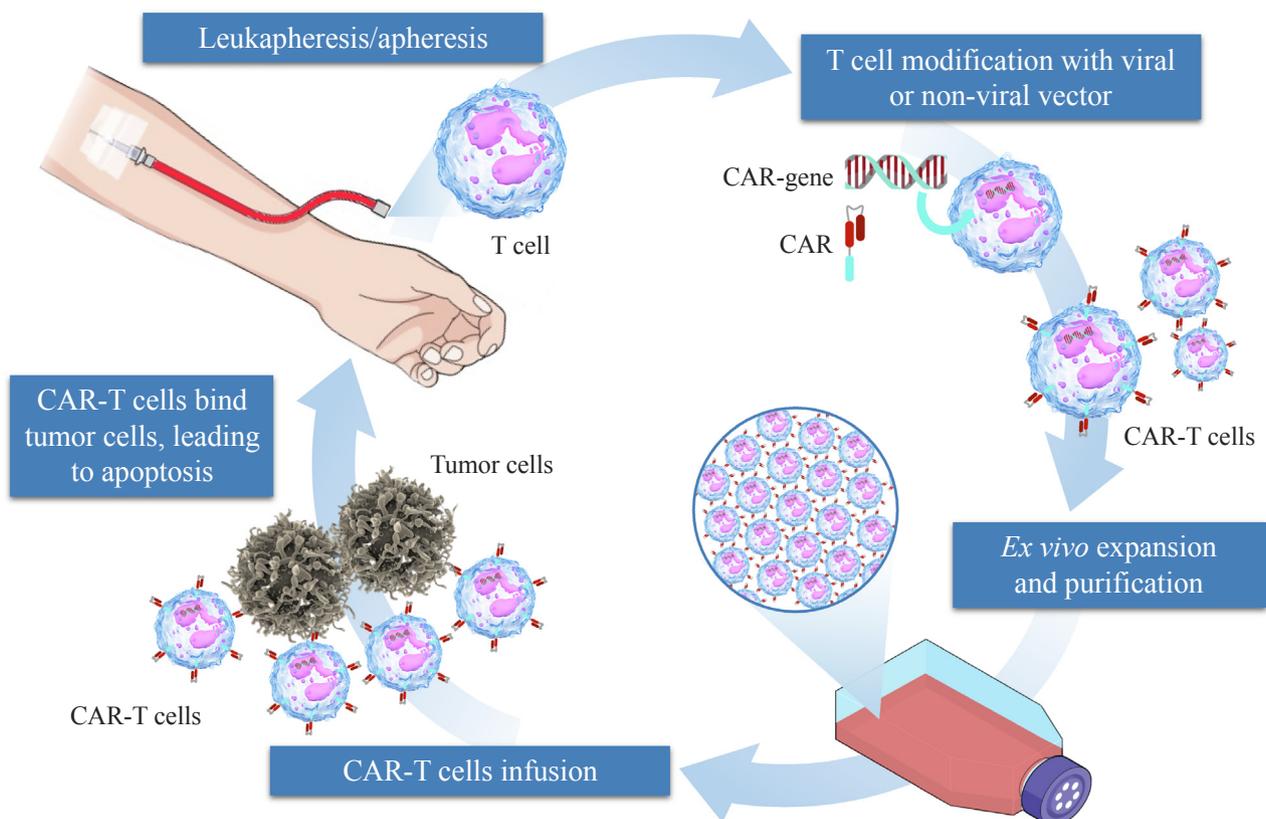


Fig. 2. Workflow of chimeric antigen receptor (CAR) T cell production. T cells from peripheral blood are collected via leukapheresis, followed by apheresis. The T cells are then transduced by viral (retroviral or lentiviral) or non-viral vector loading genes of CAR inserted artificially. Afterward, the modified CAR-T cells are expanded and purified. Eventually, cell quality and sterility will be examined before the cell products are infused into patients.

sarcomas.

HER2 expression correlates with clinical features in various sarcomas including osteosarcoma, synovial sarcoma, and Ewing's sarcoma [21–26]. In one such example, expression of HER2 correlates with worse histologic response and survival for osteosarcoma patients [27,28]. Although the anti-HER2 antibody (trastuzumab) was found to be ineffective in osteosarcoma treatment [29], there are opportunities within the realm of immunotherapy. For example, in trastuzumab-resistant medulloblastoma, HER2 CAR-T cells were able to recognize and kill low-HER2 cells [30]. It is therefore an avenue that warrants further exploration in sarcoma therapy.

HER2 CAR-T cells have induced *in vitro* therapeutic responses in osteosarcoma cell lines through IFN- γ and IL-2 [31]. In a xenograft model, HER2 CAR-T cell treatment caused tumor regression of both localized and metastatic lesions, and even prolonged survival in mice inoculated with the low-HER2 expression osteosarcoma cell line [31]. Another study showed HER2 CAR-T cells to reduce *in vitro* sarcosphere forming capacity of two osteosarcoma cell lines [32].

At the clinical level, a phase I/II trial used HER2 CAR-T cells to treat refractory or metastatic HER2-positive osteosarcoma, Ewing's sarcoma, primitive neuroectodermal tumor (PNET), and desmoplastic small round cell tumor (DSRCT) [22]. The T cells were transduced with retroviral particles encoding HER2-CD28-CD3 ζ to create second-generation CAR-T cells. Aside from one patient experiencing fever after a high dose infusion which later resolved with ibuprofen, no serious adverse events were observed [22]. HER2 CAR-T cells were detectable *in vivo* three hours after infusion within the peripheral blood by quantitative polymerase chain reaction (qPCR) analysis [22]. In terms of clinical response, 4 of 17 patients achieved stable disease for 12 weeks to 14 months [22]. Three of these patients had their residual tumor removed without additional therapy, with one specimen achieving $\geq 90\%$ tumor necrosis. Each of these three patients remained in remission at 16 months follow-up. The median overall survival was 10.3 months (5.1 to 29.1 months) with a median follow-up time of 10.1 months (1.1 to 37 months) [22]. In summary, HER2 is a promising CAR target in select sarcomas; however, future work is required to improve target specificity and to better predict patient response to therapy.

Disialoganglioside (GD2)

Disialoganglioside (GD2) is expressed on various tumors including neuroblastoma, melanoma, osteosarcoma, Ewing's sarcoma, rhabdomyosarcoma, and DSRCT, with relatively restricted expression in normal tissue [33,34]. This relative specificity makes GD2 an attractive target for cancer immunotherapy. The monoclonal anti-GD2 antibody 3F8 has been utilized in the treatment of neuroblastoma and melanoma. The other anti-GD2 antibody hu14.18K322A is currently in phase II clinical trials for the treatment of refractory or recurrent neuroblastoma in combination with chemotherapy. Of note, first generation GD2 CAR-T cells improved overall survival in patients with high-risk neuroblastoma when combined with standard treatment [35].

With regards to sarcomas, a recent study showed GD2 to be expressed in 18 of 18 (100%) patients with osteosarcoma, 7 of 35 (20%) patients with Ewing's sarcoma, and 2 of 15 (13%) patients with rhabdomyosarcoma [36]. A comparable lysis of tumor cells formed with third generation GD2 CAR-T cells incorporating 14g2a-scFv with CD28, OX40, and CD3 ζ domains, was observed in both neuroblastoma and GD2 + sarcoma cell lines [36]. Of note, a large number of monocytic and granulocytic murine myeloid-derived suppressor cells (MDSC) were observed in pediatric sarcoma xenografts, and were suspected to cause CAR-T cell inhibition. In response, the researchers combined GD2 CAR-T cells plus all-trans retinoic acid capable of reducing MDSCs and observed significantly improve antitumor response in pediatric sarcoma xenografts [36]. These results support GD2 CAR-T cells as a promising treatment for sarcoma when combined with backing immunotherapy.

Currently there is a phase I clinical trial implementing third generation GD2 CAR-T cells combined with a suicide switch caspase dimerization domain (ICD9), which enables CAR-T apoptosis in the case of serious toxicity. Eligible patients include children and young adults with solid tumors including osteosarcoma and soft tissue sarcoma (NCT02107963).

Interleukin 11 receptor, alpha subunit (IL-11RA)

Interleukin 11 (IL-11) is a stromal cell-derived cytokine with variable activity in inflammation-associated cancers [37]. The expression of IL-11 and its receptor, interleukin 11 receptor alpha subunit (IL-11RA), have been proposed to promote tumorigenesis and tumor cell growth in sarcomas such as osteosarcoma and chondrosarcoma [38–40]. Moreover, high IL-11RA expression has been demonstrated in osteosarcoma cell lines and pulmonary metastases tissues, with absent or low expression in the surrounding normal tissue [41].

Third-generation IL-11RA CAR-T cells have *in vitro* cytotoxicity to the osteosarcoma cell lines SAOS-2, LM7, CCH-OS-D, and KRIB [41]. In a mouse model mimicking pulmonary metastatic osteosarcoma, intravenous IL-11RA CAR-T cells homed to the metastatic lung lesions while sparing the surrounding healthy lung tissue. The number of visible lung metastases were also decreased [41]. Specifically, three of five mice treated with IL-11RA CAR-T cells had no visible metastases, while all five mice treated with controlled T cells had metastases. These results suggest IL-11RA CAR-T cells are promising for osteosarcoma with pulmonary metastases.

Fibroblast activation protein, alpha (FAP α)

The tumor microenvironment has emerged as a major contributor to solid tumor development. Non-malignant cells such as cancer-associated fibroblasts (CAFs) cultivate a pro-tumorigenic environment by secreting growth factors and providing physical support [42]. Therefore, the inhibition of CAFs has become increasingly recognized as a therapeutic strategy. The identification of fibroblast activation protein alpha (FAP α) on CAFs has led efforts of selective targeting. FAP α is a type II integral membrane serine protease of the prolyl oligopeptidase family [43]. FAP α functions in fibroblast growth or epithelial-mesenchymal interactions during development, tissue repair, and epithelial carcinogenesis [43]. Many studies have shown increased FAP α expression on stromal fibroblasts in malignancies such as breast, colorectal, skin, and pancreatic cancer, as well as in bone and soft tissue sarcomas [43,44].

Various FAP α -targeting modalities have been studied, including FAP α -CAR-T cell therapy. In mouse models using FAP α -CAR-T cells for mesothelioma and lung cancer treatment, there was significant antigen-specific cytotoxicity and inhibition of FAP α positive stromal cells compared to untreated and vector-control-transduced CAR-T cell-treated tumors [45]. Mechanistically, the antitumor effects of FAP α -CAR-T cell therapy can be increased with increased injection frequency, diacylglycerol kinase deficient CAR-T cells, or when combined with vaccination therapy [45]. Given FAP α is selectively overexpressed in osteosarcoma and expression correlates with clinical stage, histological grade, metastasis, and poor survival, there is significant incentive for future study aimed at identifying the mechanism and function of FAP α CAR-T cells.

Insulin-like growth factor 1 receptor (IGF-1R) and tyrosine kinase orphan-like receptor 1 (ROR1)

Insulin-like growth factor 1 receptor (IGF-1R) is a transmembrane receptor tyrosine kinase and promoter of tumor cell survival with notable prognostic significance in sarcoma. Several multidrug-resistant osteosarcoma cell lines have shown to be particularly sensitive to IGF-1R inhibition [46]. Recent clinical trials evaluating anti-IGF-1R

monoclonal antibodies in recurrent or refractory Ewing's sarcoma demonstrated a 10 – 14% overall response rate with modest median progression-free survivals of less than 2 years [47,48]. There is an ongoing phase II randomized controlled trial investigating combination chemotherapy with anti-IGF-1R antibody in Ewing's sarcoma with yet to be published results. At this time, therefore, IGF-1R CAR-T cell therapy is still within the early stages of clinical investigation.

Tyrosine kinase orphan-like receptor 1 (ROR1), also known as neurotrophic tyrosine kinase receptor-related 1 (NTRKR1), is another transmembrane protein with vital roles in cancer cell migration, invasion, and metastasis. Overexpression of ROR1 has been observed in hematologic malignancies and solid tumors alike, including Ewing's sarcoma, osteosarcoma, and rhabdomyosarcoma [49–54]. A recent study evaluated CAR-T cells targeting IGF-1R or ROR1 in sarcomas [54]. These IGF-1R and ROR1 CAR-T cells targeted sarcoma in mice xenograft models with high levels of IFN- γ , TNF- α , and IL-13 production. The IGF-1R and ROR1 CAR-T cells significantly reduced tumor growth in pre-established, localized, and systemically disseminated osteosarcoma mouse models. Moreover, IGF-1R and ROR1 CAR-T cells prolonged survival in a localized sarcoma mouse model. These data support IGF-1R and ROR1 CAR-T cells as potential treatments for sarcoma.

B7-H3

B7 homolog 3 (B7-H3, CD 276) is an inhibitory protein of the B7-CD28 superfamily with roles in inhibition of T cell activation, proliferation, and cytokine production [55]. Overexpression of B7-H3 is common in cancers such as melanoma, non-small-cell lung cancer, osteosarcoma, Ewing's sarcoma and rhabdomyosarcoma [55–57]. Notably, B7-H3 expression is very low in normal tissue. In osteosarcoma, B7-H3 expression negatively correlates with the number of TILs and promotes tumor cell invasion, indicating B7-H3 may impair T cell mediated antitumor immunity [56]. Moreover, osteosarcoma patients with high B7-H3 expression experience significantly shorter overall survival and progression-free survival times compared to those with low expression [56]. As a result of these findings, antibodies against B7-H3 have been explored for sarcoma treatment. Enoblituzumab, a humanized IgG1 monoclonal anti-B7-H3 antibody, was able to stabilize refractory B7-H3-expressing tumors such as melanoma and advanced solid tumors without dose-limiting toxicity (NCT01391143). Another ongoing study is investigating enoblituzumab in patients with B7-H3-expressing neoplasms including osteosarcoma and Ewing's sarcoma (NCT02982941).

B7-H3 CAR-T cells have recently been studied *in vivo* against xenograft osteosarcoma and Ewing's sarcoma models [58]. The B7-H3 CAR-T cells completely regressed osteosarcoma and Ewing's sarcoma xenografts, leading to significant survival improvements compared to the control mice. In the metastatic osteosarcoma model, 9 of 10 mice who received B7-H3 CAR-T cell treatment survived at least 5 months longer than the untreated mice [58]. Therefore, there is promising evidence B7-H3 CAR-T cells mediate antitumor immunity against established and metastatic osteosarcomas. In addition, an advantage of B7-H3 CAR-T cell therapy is the low B7-H3 expression found in normal tissue [58].

CD44v6

CD44 is a cell-surface glycoprotein with roles in cell proliferation, differentiation, migration, angiogenesis, cytokine presentation, and signaling for cell survival [59]. However, CD44, particularly the isoform 6 of adhesive receptor CD44 (CD44v6), is also a prominent cancer-initiating cell marker of tumorigenesis and metastasis [60]. CD44v6 expression has been reported in various malignancies and is associated with worse outcomes and metastasis [61–64]. A previous *meta*-analysis showed osteosarcoma patients with CD44v6 expression to have

significantly worse survival and metastasis rates [65]. In soft tissue sarcoma, CD44v6 is associated with a higher risk for local recurrence [66].

Recently, CD44v6 was a therapeutic target for CAR-redirection cytokine-induced killer (CIK) T cells for soft tissue sarcoma patients [67]. CD44v6 had an expression average of 40% amongst soft tissue sarcomas such as liposarcoma, fibrosarcoma, leiomyosarcoma, and undifferentiated pleomorphic sarcoma. The CAR-CIK T cells killed soft tissue sarcomas *in vitro* with much greater activity than the unmodified CIK cells. They also had much less killing activity in low CD44v6-expressing sarcomas. Moreover, the addition of anti-NKG2D antibody did not interfere with CAR-CIK killing activity but did however affect unmodified CIK cells. The *in vivo* antitumor activity of CD44v6-CAR-CIK cells is also present in subcutaneous xenografts. Significant greater delay of tumor growth in CD44v6-CAR-CIK cell-treated mice was seen compared to untreated and control-treated mice [67]. For these reasons, CD44v6 may be a favorable target for CAR-T cells in future sarcoma clinical trials.

Fetal acetylcholine receptor

Nicotinic acetylcholine receptors are polypeptides essential to neurotransmission of the nervous and musculoskeletal systems. During the development of the neuromuscular junction, the fetal type of acetylcholine receptors (fAChRs, $\alpha 2\beta\gamma\delta$) change to the adult type ($\alpha 2\beta\epsilon\delta$). fAChRs are nearly lost from mature muscle after birth; however, its expression is retained in some extraocular muscle and thymic myoid cells. They are also highly expressed in rhabdomyosarcoma [68], which has led to the advent of fAChR targeting CAR-T cell therapy in this cancer [69].

The first-generation of fAChR-directed CAR-T cells were marginally effective in killing rhabdomyosarcoma cells *in vitro* by IFN- γ secretion and > 90% rhabdomyosarcoma cell lysis [70]. Of note, an increase of fAChR expression after chemotherapy was observed in the residual tumors of patients with rhabdomyosarcoma in this same study. For these reasons, the fAChR-CAR-T cells have some potential as a treatment option for rhabdomyosarcoma, particularly post-chemotherapy. The second-generation fAChR-CAR-T cells with CD28 demonstrated activation improvement; however, rhabdomyosarcoma killing efficacy remained poor compared to CD20 + lymphoma or CEA-expressing adenocarcinoma cell lines [71]. It is likely that resolving the resistance mechanisms mitigating fAChR-CAR-T cells in rhabdomyosarcoma will improve this novel therapy.

NKG2D/NKG2DL

NK cell activating receptor group 2-member D (NKG2D) functions in antitumor immunity as a co-stimulatory transmembrane protein of the CD94/NKG2 family in NK cells, T cells, and activated macrophages [72]. While NKG2D ligands (NKG2DLs) are rarely expressed in normal tissue, they are overexpressed in infected and malignant tissue. Binding of NKG2D and its ligand triggers NK cell activation, resulting in cytotoxic granule release and tumor cell apoptosis. Importantly, NKG2DL expression has been observed in osteosarcoma and Ewing's sarcoma cell lines [73,74].

Recently, the safety and antitumor capacity of second-generation NKG2D-directed CAR-T cells against osteosarcoma has been explored [75]. The lentiviral transduction of NKG2D-1-4BB-CD3 ζ remarkably increased NKG2D expression on the CAR-T cell surface and showed strong genetic stability. These NKG2D-CAR-T cells demonstrated significantly increased *in vitro* cytotoxic activity against osteosarcoma cell lines compared to non-transduced T cells. No cytolytic activity against healthy cell lines was apparent. In an orthotopic osteosarcoma murine model, the NKG2D-CAR-T cell treated group had lower tumor burden and extended survival times compared to control groups [75]. Similarly, NKG2D-CD28- CD3 ζ CAR-T cells, which were created using a

lentivirus vector, resulted in Ewing's sarcoma cell death *in vitro* [74]. These studies defined NKG2D/NKG2DLs as promising targets for CAR-T cells in sarcoma treatment.

NY-ESO-1

Cancer-testis antigens (CTA) are proteins with roles in immunological maturation normally restricted to human male germ cells. However, these antigens can be re-expressed in various malignancies. New York esophageal squamous cell carcinoma 1 (NY-ESO-1) is a well-known CTA with re-expression in numerous cancers including sarcomas [76]. With regards to sarcomas, the most homogenous NY-ESO-1 expression has been observed in myxoid and round cell liposarcoma and synovial sarcoma [77,78]. Many immunotherapy clinical trials utilizing cancer vaccines, adoptive T cell therapy, and combination treatment with immune checkpoint inhibitors have targeted NY-ESO-1 [76]. However, most of these adoptive T cell therapies have targeted the NY-ESO-1 signal TCR transduction pathway intracellularly and in an MHC dependent manner. For traditional CAR-T cells, the TAA must be expressed on the tumor surface in order to bind with the chimeric receptor. More recently, CAR on T cells mimicking TCR function which recognize NY-ESO-1 (NY-ESO-1 CAR/TCR) in the context of HLA-A2, and HLA-A2-T cells modified to serve as NY-ESO-1 antigen presenting cells (NY-ESO-1T-APC), have both been developed and studied in multiple myeloma [79]. *In vitro* studies demonstrated NY-ESO-1 CAR/TCR T cells can be expanded when co-cultured with NY-ESO-1T-APC. In multiple myeloma mouse models, NY-ESO-1 CAR/TCR T cell treatment produced superior antitumor effects compared to control. Furthermore, combined NY-ESO-1 CAR/TCR T cell and NY-ESO-1T-APC treatment exhibited even greater anti-myeloma activity. Therefore, TCR-enhancing CAR-T cells can target intracellular NY-ESO-1 yielding antitumor effects, with enhanced efficacy seen if there is a co-infusion of NY-ESO-1T-APC tumor vaccine [79].

Cytokine release syndrome

Although a degree of immune stimulation and inflammation can be expected after ACT, severe cytokine release syndrome (CRS) has been observed with CD19-, BCMA-, and CD22 CAR-T cells and can cause considerable adverse effects. CRS is a systemic inflammatory response induced by excessive cytokine release after CAR-T cell infusion, with potential for widespread organ dysfunction [80]. Cytokines such as IL-6, IFN- γ , TNF, IL-2, IL-8, and IL-10 become elevated and cause symptoms such as fever, tachycardia, and hypotension [80]. Of note, this syndrome can be much more severe than the influenza-like syndrome commonly observed with TCR T-cell treatment. First-line treatment of CRS is aggressive supportive care and possible application of IL-6 receptor blockade with tocilizumab [80]. Corticosteroids should be reserved for cases of tocilizumab non-responsiveness and neurotoxicity [80]. Minimizing immune toxicity remains an important goal of CAR-T cell therapy.

Future directions and conclusions

The powerful clinical results seen in CAR-T cell immunotherapy in hematologic malignancies have led to an expansion of studies utilizing this therapy for sarcomas at the preclinical and clinical level. While CAR-T cells targeting multiple SAAs have been quite efficacious in sarcoma cell lines and animal models, confirmation within clinical trials is largely ongoing. As described in Table 2, there are multiple CAR-T cell clinical trials seeking to address these issues.

Compared to leukemias and lymphomas, solid tumors present several unique challenges to optimized CAR-T cell immunotherapy. For sarcomas, there is a paucity of specific and potent tumor-specific targets and high affinity CAR-domains, the tumor microenvironment is a barrier to penetration, and both intrinsic and extrinsic inhibitory

mechanisms diminish CAR-T cell longevity [81]. To offset the trafficking barrier, several works have trialed direct CAR-T cell infusion within solid tumors [82–85]. As for the intravenous infusion method, tumor to CAR-T cell chemokine receptor mismatch can cause subuded tumor infiltration; however, this can potentially be resolved with better engineered and matched CAR T cell chemokine receptors [86,87]. Of note, oncolytic viruses have been used to specifically infect and lyse tumor cells, causing tumor chemokine secretion for enhanced CAR-T cell infiltration and tumor control in neuroblastoma [88]. As an alternative, engineered CAR-T cells expressing degradative enzymes toward the extracellular matrix can improve tumor penetration and overall anti-tumor effects of CAR-T cells [89].

Even in the event of adequate infiltration, the tumor microenvironment presents a formidable barrier to CAR-T cell therapy as tumor cells and stroma produce immunosuppressive factors which dampen T cell activity and survival [90]. Specifically, there are various immune suppressor cells housed within the tumor microenvironment, such as M2 tumor-associated macrophages (M2-TAM), MDSCs, and regulatory T and B cells [90]. In response, there have been many efforts aimed towards manipulating the tumor microenvironment for anti-tumor activity via chemotherapeutic pre-conditioning, immune checkpoint inhibitors or specific antibodies, and combining MDSC depletion with CAR T cell therapy [91–95]. In one such example, armored CARs which secrete activating cytokines such as IL-2, IL-12, and IL-15 showed enhanced therapeutic efficacy in preclinical studies [96–98]. As an alternative method, CARs incorporating dominant-negative receptors (DNRs) with a nullified intracellular domain can disrupt the downstream signaling cascade ordinarily responsible for immunosuppression [99,100]. Finally, CAR-T cells can be equipped with a switchable receptor, which avoids immunosuppression by connecting the previously inhibitory immunosuppressive extracellular domain to an intracellular activating component. This is exemplified by the PD-1-CD28 switch receptor, which improves CAR-T cell infiltration and anti-tumor effects [101]. While these modalities are partially effective in dampening immunosuppression, future studies focused on combination therapy and novel CAR designs are needed to approach the results seen in hematologic malignancies.

Exhaustion of CAR-T cells occurs from chronic antigen exposure and results in loss of T cell function, proliferation, and overall poor anti-tumor effects [102]. To counteract this, PD-1 inhibitor or transduction of PD-1 DNR with CAR can reverse CAR-T cell exhaustion [100]. The co-stimulatory molecules such as CD28 and 4-1BB are also involved in T cell exhaustion and are potential therapeutic targets [103]. These pathways should, therefore, inform future CAR designs, as they can directly reduce exhaustion and improve T cell proliferation and persistence.

Aside from efficacy, another obstacle to widespread CAR-T cell therapy is patient safety. Undesired on-target on-tumor/off-tumor response and toxicity related to systemic cytokine release have been well-described, and underline the need for safety mechanisms when designing CAR-T cell therapy. Modern designs such as “chemical switches” or dual CAR constructs can control the activation-deactivation of CAR-T cells (Fig. 2). Safety modifications include suicide gene or pro-apoptotic caspase [104,105] inclusion and coupling of scFv to signal transduction domains [106]. In addition, the CAR system can be coupled to CD3 ζ and the co-stimulatory tails of scFvs, so that CAR-T cell activation occurs only upon binding both antigenic targets [107,108]. These modifications exemplify the next generation of CAR-T cell designs, and will likely expedite their specificity, efficacy, and safety profile for sarcoma patients.

CAR-T cells are an emerging, adoptive immunotherapeutic approach with the advantages of native immunity without the restrictions of MHC presentation. Until recently, clinical studies of various CAR-T cell treatments in sarcomas have produced mixed results. Ongoing efforts revolve around identifying optimal CAR constructs, tumor-associated antigens, infusion doses, lymphodepletion regimens, all while

Table 2
Selected CAR-T cell clinical trial in patients with various types of sarcomas.

Antigen	Identifier	Phase	CAR design	Disease condition	Status	Note	Reference
HER2	NCT00902044	I/II	2nd generation	HER2-positive sarcoma; osteosarcoma, Ewing's sarcoma, PNET, rhabdomyosarcoma	ongoing	No serious adverse events related to this regimen. 14 of 16 patients were observed HER2 CAR-T cells <i>in vivo</i> by qPCR analysis in the peripheral blood from dose level higher than $1 \times 10^5/m^2$, and persisted up to 18 months. 2 of 5 patients who had tumors removed had shown CD3 + T cell positivity by IHC and HER2 CAR-T cells by qPCR analysis within the tumor sites. 4 of 17 patients had SD for 12 weeks to 14 months, with one demonstrated $\geq 90\%$ tumor necrosis. The median OS is 10.3 months (5.1–29.1 months) The purpose is to find the largest safe dose, side effect, and response of GD2 CAR-T cells in combination with a varicella zoster vaccine and lymphodepleting chemotherapy.	[22]
GD2	NCT01953900	I	3rd generation	Osteosarcoma, neuroblastoma	ongoing	No result is currently available. The investigator utilized the 3rd generation GD2 CAR-T cells combined with a suicide switch comprising of a caspase dimerization domain (ICD9) to induce these CAR-T cells death in case of serious toxicity. No result is currently available.	
EGFR806	NCT03618381	I	2nd generation	Recurrent/refractory malignancies in children and young adults including bone and soft tissue sarcoma	ongoing	The purpose of this study is to evaluate the safety, feasibility, and efficacy of administering EGFR-CAR-T cells in the patients with recurrent or refractory hematologic malignancies, cancers, and sarcomas. The participants are currently enrolled.	
Sarcoma-specific	NCT03356782	I/II	4th generation	Sarcoma, osteosarcoma, Ewing's sarcoma	ongoing	The 4th generation CAR-T cells targeting sarcoma-specific antigen, such as CD133, GD2, Muc1, CD117, etc. will be utilized.	

Abbreviations: chimeric antigen receptor (CAR), desmoplastic small round cell tumors (DSRCT), epidermal growth factor receptor (EGFR), disialoganglioside (GD2), human epidermal growth factor receptor 2 (HER2), immunohistochemistry (IHC), malignant peripheral nerve sheath tumor (MPNST), overall survival (OS), primitive neuroectodermal tumor (PNET), stable disease (SD), quantitative polymerase chain reaction (qPCR).

preventing and managing toxicities. Current and future efforts will likely reveal CAR-T cell therapy to have application to sarcomas with limited therapeutic options. In addition, as technologies such as whole genome sequencing and RNA sequencing reveal new SAAs amenable to targeting, it is likely that clustered regularly interspaced short palindromic repeats (CRISPR)-Cas9 mediated genome editing will be able to engineer precise TCRs with potent affinity towards any newly discovered CAR-T antigens.

Declaration of Competing Interest

The authors declare that there is no conflict of interest.

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