

REVIEW

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# Advances on immunotherapy for osteosarcoma

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## Abstract

Osteosarcoma is the most common primary bone cancer in children and young adults. Limited progress has been made in improving the survival outcomes in patients with osteosarcoma over the past four decades. Especially in metastatic or recurrent osteosarcoma, the survival rate is extremely unsatisfactory. The treatment of osteosarcoma urgently needs breakthroughs. In recent years, immunotherapy has achieved good therapeutic effects in various solid tumors. Due to the low immunogenicity and immunosuppressive microenvironment of osteosarcoma, immunotherapy has not yet been approved in osteosarcoma patients. However, immune-based therapies, including immune checkpoint inhibitors, chimeric antigen receptor T cells, and bispecific antibodies are in active clinical development. In addition, other immunotherapy strategies including modified-NK cells/macrophages, DC vaccines, and cytokines are still in the early stages of research, but they will be hot topics for future study. In this review, we showed the functions of cell components including tumor-promoting and tumor-suppressing cells in the tumor microenvironment of osteosarcoma, and summarized the preclinical and clinical research results of various immunotherapy strategies in osteosarcoma, hoping to provide new ideas for future research in this field.

**Keywords** Osteosarcoma, Immune microenvironment, Immunotherapy

## Introduction

Osteosarcoma is the most common primary malignant bone tumor that mostly occurs in children and young adolescents [1]. It originates from mesenchymal stem cells, characterized by osteoid product [2]. Osteosarcoma exhibits a predilection to occur in the metaphysis of long extremity bones, such as distal femur, proximal tibia, proximal femur, and proximal humerus [3]. It was reported that approximately 60% of patients are between 10 and 20 years old and osteosarcoma is considered as the second leading cause of death in this age group [1].

The incidence of osteosarcoma is 3–5 per million per year [4]. Despite their rare incidence, osteosarcoma has a high disability and mortality rate. The current standard treatment for osteosarcoma is neoadjuvant chemotherapy-surgery-consolidation chemotherapy treatment mode, which achieves great clinical success for patients with localized osteosarcoma [5]. The 5-year survival rate has reached a plateau in osteosarcoma patients with localized disease ranging from 60 to 70% since the introduction of systematic chemotherapy [6]. Unfortunately, osteosarcoma is a tumor with a high tendency for metastasis, and 15–20% of newly diagnosed osteosarcoma are detected with metastasis [7]. The most common metastatic site is lung. And the patients with metastatic disease either at diagnosis or at the time of recurrence portends a dismal prognosis with 5-year survival of only 20–30% [8].

Limited progress has been made in improving the survival outcomes in patients with osteosarcoma over the

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past 40 years. Especially, the prognosis of metastatic or recurrent osteosarcoma is still unsatisfactory. Given these bottlenecks to conventional treatment regimens, there is an urgent need to exploit novel therapeutic strategies to improve the treatment situation of patients with osteosarcoma. In addition to traditional treatment methods including surgery, chemotherapy, and radiotherapy, new therapeutic approaches, such as targeted therapy and immunotherapy, are also under intense research for osteosarcoma. Currently, research on targeted therapy for osteosarcoma mainly revolves around anti-angiogenic small molecule inhibitors, with representative drugs being regorafenib and sorafenib. These anti-angiogenic drugs, do have certain effects on metastatic osteosarcoma, but they have short progression-free survival (PFS) time and insufficient objective response rate (ORR) [9].

In recent years, immunotherapies involving the application of immune checkpoint inhibitors (ICIs), genetically modified T cells, tumor vaccines, immune modulators and cytokines, have received considerable attention. Importantly, due to their impressive efficacy, ICIs are recommended for the treatment of several human malignancies, including melanoma [10], non-small-cell lung cancer [11], head and neck cancers [12] and so on. Chimeric antigen receptor T (CAR-T) cells as an adoptive cell therapy (ACT) have showed remarkable clinical outcome in the treatment of malignant hematological tumors, such as acute lymphoblastic leukemia [13], chronic lymphocytic leukemia [14], and non-hodgkin lymphoma [15]. Immunotherapy can more accurately target tumors and activate the host's anti-tumor immune response. Compared to traditional chemotherapy, immunotherapy has fewer side effects and possesses long-lasting anti-tumor activity. The microenvironment of osteosarcoma has complex heterogeneity and immunosuppressive properties, which makes immunotherapy not yet approved for indications in osteosarcoma. However, it is undeniable that immunotherapy is becoming an increasingly attractive therapy strategy for osteosarcoma. In this review, we will present on the tumor microenvironment characteristics of osteosarcoma, including immune-suppressive related cells and tumor-suppressive related cells, as well as their roles in the osteosarcoma microenvironment. We will also summarize the results of preclinical and clinical research on immunotherapy, especially ICIs and CAR-modified immune cell therapies, as well as the immuno-combination strategies, with the aim of further understanding the immune characteristics of osteosarcoma from multiple perspectives and providing new directions for the application of immunotherapy for patients with osteosarcoma.

### **Immune microenvironment of osteosarcoma**

The tumor microenvironment (TME) is a complex ecosystem with high heterogeneity, which includes tumor cells and a large number of non-tumor cells, embedded in altered extracellular matrix. In the same way, the TME of osteosarcoma also contains many components which are closely related to the escape of tumor cells from immune surveillance, uncontrolled proliferation, and metastasis. The immune cells in the TME not only play a crucial role in the occurrence and development of tumors, but also are important targets of anti-tumor immunotherapy [16]. There are various types of immune cells in osteosarcoma, mainly including tumor-associated macrophages (TAMs), tumor-associated neutrophils (TANs), myeloid-derived suppressor cells (MDSCs), T cells, B cells, natural killer cells (NK cells), and dendritic cells (DCs) and so on. In addition, molecular pathways, especially immune related molecules in the microenvironment of osteosarcoma, are closely related to the development of tumors and the prognosis of patients.

### **Characteristics of cell composition in the TME of osteosarcoma**

The TME of osteosarcoma is distinctly suppressive, with immune cells exhibiting complex and diverse functions. Understanding the roles and mechanisms of different immune cells within it, and developing corresponding therapeutic strategies targeting specific elements, can help improve the prognosis for patients with osteosarcoma.

#### ***Tumor-promoting cells in TME***

**Tumor cells** Tumor cells, as the key components of TME, which can actively interact with the immune system and promote the formation of inhibitory immune networks [17]. In recent years, circulating tumor cells (CTCs) have been frequently mentioned. Under the impact of local and systemic treatment, some tumor cells can still survive in small amounts and escape from the primary lesion into the circulatory system, causing distant metastasis or recurrence of osteosarcoma [18]. Therefore, research on these tumor cells and the development of corresponding treatment methods will be benefit to reduce the metastasis and recurrence of osteosarcoma.

**TAMs** TAMs are the most abundant immune cells in the immune microenvironment of osteosarcoma and account for approximately 50% of the immune cells [19]. The polarization state of TAMs has a significant impact on tumor cells. Generally, TAMs polarize into two types, M1 and M2, primarily due to the influence of different cytokines and signaling pathways in the tumor microenvironment [20]. In the TME of osteosarcoma, M2-type TAMs

are the main proportion and play a complex and crucial role. M2-type TAMs are typically associated with anti-inflammatory and tumor-promoting characteristics, and they participate in processes such as tumor angiogenesis, extracellular matrix remodeling, and immune defense and regulation [21]. The polarization state of M2-type TAMs can promote tumor progression by releasing specific cytokines and chemokines, such as producing anti-inflammatory cytokines like IL-10 and transforming growth factor-beta (TGF- $\beta$ ), which are involved in anti-inflammatory responses, and participate in tumor angiogenesis and extracellular matrix remodeling [22]. M2-type TAMs in the TME of osteosarcoma may also affect tumor immune evasion and drug resistance through interactions with other immune cells. For example, they may interact with cancer stem cells, supporting the function of cancer stem cells [23]. In addition, the presence and functional state of M2-type TAMs are closely related to the prognosis and therapeutic responsiveness of osteosarcoma. Studies have shown that the degree of infiltration of M2-type TAMs can serve as one of the biomarkers for judging the prognosis of osteosarcoma patients [21]. Various immune checkpoint proteins expressed on the surface of TAMs, such as PD-1 and CD47 receptor [19]. Therefore, therapeutic strategies targeting M2-type TAMs, such as promoting the polarization shift of M2-type TAMs to M1-type or targeting the immune checkpoints of TAMs, may provide new directions for targeted therapy of osteosarcoma [21]. In addition, the M3-TAMs, actually the FABP4<sup>+</sup>TAMs previously identified as the alveolar macrophages in the lung, were dominant in the lung metastatic osteosarcoma lesion [24]. High expression of FABP4 provides fatty acids for tumor cells to promote the occurrence and development of cancer [25]. There has been some research on the mechanism of FABP4 action in tumors, but the specific mechanism is not yet fully understood. M3-TAMs contribute to the metastasis and colonization of osteosarcoma cells in the lungs [26], exacerbating the malignant progression of the tumor. Developing targeted therapeutic strategies against M3-TAMs may potentially suppress the pulmonary metastasis of osteosarcoma.

**MDSCs** MDSCs are significant component in the TME of osteosarcoma, MDSCs attenuate anti-tumor immunity particularly the activity of T cells, by secreting factors such as arginase-1 (Arg-1), inducible nitric oxide synthase (iNOS), and reactive oxygen species (ROS), thereby facilitating tumor cells to evade immune surveillance [27]. MDSCs also contribute to tumor angiogenesis through the secretion of factors like vascular endothelial growth factor (VEGF) and matrix metalloproteinase 9 (MMP9) [28]. Moreover, MDSCs interact with other cells in the tumor microenvironment, such as TAMs and Tregs, to jointly promote immune evasion and tumor progression [29].

MDSCs establish pre-metastatic niches in distant organs, creating favorable conditions for the spread and colonization of tumor cells [30]. And they also secrete TGF- $\beta$  and hepatocyte growth factor (HGF), which contribute to epithelial-mesenchymal transition (EMT), thereby promoting the invasion and metastasis of osteosarcoma [31]. Recent study has discovered that CD300ld, a surface molecule on MDSCs, plays a significant role in the immunosuppressive function of MDSCs and may potentially serve as a novel therapeutic target [32]. Given the critical role of MDSCs in immune suppression and tumor progression, they have become potential targets for immunotherapy. Treatment strategies targeting MDSCs may help to lift immune suppression and enhance the efficiency of the anti-tumor immune response. However, that need more exploration before clinical application [33].

**Treg cells** In the osteosarcoma microenvironment, Treg cells exert their effects through various mechanisms. They secrete immunosuppressive cytokines such as IL-10, IL-35, and TGF- $\beta$  to inhibit the activity of effector T cells, and inhibit the formation of osteoclasts through direct cell contact-dependent manners [34]. Interaction of CD4<sup>+</sup>Tregs and osteoclasts significantly alters TME and is connected to poor prognosis of osteosarcoma [34]. And the interaction between T cell immunoglobulin and mucin-domain containing 3 (TIM-3)<sup>+</sup>T cells and monocytes, naive CD4<sup>+</sup>T cells, and Gal9-expressing CD4<sup>+</sup>CD25<sup>+</sup>Tregs could resulting in progressive suppression of Th1 responses [35]. Moreover, Zhou et al. Report that infiltration of T cell immunoreceptor with Ig and ITIM domains (TIGIT) expressing Treg cells in osteosarcoma tissue, providing a new target for the immunotherapy of osteosarcoma [4]. Treg cells not only help tumor cells evade immune surveillance but also participate in promoting tumor angiogenesis [36]. Furthermore, the heterogeneity of Treg cells in the osteosarcoma microenvironment and their proliferation and activation status are closely related to tumor development, potentially serving as targets for immunotherapy.

**TANs** TANs are a heterogeneous and functionally diverse subset of neutrophils that infiltrate the tumor microenvironment. TANs could also be polarized to the anti-tumor N1 phenotype and pro-tumor N2 phenotype. Infiltrating neutrophils combat tumor cells by orchestrating the recruitment of immune cells and also by facilitating antibody-dependent cellular cytotoxicity [37]. Yang et al. identified that neutrophils are more abundant at the primary tumor site than those at metastatic sites by detecting the specific marker CD11b [38]. It is reported that TANs significantly decreased in the highly hypoxic tumor microenvironment. Hypoxia may downregulate immune cells, thereby facilitating tumor immune evasion and

metastasis [39]. Web-like chromatin structures known as neutrophil extracellular traps (NETs) plays important roles in immune protection, inflammatory and autoimmune diseases and cancer [40]. In osteosarcoma, a signature derived from NETs is associated with tumor recurrence and metastasis, and can predict patient prognosis [41, 42]. Currently, research on TANs in osteosarcoma is in its infancy, and there is a long road ahead in developing anti-tumor therapeutic strategies associated with TANs.

**Cancer stem cells (CSCs)** CSCs are a subset of tumor cells that play a pivotal role in various stages of cancer development, including tumorigenesis, drug resistance, recurrence and metastasis [43, 44]. Extensive research suggests that osteosarcoma may arise from mesenchymal stem cells (MSCs), which contribute to modulate immune responses, facilitate cell fusion and differentiation [45]. MSCs are capable of secreting anti-inflammatory factors while suppressing pro-inflammatory agents, thereby aiding osteosarcoma cells in evading immune surveillance. This process is mediated by extracellular vesicles (EVs), particularly exosomes, which can be released through autocrine or paracrine mechanisms. MSC-EVs inhibit T cell proliferation and the immunological effects of B cells [46, 47]. TGF- $\beta$  and interferon- $\gamma$  (IFN- $\gamma$ ) associated with MSC-EVs can facilitate the transformation of mononuclear cells into Tregs [48]. Furthermore, MSCs can promote the polarization of TAMs towards the M2 phenotype by secreting IL-6 [49].

**Tumor-associated fibroblasts (TAFs)** TAFs comprise a large proportion of cells in the microenvironment and produce components of the extracellular matrix that facilitate communication between cells, promote cell adhesion, and stimulate proliferation [50]. TAFs can be reprogrammed in lung under the influence of TGF- $\beta$ 1 in osteosarcoma-derived EVs, which promotes metastatic progression of osteosarcoma [51]. The interaction of osteosarcoma cells expressing CXCL14 and fibroblasts expressing integrin  $\alpha$ 11 $\beta$ 1 promotes metastasis and demonstrate that targeting the CXCL14-integrin  $\alpha$ 11 $\beta$ 1 axis is a potential strategy to inhibit osteosarcoma lung metastasis [52].

#### ***Tumor-suppressing cells in TME***

**T cells** T cells, particularly cytotoxic T cells, play a significant role in the immune microenvironment of osteosarcoma. T cell infiltration is the first step in its anti-tumor effectiveness. Analyses of TME of osteosarcoma demonstrate an immune cell infiltrate consisting of both macrophages and T cells [53, 54]. TIM-3(+)-PD-1(+) T cells-specific immunosuppression that was amplified by M2-type tumor-associated macrophages. depletion of CD163(+) macrophages significantly improved T cell proliferation and proinflammatory cytokine production [55]. In osteo-

sarcoma, the number of T cells in metastatic lesions is significantly higher than that in primary and recurrent lesions in situ [56]. An increased number of tumour-infiltrating T cells and PD-L1 expression in metastases compared with primary tumours, suggesting accessibility for T cells, could imply that osteosarcoma patients with metastatic disease may benefit from T-cell-based immunotherapy [56]. Tumor infiltrating lymphocytes (TILs) are mainly distributed in the region expressing human leukocyte antigen Class I, whereas CD4<sup>+</sup> and CD8<sup>+</sup> T cells concentrated at the pulmonary metastases interface [33]. The CD8<sup>+</sup> T cells in TME of osteosarcoma are functionally inactive with increased expression of PD-1 and TIM-3. TIM-3 blockade restored the T cell alloreactive function of the CD8<sup>+</sup> T cells in vitro and in vivo [57]. A study claimed that CD8<sup>+</sup> T cells have a positive association with prognosis, whereas  $\gamma\delta$ T cells are associated with poor prognosis [58]. As the main killer of tumor cells in the microenvironment, T cells have become the most important research object for developing immunotherapy.

**DCs** DCs, as professional antigen-presenting cells (APCs), act crucial roles between innate and adaptive immunity [59]. DCs detect tumor antigens and present them to helper cells and cytotoxic T cells, thereby triggering the anti-tumor immune response. During this process, DCs also transform from an immature state to a mature state. During the initial phases, DCs engage in robust proliferation and maturation, which is essential for the activation of both helper and cytotoxic T cells. However, as the tumor progresses, osteosarcoma cells evolve to generate variants that are resistant to the actions of DCs and phagocytes. This resistance results in diminished DC activation, ultimately allowing the tumor to evade the immune surveillance [60]. A study found that in group with high immune scores, the quantity of resting DCs was significantly higher than that in the low immune score group, and the degree of DC activation was positively related with anti-tumor therapy response [61]. The role of DCs in the immune microenvironment is complex. A study reported that DCs drive the pathogenesis of osteosarcoma through oncogenes and the tumor suppressor glutamate metabotropic receptor 4 (GRM4) [62]. Compared with wild-type DCs, GRM4-knockout DCs secrete more IL-23 and IL-12, leading to rapid tumor growth and accelerated progression in vivo [63]. The proportion of CD1c<sup>+</sup> DCs is larger in lung metastatic lesions than that in primary and recurrent lesions [4]. At present, the most popular therapeutic strategy for DCs is vaccination. In osteosarcoma, researchers tested the efficacy of DC vaccines in preclinical studies and demonstrated that DCs vaccines induced tumor suppression [64]. However,

in clinical trials, DC vaccines showed limited effect for the treatment of osteosarcoma [65, 66].

**NK cells** NK cells play key roles in the killing of tumor cells by releasing perforin, granzyme, and tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) and expressing FasL [67]. But the anti-tumor effects of NK cells are also affected by the PD-1/PD-L1 interactions [68]. In the osteosarcoma microenvironment, NK cells were suppressed, but TGF- $\beta$  expression increased. TGF- $\beta$  plays a key role in blunting the NKG2D-mediated tumor surveillance [69]. TIGIT also is expressed on NK cells. TIGIT inhibits human NK cytotoxicity against the tumor by interacting with the ligands, including CD155 and CD112 [70]. Therefore, targeting immune checkpoints and/or TGF- $\beta$  pathway maybe can reinvigorate tumor elimination efficiency of NK cells. Additionally, stimulating the inherent tumor-killing function of NK cells is also a promising strategy. Adoptive NK cells showed initial success in the treatment of osteosarcoma [71].

**B cells** B cells are not only the protagonists of humoral immunity by producing antibodies but are also a type of antigen-presenting cells involved in immunoregulation. Regulatory B cells are a type of B cells with immunosuppressive effects. Regulatory B cells inhibit CD4<sup>+</sup> T cells, cytotoxic T lymphocytes (CTLs), macrophages, and DCs by secreting inhibitory cytokines such as IL-10, TGF- $\beta$ , IL-35, and expressing membrane surface regulatory molecules such as FasL and CD1d, and promote the transformation of T cells into Tregs, thus weakening anti-tumor immune responses [72]. Osteosarcoma patients with high infiltration of B cells had a better prognosis and

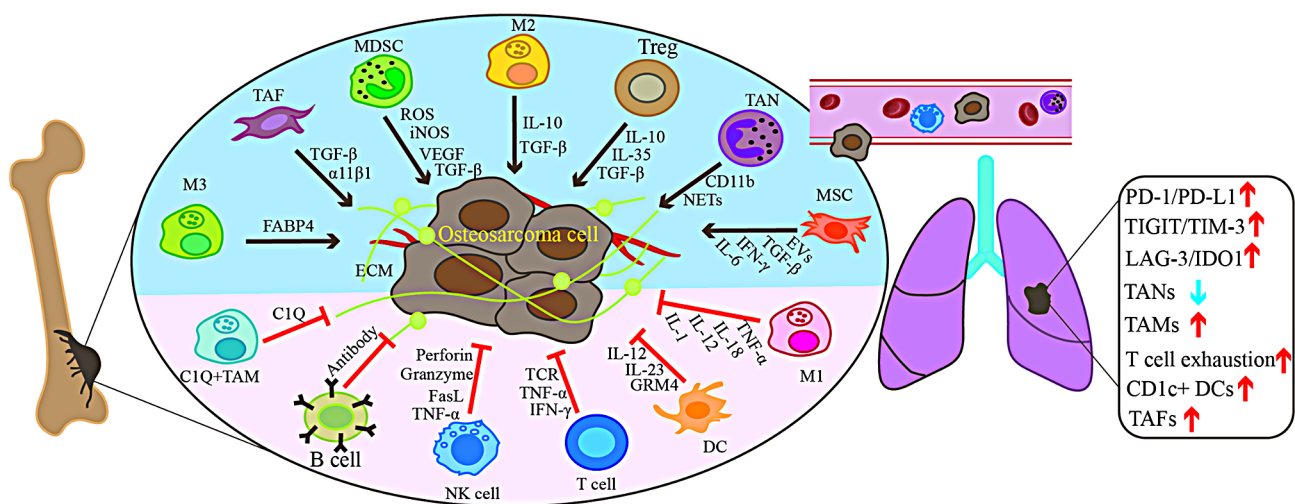
activated B cells were positively correlated with survival [73]. Research on B cells in osteosarcoma is still very limited, and new therapies based on B cells lack satisfactory results.

**M1-type TAMs and C1Q+TAMs** TAMs are typed into M1 and M2. Generally, M1 macrophages participate in anti-tumor effects, while M2 macrophages promote carcinogenesis [20]. Although M2-TAMs are predominant in the osteosarcoma microenvironment, M1 macrophages also play their respective roles. M1 macrophages release pro-inflammatory mediators such as IL-1, IL-12, IL-18, and TNF- $\alpha$ , which have anti-tumor effects. In addition, an analysis of single-cell RNA datasets and bulk RNA datasets demonstrate that complete component 1q (C1Q)-positive TAMs have anti-tumor effect and predict a better prognosis for patients with osteosarcoma [74].

The cellular components infiltrating the tumor microenvironment are diverse (Fig. 1), and their functions are not singular but also varied. Immune cells in different states may primarily promote tumor progression, or they may exert anti-tumor effects when the surrounding environment changes. Therefore, it is necessary to view the cells in the microenvironment dialectically and develop appropriate anti-tumor treatment strategies based on their characteristics.

**Molecular characteristics of osteosarcoma microenvironment**

The bone microenvironment is closely related to the progression and malignancy of osteosarcoma. The analysis of TME landscapes in osteosarcoma suggested that patients with non-metastasis showed higher immune scores and



**Fig. 1** Schematic illustration of the cellular components infiltrated in the osteosarcoma microenvironment. The TME of osteosarcoma contains tumor cells and a large number of non-tumor cells, embedded in altered extracellular matrix. The cells in the TME can be divided into tumor-promoting cells and tumor-suppressing cells. They exert their effects through different mechanisms in the microenvironment, but their functions are dynamically changing. Compared to the primary lesion, the expression of immune checkpoint molecules in the lung metastatic foci is increased, and there is an increase in immunosuppressive cells

better prognosis compared with those with metastatic osteosarcoma. In addition, high levels of tumor-infiltrating immune cells may suppress metastasis [38]. Wu et al. conducted a comprehensive genomic and immune characterization of post-treatment primary, local recurrence, and metastasis osteosarcoma specimens from 48 pediatric and adult patients. The results suggested that the high levels of genomic rearrangements and moderate point mutation burdens in osteosarcoma, which was not associated with immune infiltrate levels and neoantigen expression. Besides, there are likely multiple immune-suppressive mechanisms in play in osteosarcoma [75]. The immunosuppressive mechanisms in osteosarcoma, including (1) Highly mutated and rearranged osteosarcoma genome may not generate sufficient neoantigens to elicit an immune response, (2) Aneuploidy and copy number changes alterations and (3) Deregulation of tumor-intrinsic immunosuppressive pathways such as IFNG, MAPK/PI3K/mTOR, and JAK/STAT signal pathways [76]. Yang et al. summarized the factors, including mesenchymal stem cells, hypoxia and acidic condition, chemokines and extracellular vesicles, that affect osteosarcoma metastasis in bone microenvironment through several signaling pathways including the PI3K/Akt, Wnt/ $\beta$ -Catenin, MAPK/ERK, Hedgehog, and Notch signaling pathways [PMID: 32977425]. TAM-derived cathepsins may mediate the activation of the nuclear factor-kappa B (NF- $\kappa$ B) signaling pathway and the signal transducer and activator of transcription 3 (STAT3) to facilitate therapeutic resistance [77]. Sundara et al. studied 85 formalin-fixed, paraffin-embedded blocks from 25 osteosarcoma patients and found that the high density of tumor-infiltrating T cells in metastatic osteosarcoma lesions compared to primary tumors and local relapses. Besides, positive PD-L1 expression was found in 13% of primary tumors, 25% of relapses and 48% of metastases and correlated with a high T-cell infiltration [56]. Wan et al. identified five immune subtypes in 87 osteosarcoma samples, and each of them was associated with distinct molecular characteristics and clinical outcomes [78]. The study of RNA sequencing of 100,987 individual cells from seven primary, two recurrent, and two lung metastatic osteosarcoma lesions provides a deeper insight into the cellular and molecular characteristics of osteosarcoma and its TME properties. The results showed that proinflammatory FABP4<sup>+</sup> macrophages infiltration was found in lung metastatic osteosarcoma. And a large number of inactivated and exhausted T cells were observed, especially CD8<sup>+</sup>T cells that highly express the inhibitory receptor TIGIT and lymphocyteactivation gene 3 (LAG-3). Compared to primary lesions, pulmonary metastatic lesions expressed more immune checkpoint and immunoregulatory molecules, including PD-1, PD-L1, LAG-3, TIM-3, indoleamine 2,3-dioxygenase (IDO1) and IFN- $\gamma$  [33].

Given the suppressive immune microenvironment and high heterogeneity of osteosarcoma, achieving satisfactory therapeutic outcomes with immunotherapy may require approaches from the following aspects: (1) enhancing the immunogenicity of tumors to allow more immune cells to infiltrate the tumor tissue; (2) revitalizing the activity of anti-tumor immune cells within the microenvironment, turning “cold” tumors into “hot” tumors; (3) identifying biomarkers that can predict the responsiveness to immunotherapy, to select a population of osteosarcoma patients who are likely to benefit from such treatments; (4) considering the diverse immunosuppressive pathways in osteosarcoma, a single immunotherapy might have limited effects, while combined therapies could potentially break through this bottleneck.

#### **The relationship between TME and prognosis in osteosarcoma**

In order to detect the correlation between microenvironment and clinical parameters, Hong et al. calculated immune and stromal scores based on the ESTIMATE algorithm in 83 osteosarcoma samples and found that 137 genes were dysregulated, including 134 upregulated genes and three downregulated genes [79]. Hu et al. analyzed the data of 89 osteosarcoma samples and identified 769 TME-related genes by comparing the high-immune score and low-immune score osteosarcoma patients, which confirmed that patients with higher immune score had a favorable overall survival (OS) and disease free survival (DFS) [80]. Sheng et al. reviewed the potential mechanisms underlying osteosarcoma metastasis including microenvironment, osteoclast, angiogenesis, metabolism, immunity, and noncoding RNAs. For the immunity and metastasis, TAMs, TILs interactions between of PD-1 and PD-L1 putatively participate in immune response during osteosarcoma metastatic progression [81]. In addition, analysis of 85 osteosarcoma samples from TCGA dataset indicated that osteosarcoma cases with elevated immune cell infiltration in the microenvironment showed better prognosis [61].

In general, the TME of osteosarcoma is immunosuppressive, as increased expression of immunosuppressive molecules such as PD-1 and PD-L1 has been detected, especially in metastatic lesions. In theory, treatment with ICIs can be effective for osteosarcoma patients. Besides, osteosarcoma is a low immunogenic tumor that cannot attract a sufficient number of immune cells and tumor-specific T cells to infiltrate the tumor microenvironment. Therefore, combination strategies to improve the immunogenicity of osteosarcoma and then administering immunotherapy may have stronger anti-tumor effects. And discovering more potential tumor antigen that is specifically or at least relatively exclusively expressed in

osteosarcoma will be beneficial for the development of immunotherapy strategies based on these targets.

### Studies of ICIs for osteosarcoma

#### PD-1/PD-L1

Osteosarcoma has been shown to have variable PD-L1 expression and frequent deletions of MHC class I, which may promote immune evasion [56]. Metastatic osteosarcoma express higher PD-L1 than primary tumors. Blockade of PD-1/PD-L1 interactions significantly promotes the anti-tumor activity of tumor-infiltrating CTLs in vitro and in vivo [82]. Nivolumab (anti-PD-1 antibody) inhibits osteosarcoma metastasis in human PBMC-engrafted mouse models. The further study of mechanisms indicated that nivolumab promotes tumor lymphocyte infiltration (CD4<sup>+</sup> and CD8<sup>+</sup> lymphocytes) and enhances the cytolytic activity of CD8 lymphocytes in lung metastases [83]. Davis et al. initiated a phase I/II clinical study to test the safety, pharmacokinetics, and anti-tumor activity of nivolumab in children and young adults with recurrent or refractory non-CNS solid tumors or lymphoma [84]. The preliminary research results showed that nivolumab only has 10–30% response rate in lymphoma but no response in other tumor types (including osteosarcoma), although the treatment related side effects were acceptable [84]. SARC028 trial (NCT02301039) is the first study to assess the activity and safety of anti-PD-1-antibodies in the treatment of soft-tissue sarcoma and bone sarcoma. This trial recruited 86 patients, and 80 of whom were treated with 200 mg intravenous pembrolizumab every three weeks. The results showed that two (5%) of 40 patients with bone sarcoma had an objective response, including one (5%) of 22 patients with osteosarcoma and one (20%) of five patients with chondrosarcoma. And the adverse events were acceptable [85]. Another single-arm, open-label, phase II trial (NCT03013127) was performed to evaluate the anti-tumor activity and safety of the PD-1 antibody pembrolizumab in patients with unresectable, relapsed osteosarcoma. Total 12 patients were enrolled in this study and accepted treatment of pembrolizumab 200 mg every 21 days. The results demonstrated that pembrolizumab was well tolerated but did not show clinical benefit [86]. Besides, a phase II study (NCT02406781) was conducted to test the safety and anti-tumor effects of pembrolizumab in combination with metronomic cyclophosphamide in patients with advanced osteosarcoma. It is a pity that only one patient experienced partial response (PR). Expression level of PD-L1 is not directly related to anti-tumor efficacy [87]. PD-1/PD-L1 antibodies not only block the interaction of PD-1/PD-L1, but also show anti-tumor effect through other ways. Liu et al. reported that atezolizumab (anti PD-L1 antibody) inhibits proliferation and induces immune-independent apoptosis of osteosarcoma cells through increasing the release

of ROS and cytochrome-c. The excessive release of ROS could induce autophagy. They performed further studies in vitro and in vivo and demonstrated that blocking the protective autophagy induced by atezolizumab could significantly amplify its anti-tumor effect on osteosarcoma cells [88]. The safe dose of atezolizumab was confirmed in pediatric and young adult patients, supportive of weight-based dosing in pediatric patients [89]. However, the therapeutic effect of atezolizumab monotherapy was limited [90].

#### CTLA-4

Cytotoxic T lymphocyte-associated antigen-4 (CTLA-4) participates in the negative regulation of T cell activation and proliferation, which suppresses anti-tumor response [91]. The study suggested that CTLA-4 genetic polymorphisms also potentially associated with osteosarcoma risk [92, 93]. A phase I clinical trial was conducted by Merchant et al. to test the safety, pharmacokinetics, and immunogenicity of ipilimumab (anti-CTLA-4 antibody) in pediatric patients with advanced solid tumors, including osteosarcoma. The results suggested that ipilimumab showed incredible safety and increased numbers of activated and cycling T cells, but not regulatory T cells [94].

#### CD47

There is a large amount of macrophage infiltration in osteosarcoma, and the phagocytic effect of macrophages on tumor cells is inhibited by CD47 molecule. CD47 as a transmembrane protein inhibits macrophage phagocytosis when it binds to macrophage SIRP $\alpha$  [95]. Under physiological conditions, this is protection for normal tissues. But for tumors, CD47 is a checkpoint molecule that is overexpressed on tumor cells and inhibits macrophage anti-tumor activity. CD47 is also highly expressed in osteosarcoma and associated with the progression of tumor [96]. In vitro and in vivo osteosarcoma models demonstrated that anti-CD47 monoclonal antibody can block the CD47-SIRP $\alpha$  signaling pathway, thus enhancing the anti-tumor ability of macrophages [96].

#### TIM-3

TIM-3 was originally found to be expressed on the surface of type 1 T helper (Th1) cells [97]. TIM-3, as a negative regulator, binds to its ligand galectin-9 (Gal-9) to induce the depletion of Th1 cells [98]. Subsequently, TIM-3 was found to be expressed on the surface of various immune cells, including CD8<sup>+</sup>T, CD4<sup>+</sup>T, Treg, macrophage, NK cells and DCs, playing different roles [98]. Blocking the TIM-3/Gal-9 signaling pathway can significantly increase the function of T cells. Therefore, the negative regulation of TIM-3 has attracted much attention for the anti-tumor therapy. TIM-3 and Gal-9 are expressed in osteosarcoma tissues, and the interaction

of TIM-3 and Gal-9 promotes the apoptosis of CD4<sup>+</sup> and CD8<sup>+</sup> T cells in the TME of osteosarcoma, which is related with poor prognosis in osteosarcoma patients [99]. TIM-3 blockade impaired tumor growth in osteosarcoma models and decreased the number of tumor-infiltrating CD4<sup>+</sup> T cells while increasing the numbers and functional activation of tumor-infiltrating CD8<sup>+</sup> T cells [57]. To date, clinical research outcomes regarding TIM-3 inhibitors have not been published for osteosarcoma. But TIM-3 is a significant potential target for future advancements.

In addition to TIM-3, the expression of LAG-3 and IDO1 are significantly expressed in pulmonary metastatic foci of osteosarcoma and is closely related to immune suppression [33]. Another inhibitory molecule HHLA2, a newly defined B7 family member, widely expressed in osteosarcoma, which is associated with metastases and worse survival [100]. And the immunoreceptor inhibitory checkpoint marker TIGIT was highly expressed by the CD8<sup>+</sup> T, CD4<sup>+</sup> T, Treg and NKT cells in osteosarcoma lesions. Blocking TIGIT signaling can significantly enhance the cytotoxicity effects of CD3<sup>+</sup> T cells with high expression of TIGIT against osteosarcoma cells, indicating potential therapeutic value of targeted TIGIT therapy for osteosarcoma [4]. Currently, there is more research on traditional immune checkpoints, such as PD-1, PD-L2 and CTLA-4, but emerging immune checkpoint molecules have tremendous potential and are worth further investigation in osteosarcoma. We summarized the clinical trials of ICIs in patients with osteosarcoma (Table 1). But the current research results show that immune checkpoint inhibitors have unsatisfactory anti-tumor effects in osteosarcoma. Perhaps exploring the underlying mechanisms of immunotherapy at a deeper level can provide new directions and assistance for the application of immunotherapy in osteosarcoma.

## Studies of CAR-modified cellular therapies for osteosarcoma

### CAR-T therapy

Adoptive T cell transfer, particularly CAR-engineered T cells, is one of the current research hotspots. At present, research on adoptive cell therapy represented by CAR-T is flourishing in various tumors, especially in hematological tumors, which have achieved good therapeutic effects. CAR-T also has shown great potential in the treatment of osteosarcoma. Targeting suitable molecules is the prerequisite for the anti-tumor effects of CAR-T. Human epidermal growth factor receptor-2 (HER2) CAR-T cells, disialoganglioside (GD2) CAR-T cells, and B7H3 CAR-T cells have been discussed the most in osteosarcoma [101].

### HER2

HER2 expression has been reported in 60-70% of primary osteosarcoma [102] and is associated with adverse clinical outcomes [103]. Due to the low expression level of HER2, anti-HER2 monoclonal antibodies were ineffective for osteosarcoma patients with HER2-positive [104]. Therefore, Ahmed et al. developed HER2-specific CAR-T cells and tested their ability to proliferate, produce immunostimulatory cytokines, and kill HER2-positive osteosarcoma cell lines in vitro. The HER2-positive CAR-T cells showed markedly regression of osteosarcoma both in locoregional and lung metastatic models [105]. In addition, HER2-specific CAR-T cells also decreased sarsosphere formation of drug resistant tumor-initialing cells [106]. In 2015, Ahmed et al. reported the results of a phase I/II clinical trial (NCT00902044) which to evaluate the safety and efficacy of HER2-CAR-T cells in patients with refractory or metastatic HER2-positive sarcoma. This trial included 19 patients with HER2-positive tumors (16 osteosarcomas, one Ewing sarcoma, one primitive neuroectodermal tumor, and one desmoplastic small round cell tumor). Patients received escalating doses of HER2-CAR-T cells ( $1 \times 10^4$  to  $1 \times 10^8$  /m<sup>2</sup>) without the administration of IL-2 or lymphodepleting chemotherapy. The results demonstrated that infusion of up to  $1 \times 10^8$  /m<sup>2</sup> HER2-CAR-T cells was well tolerated, and 4 of 17 evaluable patients had stable disease for 12 weeks to 14 months. After removal of residual metastasis, three patients remain in remission at 6, 12, and 16 months, and one showing >90% necrosis. The median OS time of all 19 patients is 10.3 months (range 5.1 to 29.1 months). Further analysis found that the CAR-T cells persisted for up to 18 weeks in peripheral blood, and they were detected in tumor sites [107].

### GD2

Osteosarcoma cells express high level GD2 antigen that can be a potential target [108]. GD2-specific CAR-T cells exhibited therapeutic potency in Ewing sarcoma preclinical models [109]. Chulanetra et al. constructed CAR modified T cells targeting GD2 and demonstrated that the osteosarcoma cells expressing high levels of GD2 were be effectively targeted and killed by GD2-CAR-T cells in vitro. Besides, the combination of GD2-CAR-T cells with suboptimal chemotherapeutic treatment with docorubicin showed stronger anti-tumor efficacy. Unfortunately, further analysis suggested that the osteosarcoma cells and GD2-CAR-T cells were induced to up-regulate expression of PD-L1 and PD-1, respectively. And the interaction of PD-1 and PD-L1 promoted apoptosis of CAR-T cells [110]. This phenomenon suggests that the combination of PD-1/PD-L1 antibody and CAR-T cells may exhibit better anti-osteosarcoma effects.



**Table 1** Summary of clinical trials of ICIs in osteosarcoma

| Registration NO | Clinical Trial   | Target   | Participant Group  | State                  | Stage     |
|-----------------|--|--|--|------------------------|-----------|
| NCT03013127     | Pembrolizumab in Patients With Relapsed Or Metastatic Osteosarcoma Not Eligible for Curative Surgery   | PD-1   | Pembrolizumab  | Terminated             | Phase 2   |
| NCT03006848     | Avelumab in Patients With Recurrent or Progressive Osteosarcoma  | PD-L1  | Avelumab   | Has results            | Phase 2   |
| NCT04751383     | Magrolimab and Dinutuximab in Patients With Relapsed or Refractory Neuroblastoma or Relapsed Osteosarcoma  | CD47<br>GD2  | Arm A: Dinutuximab + Magrolimab<br>Arm B: Dinutuximab + Magrolimab + Surgery<br>Biological: Atezolizumab<br>Drug: Cabozantinib | Suspended              | Phase 1   |
| NCT05019703     | Atezolizumab and Cabozantinib in Adolescents and Young Adults Patients With Recurrent or Metastatic Osteosarcoma   | PD-L1<br>VEGFR2/MET  | Arm A: Famitinib + Camrelizumab<br>Arm B: Famitinib<br>Arm C: Famitinib + Ifosfamide   | Recruiting             | Phase 2   |
| NCT04044378     | Famitinib Plus Camrelizumab & Famitinib Alone & Famitinib Plus Ifosfamide in Patients With Advanced Osteosarcoma   | PD-1<br>VEGFR2/<br>PDGFR/c-kit                               | Oleclumab + Durvalumab   | Withdrawn              | Phase 1/2 |
| NCT04668300     | Oleclumab and Durvalumab in Patients With Recurrent, Refractory, or Metastatic Sarcoma   | PD-L1<br>CD73  | Arm A: Nivolumab<br>Arm B: Nivolumab + Ipilimumab  | Recruiting             | Phase 2   |
| NCT02500797     | Nivolumab With or Without Ipilimumab in Patients With Metastatic or Unresectable Sarcoma   | PD-1<br>CTLA-4   | Arm A: Nivolumab<br>Arm B: Nivolumab + Ipilimumab  | Has results            | Phase 2   |
| NCT02304458     | Nivolumab With or Without Ipilimumab in Younger Patients With Recurrent or Refractory Solid Tumors or Sarcomas   | PD-1<br>CTLA-4   | Arm A: Nivolumab<br>Arm B: Nivolumab + Ipilimumab  | Has results            | Phase 1/2 |
| NCT02301039     | Pembrolizumab in Patients With Advanced Sarcomas   | PD-1   | Pembrolizumab  | Has results            | Phase 2   |
| NCT02982486     | Nivolumab Plus Ipilimumab in Patients With Non-resectable Sarcoma and Endometrial Carcinoma  | PD-1<br>CTLA-4   | Nivolumab + Ipilimumab   | Unknown                | Phase 2   |
| NCT02406781     | MK3475 and Metronomic Cyclophosphamide in Patients With Advanced Sarcomas  | PD-1   | Pembrolizumab + Metronomic Cyclophosphamide  | Has results            | Phase 2   |
| NCT03359018     | Apatinib Plus Anti-PD1 Therapy in Patients With Advanced Osteosarcoma  | PD-L1<br>VEGFR   | Apatinib + SHR-1210  | Completed              | Phase 2   |
| NCT02982941     | Enoblituzumab in Children With B7-H3-expressing Solid Tumors   | B7-H3  | Enoblituzumab  | Completed              | Phase 1   |
| NCT03006848     | Avelumab in Patients With Recurrent or Progressive Osteosarcoma  | PD-L1  | Avelumab   | Has results            | Phase 2   |
| NCT03628209     | Nivolumab or Nivolumab and Azacitidine in Patients With Recurrent, Resectable Osteosarcoma   | PD-1   | Arm A: Nivolumab<br>Arm B: Nivolumab + Azacitidine   | Recruiting             | Phase 1/2 |
| NCT02541604     | Atezolizumab in Pediatric and Young Adult Participants With Solid Tumors   | PD-L1  | Atezolizumab   | Has results            | Phase 1/2 |
| NCT02815995     | Immunotherapeutic Agents in Multiple Sarcoma Subtypes  | PD-L1<br>CTLA-4  | Durvalumab + Tremelimumab  | Completed              | Phase 2   |
| NCT03190174     | Nivolumab Plus ABL-009 in Patients With Advanced Sarcoma and Certain Cancers   | PD-1<br>mTOR   | Nivolumab + ABL-009  | Completed              | Phase 1/2 |
| NCT03277924     | Sunitinib and/or Nivolumab Plus Chemotherapy in Patients With Advanced Soft Tissue and Bone Sarcomas   | PD-1<br>VEGFR/PDGFR $\beta$ /FLT3/RET/CSF-1R<br>chemotherapy | Arm A: Sunitinib + Chemotherapy<br>Arm B: Sunitinib + Nivolumab + Chemotherapy   | Recruiting             | Phase 1/2 |
| NCT03449108     | LN-145 or LN-145-S1 in Treating Patients With Relapsed or Refractory Ovarian Cancer, Triple Negative Breast Cancer (TNBC), Anaplastic Thyroid Cancer, Osteosarcoma, or Other Bone and Soft Tissue Sarcomas | PD-1<br>CTLA-4   | Autologous Tumor Infiltrating Lymphocytes + Nivolumab + plilimumab   | Active, not recruiting | Phase 2   |

**Table 1** (continued)

| Registration NO | Clinical Trial  | Target   | Participant Group  | State                  | Stage         |
|-----------------|---|--|--|------------------------|---------------|
| NCT03676985     | PD-L1 Antibody in Limited Stage of High-grade Osteosarcoma  | PD-L1  | ZKAB001  | Unknown                | Phase 1/2     |
| NCT04074564     | MASCT-I Combined With Apatinib and/or Camrelizumab in Bone and Soft Tissue Sarcoma  | PD-1<br>VEGFR2                                     | MASCT-I+ Camrelizumab + Apatinib   | Recruiting             | Early Phase 1 |
| NCT04294511     | Camrelizumab in Combination With Neoadjuvant Chemotherapy in Osteosarcoma   | PD-1<br>chemotherapy                               | Camrelizumab + chemotherapy  | Unknown                | Phase 2       |
| NCT04351308     | MAPI + Camrelizumab Versus API + Apatinib Versus MAPI in Patients With a Poor Response to Preoperative Chemotherapy for Newly Diagnosed High-grade Osteosarcoma                                   | PD-1<br>VEGFR2                                     | Arm A: chemotherapy<br>Arm B: chemotherapy + Apatinib<br>Arm C: chemotherapy + Camrelizumab  | Unknown                | Phase 2       |
| NCT04359550     | ZKAB001 for Maintenance Therapy in Patients With High-grade Osteosarcoma After Adjuvant Chemotherapy  | PD-L1  | ZKAB001  | Unknown                | Phase 3       |
| NCT04544995     | Niraparib and Dostarlimab in Pediatric Participants With Solid Tumors   | PD-1<br>PARP                                       | Niraparib + Dostarlimab  | Suspended              | Phase 1       |
| NCT04730349     | Bempegaldesleukin in Combination With Nivolumab in Children, Adolescents and Young Adults With Recurrent or Treatment-resistant Cancer  | PD-1<br>CD122                                      | Nivolumab + NKTR-214   | Has results            | Phase 1/2     |
| NCT04803877     | Regorafenib and Nivolumab in Osteosarcoma   | PD-1<br>VEGFR1/VEGFR2/VEGFR3/PDGFR-β/Kit/RET/Raf-1 | Nivolumab + Regorafenib  | Active, not recruiting | Phase 2       |
| NCT05182164     | Pembrolizumab and Cabozantinib in Patients With Advanced Sarcomas   | PD-1<br>VEGFR2/MET                                 | Pembrolizumab + Cabozantinib   | Recruiting             | Phase 2       |
| NCT05302921     | Neoadjuvant Dual Checkpoint Inhibition and Cryoablation in Relapsed/Refractory Pediatric Solid Tumors   | PD-1<br>CTLA-4                                     | Nivolumab + Ipilimumab + Cryoablation  | Active, not recruiting | Phase 2       |
| NCT03307616     | Nivolumab With and Without Ipilimumab and Radiation Therapy in Treating Patients With Recurrent or Resectable Undifferentiated Pleomorphic Sarcoma or Dedifferentiated Liposarcoma Before Surgery | PD-1<br>CTLA-4                                     | Arm A: Nivolumab<br>Arm B: Nivolumab + Ipilimumab<br>Arm C: Nivolumab + Radiation Therapy<br>Arm D: Nivolumab + Ipilimumab + Radiation Therapy | Active, not recruiting | Phase 2       |
| NCT03282344     | NKTR-214 in Combination With Nivolumab in Patients With Metastatic and/or Locally Advanced Sarcoma  | PD-1<br>CD122                                      | NKTR-214 + Nivolumab   | Active, not recruiting | Phase 2       |

### ALCAM

Activated leukocyte cell adhesion molecule (ALCAM, CD166) is a 105 kDa trans-membrane glycoprotein that belongs to the immunoglobulin superfamily. The study demonstrated that ALCAM was associated with tumorigenesis in osteosarcoma [111]. ALCAM acts as a potential therapeutic target for osteosarcoma patients with ALCAM positivity. Wang et al. developed CAR-T cells targeting ALCAM and evaluated the anti-tumor efficacy. ALCAM-CAR-T cells exhibited specific and potent cytotoxicity against human osteosarcoma cells *in vitro*; the cytotoxicity was positively correlated with the level of ALCAM expression on the osteosarcoma cells. In murine models, ALCAM-CAR-T cells inhibited the tumor growth with no obvious toxicity [112].

### B7-H3

B7-H3 (CD276) is a checkpoint molecule that contributes to tumor immune evasion, metastasis and poor prognosis [113, 114]. B7-H3 highly expressed on pediatric solid tumors, including osteosarcoma. Majzner et al. constructed a novel second-generation CAR-T cells directing B7-H3 and tested the anti-tumor efficacy in a MG63.3 xenograft model of osteosarcoma with strong metastatic potential. The results demonstrated that B7-H3-CAR-T cells mediated complete regression, eradication of osteosarcoma and lead to a significant survival advantage compared with control group. In addition, they found that CAR-T cell activity is dependent on B7-H3 molecule density, which reduced the off-target effects of CAR-T cells [115]. Zhang et al. conducted the third-generation CAR-T cells targeting B7-H3 and proved the killing effects for tumor cells *in vitro* and *in vivo* using the patient-derived xenografts (PDXs) model of osteosarcoma [116–125].

### EphA2

EphA2 is a tyrosine kinase receptor for Ephrin signaling during embryonic development. In osteosarcoma, overexpression of EphA2 has association with oncogenic signalling, the promotion of angiogenesis and tumor progression [117]. EphA2 is a promising target because of the high expression in tumors and low expression in normal tissue [118]. Hsu et al. generated EphA2-CAR-T cells that effectively killed EphA2-positive osteosarcoma cell lines *in vitro*. And in subcutaneous osteosarcoma mouse models, EphA2-CAR-T cells induced significant reduction or elimination of osteosarcoma and extended survival in a dose and delivery route-dependent manner. Besides, EphA2-CAR-T cells showed superior efficacy for liver and lung metastatic osteosarcoma cells when delivered systemically [119].

### IL-11R $\alpha$

Interleukin-11 (IL-11), a member of a family of pleiotropic cytokines [120], specifically binds to IL-11 receptor  $\alpha$ -chain (IL-11R $\alpha$ ) and active the signaling pathways involved in adipogenesis, osteoclastogenesis, neurogenesis, and megakaryocyte maturation and platelet production [121]. The overexpression of IL-11R $\alpha$  was found in several cancer types, including osteosarcoma [122]. Based on that, Huang et al. developed IL-11R $\alpha$ -CAR-T cells and tested the tumor suppression activity on osteosarcoma *in vitro* and *in vivo*. IL-11R $\alpha$ -CAR-T cells not only killed the osteosarcoma cell lines expressing IL-11R $\alpha$ , but also results in the regression of osteosarcoma lung metastases. Besides, the cytotoxicity of IL-11R $\alpha$ -CAR-T cells correlated with level of IL-11R $\alpha$  expression on osteosarcoma cells [123].

### IGF1R and ROR1

Insulin-like growth factor 1 receptor (IGF1R) is a tetrameric transmembrane receptor tyrosine kinase and widely expressed by several solid tumors and hematologic malignancies. The binding of ligand and IGF1R contributes to the proliferation, survival, transformation, metastasis, and angiogenesis [124]. Overexpression of tyrosine kinase-like orphan receptor 1 (ROR1) involved in tumor cell migration and invasiveness [125]. And the limited expression of ROR1 in normal tissues is another advantage for it to be an attractive therapeutic target in sarcomas [126]. The preclinical study conducted by Huang et al. demonstrated that IGF1R and ROR1 CAR-T cells exhibited significantly tumor growth inhibition and survival extension on sarcoma [127].

### NKG2D

CAR-T cells therapies are increasingly attractive for various malignant tumor. However, the severe cytokine release syndrome (CRS) caused by T-cell activation and expansion limited the clinical application. To solve the problem, Fernandez et al. used memory T cells to express an CAR. They chose natural killer cell group 2D (NKG2D) as the target of CAR-T cells [128]. NKG2D receptor is heterogeneously expressed in primary and metastatic osteosarcoma cells, but is rarely expressed by healthy tissues [129]. CD45RA<sup>+</sup> memory T cells expressing an NKG2D CAR effectively killed the osteosarcoma cells *in vitro* and inhibited the tumor growth in mouse models of metastatic osteosarcoma with no toxicity to healthy tissues [130]. Similarly, to diminish the risk of CRS, Lu et al. developed a CAR-T adaptor molecule (CAM)-based therapy using a bispecific low-molecular weight ligand EC17 (FITC- folic acid). EC17 penetrates solid tumors within minutes and strongly bind with folate receptor (FR), whereas unbound EC17 will be rapidly cleared [131]. Administration of intermitte dosing and/

or dose-titration of EC17 CAM could drive CAR-T cell activation, proliferation, and persistence and control the amount of cytokine releasing, which is a safety mechanism for the application of CAR-T cells. For FR<sup>+</sup> osteosarcoma cells, EC17 controlled CAR-T cells showed potent anti-tumor activity in mice models [132].

#### **CAR-M therapy**

Macrophages, as the most abundant immune cell component infiltrating the osteosarcoma microenvironment, hold great potential as a tool for adoptive immunotherapy. Compared with other immune cells, one major advantage of using macrophages for ACT is the propensity in migration and infiltration into tumors [133]. CAR macrophage (CAR-M) studies are mainly at the nascent stage with one phase I trial ongoing which uses autologous CAR-M targeting HER2 overexpressing solid tumors [134]. Pre-clinical study showed that anti-CD19 and anti-HER2 CAR-Ms phagocytose antigen-bearing tumor cells in an antigen-specific manner *in vitro*. In lung metastases mouse models, CAR-M treatment significantly inhibited tumor growth and prolonged the overall survival of the tumor-bearing mice [134]. In addition, CAR-Ms promoted an inflammatory state within the TME and were capable of antigen cross-presentation to tumor-specific CD8<sup>+</sup> T cells [135]. In osteosarcoma, CAR-M is a promising tool, which specifically recognize and eliminate tumor cells [136, 137]. Moreover, engineered CAR-Ms have led to increase of anti-tumor cytokines, such as IL-6, and chemokines, such as CXCL18, in the TME of osteosarcoma [138, 139]. The production of these beneficial cytokines fosters help to convert cold tumors into hot tumors [140].

#### **CAR-NK therapy**

As previously mentioned, NK cells possess potent anti-osteosarcoma effects, but NK cells lack the appropriate machinery to recognize tumor antigens without binding Fc- $\gamma$  receptors through CD16 to IgG-coated targets. When the NK cells are loaded a CAR targeting specific osteosarcoma antigen, this limitation can be overcome [141]. Currently, there are a number of clinical trials involving CAR-NK that have been planned or are ongoing. All these clinical trials are at the phase I/II trial stage [142]. At present, the early clinical trial results have not yet demonstrated consistent efficacy and safety. The trial in adults with chronic lymphocytic leukemia demonstrated that CAR-NK targeting CD19 are safe and effective [143]. However, the results from studies on targets other than CD19 in CAR-NK have been less than satisfactory, including both *in vivo* studies and clinical research. GD2-specific CAR-NK cells for Ewing sarcoma failed to eliminate GD2-positive sarcoma in xenografts models [71]. NK cells have a relatively short half-life,

typically less than 10 days [144], which is a double-edged sword in CAR-NK therapy. This confers an advantage in case severe toxicity occurs, but also creates a challenge that repeated administrations may be needed to achieve durable response. Compared with CAR-T cells, CAR-NK therapy show several advantages, including (1) the ability to be derived from established cell lines or allogeneic NK cells without the major histocompatibility complex (MHC) restriction; (2) CAR-NK cells eliminate cancer cells through both CAR-dependent and CAR-independent manners; (3) reduced toxicity, particularly in terms of cytokine-release syndrome and neurotoxicity.

So far, CAR-NK and CAR-M therapies have not made promising progress in the research of osteosarcoma. It need to point out, these two therapies could theoretically compensate for the shortcomings of CAR-T treatment, but there is still a long way to go before their clinical application. At present, CAR-T has shown clear anti-tumor effects in preclinical studies for osteosarcoma, and a large number of clinical trials are still ongoing, with GD2, HER2, and B7-H3 as the main targets (Table 2). Compared to other cellular adoptive therapies, CAR-T research in osteosarcoma may achieve breakthrough progress first. We are looking forward to having inspiring results reported in the future.

#### **Other immunotherapies for osteosarcoma**

GD2 and HER2 as candidate target antigens due to their high expression level on osteosarcoma cells. However, clinical trials of trastuzumab (anti-HER2) or dinutuximab (anti-GD2) for metastatic or refractory osteosarcoma were not successful [145, 146]. Therefore, Park et al. constructed the bispecific antibodies (BsAbs) targeting CD3 and GD2 or HER2. GD2-BsAb and HER2-BsAb successfully directed T cells into tumor tissues and exerted potent anti-tumor activity against osteosarcoma. Besides, PD-1/PD-L1 blockade significantly enhanced anti-tumor activity of GD2-BsAb and HER2-BsAb [147]. Currently, only a small number of clinical studies of BsAbs have been conducted in osteosarcoma, summarized in Table 3. Besides, Mason et al. performed a phase I trial in canine osteosarcoma to evaluate the anti-tumor activity of recombinant *Listeria* vaccines expressing a chimeric human HER2/neu fusion protein (ADXS31-164). They found that ADXS31-164 induced HER2/neu-specific immunity and reduced the incidence of metastatic disease and prolonged OS [148]. DCs are the most potent professional antigen-presenting cells and play a crucial role in the osteosarcoma microenvironment. Enhancing the anti-tumor capabilities of DCs holds promise as a novel therapeutic approach for osteosarcoma. Kawano et al. suggested that combined DCs and anti-TGF- $\beta$  antibodies to treat osteosarcoma and detected enhanced systematic immune responses in mice models [149].

**Table 2** Summary of clinical trials of adoptive cell therapies in osteosarcoma

| Registration NO | Clinical Trial   | Target                                  | Participant Group  | State                  | Stage         |
|-----------------|--|---|--|------------------------|---------------|
| NCT04539366     | GD2-Targeted Modified T-cells in Children, Adolescents, and Young Adults With Relapsed/Refractory Osteosarcoma and Neuroblastoma               | GD2                                     | GD2-CART cells   | Suspended              | Phase 1       |
| NCT02107963     | T Cells Expressing an Anti-GD2 Chimeric Antigen Receptor in Children and Young Adults With GD2+ Solid Tumors                                   | GD2                                     | GD2-CART cells   | Completed              | Phase 1       |
| NCT03209869     | Ex-Vivo Expanded and Activated Haploidentical NK Cells and Hu14.18-IL2 for Patients With Relapsed or Refractory Neuroblastoma and Osteosarcoma | GD2                                     | Ex vivo Expanded and Activated Haploidentical Donor NK Cells + hu14.18-IL2                       | Withdrawn              | Phase 1       |
| NCT03635632     | C7R-GD2.CART Cells for Patients With Relapsed or Refractory Neuroblastoma and Other GD2 Positive Cancers                                       | GD2                                     | C7R-GD2.CART cells   | Recruiting             | Phase 1       |
| NCT01953900     | iC9-GD2-CAR-VZV-CTLs for Refractory or Metastatic GD2-positive Sarcoma and Neuroblastoma   | GD2                                     | GD2-CART cells + VZV vaccine   | Active, not recruiting | Phase 1       |
| NCT02107963     | T Cells Expressing an Anti-GD2 Chimeric Antigen Receptor in Children and Young Adults With GD2 + Solid Tumors                                  | GD2                                     | GD2-CART cells   | Completed              | Phase 1       |
| NCT03373097     | Anti-GD2 CART Cells in Pediatric Patients Affected by High Risk and/or Relapsed/Refractory Neuroblastoma or Other GD2-positive Solid Tumors    | GD2                                     | GD2-CART cells   | Recruiting             | Phase 1/2     |
| NCT03721068     | CAR T-Cells Targeting the GD2 With IL-15 + iCaspase9 for Relapsed/Refractory Neuroblastoma or Relapsed/Refractory Osteosarcoma                 | GD2                                     | iC9.GD2.CAR.IL-15 T cells  | Recruiting             | Phase 1       |
| NCT00902044     | HER2 Chimeric Antigen Receptor Expressing T Cells in Advanced Sarcoma  | HER2                                    | HER2-CART cells  | Active, not recruiting | Phase 1       |
| NCT04995003     | HER2 Chimeric Antigen Receptor T Cells in Combination With Checkpoint Blockade in Patients With Advanced Sarcoma                               | HER2                                    | Arm A: HER2-CART cells + Pembrolizumab<br>Arm B: HER2-CART cells + Nivolumab                     | Recruiting             | Phase 1       |
| NCT04483778     | B7H3 CART Cell Immunotherapy for Recurrent/Refractory Solid Tumors in Children and Young Adults  | B7-H3                                   | Arm A: SCRI-CARB7H3(s)<br>Arm B: SCRI-CARB7H3(s)x19<br>Arm C: SCRI-CARB7H3(s)x19 + Pembrolizumab | Active, not recruiting | Phase 1       |
| NCT04897321     | B7-H3-Specific Chimeric Antigen Receptor Autologous T-Cell Therapy for Pediatric Patients With Solid Tumors                                    | B7-H3                                   | B7-H3 CART cells   | Recruiting             | Phase 1       |
| NCT04864821     | CD276 Targeted Autologous Chimeric Antigen Receptor T Cell Infusion in Patients With CD276 Positive Advanced Solid Tumor                       | B7-H3                                   | CD276-CART cells   | Unknown                | Early Phase 1 |
| NCT03618381     | EGFR806 CAR T Cell Immunotherapy for Recurrent/Refractory Solid Tumors in Children and Young Adults  | EGFR                                    | Arm A: EGFR 806CAR-EGFRt<br>Arm B: EGFR 806CAR-EGFRt and CD19CAR-T2A-HER2tG                      | Recruiting             | Phase 1       |
| NCT03462316     | NY-ESO-1-specific T Cell Receptor T Cell in Sarcoma  | NY-ESO-1                                | NY-ESO-1 TCR Specific T cell   | Active, not recruiting | Phase 1       |
| NCT03356782     | 4th Generation Safety-engineered CART Cells Targeting Sarcomas   | CD133, GD2, Muc1, CD117 or other marker | Sarcoma-specific CART cells  | Recruiting             | Phase 1/2     |

**Table 2** (continued)

| Registration NO | Clinical Trial  | Target                                  | Participant Group   | State                  | Stage     |
|-----------------|---|---|---|------------------------|-----------|
| NCT04433221     | Combination Immunotherapy Targeting Sarcomas  | GD2, PSMA, HER2, CD276 or other markers | Multiple sarcoma-specific CART cells and sarcoma vaccines     | Recruiting             | Phase 1/2 |
| NCT05312411     | FITC-E2 CART Cells in Combination With Parenterally Administered Folate-Fluorescein (UB-TT170) For Osteogenic Sarcoma   |   | UB-TT170 following SCRI-E2CAR EGFRtvl                         | Recruiting             | Phase 1   |
| NCT05621668     | T-Cell Membrane-Anchored Tumor Targeted III2 (AttII2)- T-Cell Therapy in Subjects With Advanced/Metastatic Soft Tissue and Bone Sarcoma   |   | AttII2-T cell therapy   | Recruiting             | Phase 1   |
| NCT05703854     | CAR.70-engineered IL15-transduced Cord Blood-derived NK Cells in Conjunction With Lymphodepleting Chemotherapy for the Management of Advanced Renal Cell Carcinoma, Mesothelioma and Osteosarcoma   |   | CAR.70/IL15-transduced CB-derived NK cells                    | Recruiting             | Phase 1/2 |
| NCT05208038     | α/β CD19 + Depleted Haploidentical Transplantation + Zometa for Pediatric Hematologic Malignancies and Solid Tumors   |   | TCRαβ+/CD19 + depleted Haploidentical HSCT + Zoledronate      | Recruiting             | Phase 1   |
| NCT03449108     | LN-145 or LN-145-S1 in Treating Patients With Relapsed or Refractory Ovarian Cancer, Triple Negative Breast Cancer, Anaplastic Thyroid Cancer, Osteosarcoma, or Other Bone and Soft Tissue Sarcomas |   | Autologous Tumor Infiltrating Lymphocytes LN-145 or LN-145-S1 | Active, not recruiting | Phase 2   |
| NCT02100891     | Haploidentical Transplant and Donor Natural Killer Cells for Solid Tumors   |   | Allogeneic HCT + Donor NK Cell Infusion                       | Active, not recruiting | Phase 2   |

The synergistic action of DCs and agonistic antibodies against the glucocorticoid-induced tumor necrosis factor receptor (anti-GITR) can significantly amplify the systemic immune response. This strategy not only facilitates the elimination of regulatory T cells but also effectively inhibites osteosarcoma growth in vivo [150]. A variety of vaccines have shown encouraging results, including the CD1c<sup>+</sup> DC vaccine, which harnesses the power of antigen-presenting DCs, and the innovative approach of vaccinating with polyinosinic: polycytidylic acid (poly I: C). This treatment activates and loads tumor antigens onto CD103<sup>+</sup> myeloid/conventional DC1s, enhancing the immune system's capacity to target cancer cells [151].

Research on immunotherapy for osteosarcoma is mostly in the early stages, and current results show that these treatment strategies have some therapeutic potential, but further validation and improvement are still needed. We present the current immunotherapy in osteosarcoma in Fig. 2.

**Combination therapy strategies**

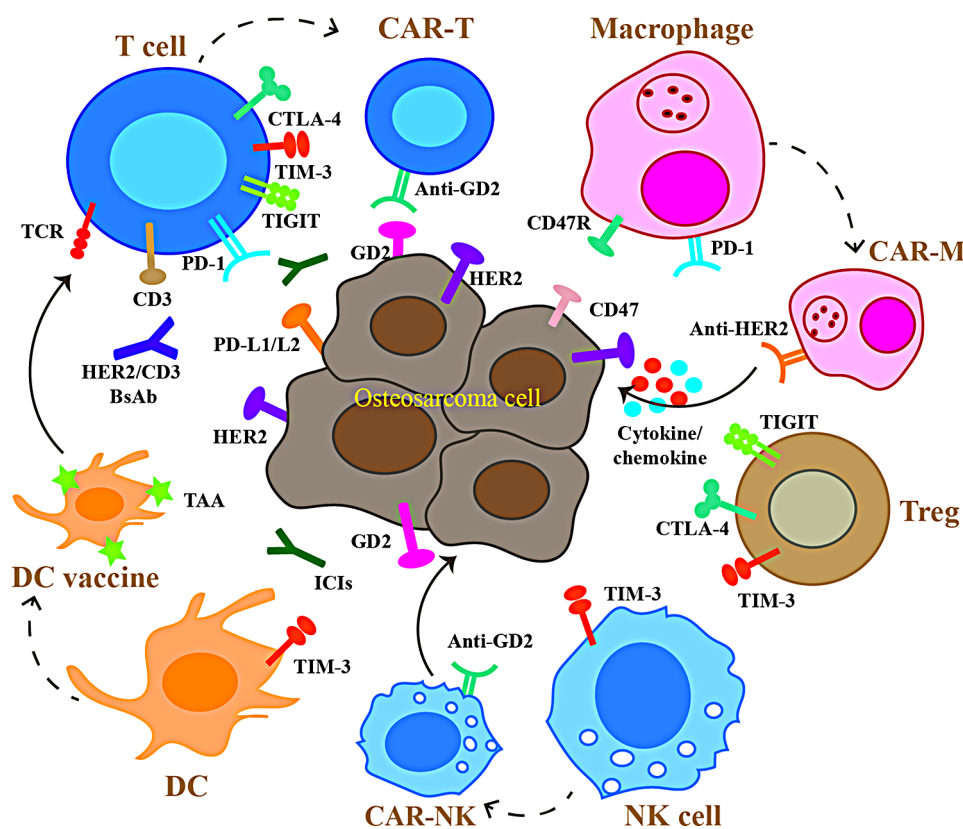
**Combination of anti-PD-1/PD-L1 and anti-CTLA-4 treatment**

Due to the presence of various immunosuppressive pathways in the microenvironment of osteosarcoma, treatment with a single-target ICI has not achieved satisfactory results. Like other solid tumors, trying a combination therapy of two ICIs in osteosarcoma may have better anti-tumor effects. At present, the combined application of ICIs is still mainly focused on classic anti-PD-1/PD-L1 and anti-CTLA-4 antibodies. Although combination therapy may bring breakthroughs in efficacy, its side effects also need to be paid more attention.

A study demonstrated that anti-PD-L1 treatment for metastatic osteosarcoma upregulate additional inhibitory receptors including CTLA-4, which contributed to the resistance of anti-PD-L1 therapy. Therefore, a combination therapy of anti-PD-L1 with anti-CTLA-4 antibody completely controlled metastatic osteosarcoma in an osteosarcoma mouse model [152]. A case report demonstrated that the combination of an anti-PD-1 antibody (nivolumab) and an anti-CTLA-4 antibody (ipilimumab) inhibited the progression of metastatic tumor for a young man with a metastatic osteosarcoma not responsive to several lines of standard chemotherapy. Before the application of dual checkpoint inhibition treatment, they detected the amplification of PD-L1 and PD-L2 in resected tumor tissue through next-generation sequencing. Thus it can be seen, it is important that identifying biomarkers to select patients with high response rates to immunotherapy [153]. The trial compared the efficacy of anti-PD-1 antibody only and the combination therapy of anti-PD-1 antibody plus anti-CTLA-4 antibody. Enrolled patients were allocated to receive either nivolumab

**Table 3** Summary of clinical trials of BsAbs in osteosarcoma

| Registration NO | Clinical Trial  | Target               | Participant Group           | State      | Stage     |
|-----------------|---|----------------------|-----------------------------|------------|-----------|
| NCT03860207     | Humanized 3F8 Bispecific Antibody (Hu3F8-BsAb) in Patients With Relapsed/Refractory Neuroblastoma, Osteosarcoma and Other Solid Tumor Cancers | GD2<br>CD3           | Humanized 3F8-BsAb          | Terminated | Phase 1/2 |
| NCT02173093     | Activated T Cells Armed With GD2 Bispecific Antibody in Children and Young Adults With Neuroblastoma and Osteosarcoma                         | GD2<br>CD3           | IL-2 + GM-CSF + GD-2Bi-aATC | Unknown    | Phase 1/2 |
| NCT03406949     | MGD009/MGA012 Combination in Relapsed/Refractory Cancer   | B7-H3<br>CD3<br>PD-1 | Obrindatamab + Retifanlimab | Completed  | Phase 1   |
| NCT02628535     | MGD009 in B7-H3-expressing Tumors   | B7-H3<br>CD3         | Obrindatamab                | Terminated | Phase 1   |



**Fig. 2** The main immunotherapy strategies in osteosarcoma. In osteosarcoma, the relatively well-studied immunotherapies include: immune checkpoint inhibitors (with targets such as PD-1/PD-L1, CTLA-4, TIM-3, CD47 and TIGIT), adoptive cell therapies (such as CAR-T, CAR-M and CAR-NK), dendritic cell vaccines, and bispecific antibodies (targeting HER2 or GD2 and CD3). TAA: tumor-associated antigen

monotherapy (43 patients) or nivolumab plus ipilimumab (42 patients). Nivolumab monotherapy showed limited efficacy for patients with advanced sarcoma. Nivolumab combined with ipilimumab demonstrated promising response rate with a manageable safety profile, which need further study [154]. 62 patients diagnosed as advanced or metastatic sarcoma were enrolled into the clinical trial (NCT02815995) to evaluate the safety and efficacy of the combination of durvalumab (anti-PD-L1 antibody) and tremelimumab anti-CTLA-4 antibody) [155]. Patients received 1500 mg intravenous durvalumab and 75 mg intravenous tremelimumab for four cycles,

followed by durvalumab alone every 4 weeks for up to 12 months. The PFS at 12 weeks was 49%. Grade 3–4 treatment-related adverse events were reported in 21 patients [155]. It can be supposed that the combination therapy of different immune checkpoint inhibitors is a promising option for patients with osteosarcoma who have progressed after multi line therapy.

**Combination of anti-PD-1/PD-L1 and anti-angiogenic treatment**

Angiogenesis is significantly correlated with tumor progression and is an important part of the formation of the

immunosuppressive microenvironment in osteosarcoma. Combining anti-angiogenic therapy with immune checkpoint inhibitor treatment is beneficial for lifting immunosuppression. At the same time, anti-angiogenic therapy promotes the normalization of tumor blood vessels, helping more anti-tumor drugs and immune cells to enter the tumor microenvironment and exert their effects.

Immunosuppression promoted by tumor angiogenesis, and more immune cells are related with angiogenesis [156]. Therefore, simultaneously targeting tumor blood vessels and antagonizing immune suppression may propose new therapeutic strategies for sarcoma. At 2020, Martin-Broto et al. reported the results of a phase Ib/II trial evaluating nivolumab (anti-PD-1 antibody) and sunitinib (inhibitor of anti-angiogenesis) combination in advanced soft tissue sarcomas (NCT03277924). 16 sarcoma patients (including osteosarcoma) entered into phase Ib study, and the results determined the recommended dose of sunitinib for phase II was 37.5 mg as induction and then 25 mg in combination with nivolumab. The 6-month PFS rate for soft-tissue sarcoma patients was 48% (95% CI: 41–55%). The most frequent treatment-related toxicities were fatigue (63.5%) and increased aspartate aminotransferase (48.0%). The most common grade 3 or 4 side effects were transaminitis (17.3%) and neutropenia (11.5%) [157]. We look forward to the publication of overall survival data of soft-tissue sarcoma patients and research results on osteosarcoma cohort. Yao et al. conducted a phase I trial (NCT04074564) to assess the safety and efficacy of multi-antigen stimulated cell therapy-I in combination with camrelizumab (anti-PD-1 antibody) and apatinib (a highly selective inhibitor targeting VEGFR2) in patients with unresectable recurrent or metastatic bone and soft-tissue sarcoma who had previously undergone at least one line of systemic therapy. Totally, 19 patients, including 6 osteosarcoma patients, were enrolled into the study. For the 6 patients with osteosarcoma, the ORR was 33.3%, the disease control response (DCR) was 50.0%, and median PFS was 5.7 months. The most common treatment-related adverse event was decreased neutrophil count. 11 (57.9%) experienced grade  $\geq 3$  treatment-related adverse events. No treatment-related deaths occurred [158]. Besides, Xie et al. reported the results of a single-arm phase II trial evaluating the safety and efficacy of apatinib (anti-VEGFR2 inhibitor) plus camrelizumab (anti-PD-1 antibody) in patients with chemotherapy-refractory osteosarcoma (NCT03359018). 43 patients enrolled this trial, and the ORR was 20.9% and two patients with durable disease control were observed. 13 of 43 patients were progression free at 6 months and the 6-month PFS rate was 50.9% [159]. In addition to ICIs, Park et al. conducted the preclinical study demonstrating that VEGF blockade enhanced the infiltration of

tumor-antigens (GD2/HER2/GPC3) armed T cells into tumor microenvironment and increased CD8<sup>+</sup> T cells survival and dispersion [160].

#### **Combination of target-specific and non target-specific treatment**

Target-specific therapies are notable for their strong targeting capabilities and significant effects, while non target-specific therapies enhance the overall immune response to fight cancer, potentially offering a broader range of applications. Non target-specific immune response, such as vaccine, DCs, IL-2 and TGF- $\beta$ , combined with target-specific immunotherapy may exhibit stronger anti-tumor efficacy.

A study examined the anti-tumor efficacy of the combination of tumor lysate-pulsed DCs and CTLA-4 antibody in a C3H mouse osteosarcoma model. The combination therapy potently inhibited the accumulation of Tregs and promoted the infiltration of CD8<sup>+</sup> T cells inside the metastatic settings. The synergistic effect of tumor lysate-pulsed DCs and CTLA-4 blockade is manifested in inhibiting the growth of metastatic tumors and prolonging survival time [161]. Guo et al. developed a new bifunctional fusion protein composed of a monoclonal antibody against PD-L1 fused with the extracellular domain of TGF- $\beta$  receptor. However, the results of the phase Ib study showed no significantly improvement of the ORR in patients with recurrent osteosarcoma, while the side effects are acceptable [162]. Bempegaldesleukin, a CD122-preferential IL-2 pathway agonist, is associated with increased proliferation and activation of TILs among patients with solid tumors [163]. Bempegaldesleukin may be beneficial for optimizing the responsiveness of metastatic sarcoma to checkpoint blockade. Based on that, a study was conducted to evaluate the safety and efficacy of bempegaldesleukin combination with nivolumab for 84 patients with selected locally advanced or metastatic high-grade sarcoma, including 10 osteosarcoma patients. The combination strategy showed limited activity for most sarcoma. In other sarcoma subtypes, CD8<sup>+</sup> T cell infiltration, PD-1 expression and upregulation of immunerelated pathways correlated with improved ORR. However, in osteosarcoma, although it has a higher tumor mutational burden, there is no significant response to the combination therapy [164].

As mentioned earlier, T cells are edited to express specific tumor antigens, allowing them to recognize and kill tumor cells. GD2-CAR-T therapy is being explored in extensive research in osteosarcoma, but the survival time of CAR-T cells in body is indeed limited. When T cells recognize viruses, they can exist in the body for many years. And when the same virus or virus vaccine enters the body again, these T cells can be quickly activated and cleared of the virus. Tanaka et al. generated the



GD2-CAR-modified varicella zoster virus (VZV)-specific T cells and conducted a phase I trial (NCT01953900) to evaluate the activity of combined application of CAR-T cells and vaccine in advanced osteosarcoma and neuroblastoma [165]. At present, the results of this study have not been reported. It is hoped that through the multifaceted exploration of researchers, strategy can be found to control tumors for osteosarcoma patients, especially those with recurrent or metastatic disease.

Immunotherapy-combination strategies have certain therapeutic potential, and discovering and verifying effective treatment combinations that achieve a “1+1>2” synergistic effect remains challenging. The exploration of advantageous combinations for immunotherapy-combination strategy is a long and arduous task.

### Conclusions and future directions

Osteosarcoma is the most common primary bone malignancy for children and young adults. The current standard care has improved the prognosis of patients with localized osteosarcoma, but the overall survival has not substantially improved over the past decades. For metastatic or relapsed osteosarcoma, even active treatment results in a 5-year survival rate of 20%. Therefore, it is urgent to find new and effective strategies to further improve the survival rate of osteosarcoma patients. The immunosuppressive TME of osteosarcoma, especially the lung metastatic foci, is infiltrated with a variety of cells that promote immunosuppression and express numerous immunosuppressive molecules, providing opportunities for the application of immunotherapy in osteosarcoma. However, it is precisely due to the unique inhibitory immune microenvironment of osteosarcoma, the responsiveness to immunotherapy is unsatisfactory. The immune checkpoint blockers (such as anti-PD-1/PD-L1 antibody and anti-CTLA-4 antibody) treatment only showed a limited therapeutic effect on osteosarcoma. CAR-T therapy in osteosarcoma is still in early stages of research. CAR-T therapy has not yet achieved the same success in clinical trials for osteosarcoma as it has in hematological malignancies, and other CAR-engineered adoptive cell therapies, such as CAR-NK and CAR-M, are still in the preclinical research phase. Based on current preclinical and clinical data on immunotherapy, there is still a long way to go for the application of immunotherapy in osteosarcoma. We need to explore more other biomarkers to predict the efficacy of immunotherapy in patients and choose the subpopulations that will be beneficial from immunotherapy. In addition, a single immunotherapy has low response rate in osteosarcoma. Combination therapy based on immunotherapy that converting a immune cold into an inflamed microenvironment may be a future direction.

### Abbreviations

|                 |   |
|-----------------|---|
| PFS             | Progression-free survival                                   |
| ORR             | Objective response rate                                     |
| ICIs            | Immune checkpoint inhibitors                                |
| CAR-T           | Chimeric antigen receptor T                                 |
| ACT             | Adoptive cell therapy                                       |
| TME             | Tumor microenvironment                                      |
| TAMs            | Tumor-associated macrophages                                |
| TANs            | Tumor-associated neutrophils                                |
| MDSCs           | Myeloid-derived suppressor cells                            |
| NK cells        | Natural killer cells  |
| DCs             | Dendritic cells   |
| CTCs            | Circulating tumor cells                                     |
| TGF- $\beta$    | Transforming growth factor-beta                             |
| Arg-1           | Arginase-1  |
| iNOS            | Inducible nitric oxide synthase                             |
| ROS             | Reactive oxygen species                                     |
| VEGF            | Vascular endothelial growth factor                          |
| HGF             | Hepatocyte growth factor                                    |
| EMT             | Epithelial-mesenchymal transition                           |
| TIM-3           | T-cell immunoglobulin and mucin domain-containing protein 3 |
| TIGIT           | T cell immunoreceptor with Ig and ITIM domains              |
| NETs            | Neutrophil extracellular traps                              |
| CSCs            | Cancer stem cells   |
| MSCs            | Mesenchymal stem cells                                      |
| EVs             | Extracellular vesicles                                      |
| IFN- $\gamma$   | Interferon- $\gamma$  |
| TILs            | Tumor-infiltrating lymphocytes                              |
| APCs            | Antigen-presenting cells                                    |
| TNF- $\alpha$   | Tumor necrosis factor- $\alpha$                             |
| CTLs            | Cytotoxic T lymphocytes                                     |
| C1Q             | Complete component 1q                                       |
| NF- $\kappa$ B  | Nuclear factor-kappa B                                      |
| STAT3           | Signal transducer and activator of transcription 3          |
| LAG-3           | Lymphocyteactivation gene 3                                 |
| IDO1            | Indoleamine 2,3-dioxygenase                                 |
| OS              | Overall survival  |
| DFS             | Disease free survival                                       |
| PR              | Partial response  |
| CTLA-4          | Cytotoxic T lymphocyte-associated antigen-4                 |
| Th1             | Type 1 T helper   |
| Gal-9           | Galectin-9  |
| HER2            | Human epidermal growth factor receptor-2                    |
| GD2             | Disialogangliosides   |
| ALCAM           | Activated leukocyte cell adhesion molecule                  |
| PDXs            | Patientderived xenografts                                   |
| IL-11           | Interleukin-11  |
| IL-11R $\alpha$ | IL-11 receptor $\alpha$ -chain                              |
| IGF1R           | Insulin-like growth factor 1 receptor                       |
| ROR1            | Tyrosine kinase-like orphan receptor 1                      |
| CRS             | Cytokine release syndrome                                   |
| NKG2D           | Natural killer cell group 2D                                |
| CAM             | CART adaptor molecule                                       |
| FR              | Folate receptor   |
| CAR-M           | CAR macrophage  |
| MHC             | Major histocompatibility complex                            |
| BsAbs           | Bispecific antibodies                                       |
| GITR            | Glucocorticoid-induced tumor necrosis factor receptor       |
| DCR             | Disease control response                                    |
| VZV             | Varicella zoster virus                                      |

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