Treating Osteosarcoma with CAR T cells

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List of abbreviations:
CAR; Chimeric Antigen Receptor
OS; Osteosarcoma
scFv, single chain variable fragment

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Abstract

Novel therapies to treat patients with solid cancers that have developed resistance to chemotherapy represent unmet needs of considerable dimensions. In the present review, we will address the attempts to develop Chimeric Antigen Receptor (CAR) targeted immunotherapy against osteosarcoma (OS). This aggressive cancer displays its peak incidence in children and young adults. The main cause of patient death is lung metastases with a 5-year survival as low as 5-10% in the primary metastatic setting and 30% in the relapse situation, respectively. Effective adjuvant combination chemotherapy introduced more than 40 years ago improved the survival rates from below 20% to around 60% in patients, however, since then, no major breakthroughs have been made. The use of immune checkpoint inhibitors has been disappointing in OS, while other types of immunotherapies such as CAR T cells remain largely unexplored. Indeed, for CAR T-cell therapy to be efficacious, two main criteria need to be fulfilled: (1) CAR T cells should target an epitope selectively expressed on the cell surface of OS in order to prevent toxicities in normal tissues (2) the target should also be widely expressed on OS metastases. These challenges have already been undertaken in OS and illustrate the difficulties in developing tomorrow's CAR-T treatment in a solid tumour. We will discuss the experiences with CAR-T therapy development and efficacy to combat the clinical challenges in OS.

Keywords: Solid tumors, Cell Therapy, T cells, Antibodies, Cytotoxicity
Introduction

The spectrum of available immunotherapies to combat cancer is broadening each year. Some of these have been around for decades, while others have just recently received clinical approval. They can be divided into three main categories; vaccines, adoptive therapies and immune checkpoint blockade [1]. Certain treatment strategies have led to dramatic improvements in the clinical outcome of some cancer types. A prime example is one of the best-known developments in the field: Chimeric antigen receptor (CAR) engineered T cells that belong to the category of adoptive cell therapy. The concept behind this method is to fuse a specific antibody derived single-chain variable fragment (scFv) with T-cell signalling domains to make an artificial receptor which recognizes antigen via scFv to get activation signal and release effector function upon target cell binding [2]. The advantage of CAR over its natural counterpart T-cell receptor (TCR) is that CAR does not rely on Human leukocyte antigen (HLA)-restriction. CARs are categorized depending on the choice of signalling compartments; The first generation CAR only had a CD3ζ tail whereas the second and third generation CARs have one or two co-stimulatory domains added, respectively [3] (Figure 1). In these designs, the inclusion of one or more co-stimulatory domains such as CD28, 4-1BB, and OX40 improves survival, proliferation and persistence of T cells compared to their first generation counterpart [4]. As mentioned later, however, adding more than two signalling domains seem to affect the fitness of the effector cells. For the treatment of solid tumours, although the three types of design are being exploited, the second generation seems to be predominantly used [5]. The topic of the present mini-review will be restricted to CAR development, but it is worth noting that therapeutic TCRs are also in development, some of which have already been tested in the clinic and/or which a comprehensive list can be found here [6].

Recently, CAR-based therapies have received tremendous attention due to the increased complete response rates (CRR) observed in some haematological malignancies, leading to FDA approval for use in B-cell malignancies (Tisagenlecleucel for pediatric B-ALL and Axicabtagene ciloleucel for adult refractory/relapsed large B-cell lymphoma) last year [7]. CARs targeting the B-lymphocyte antigen CD19 achieved 80% CRR in B-cell acute lymphoblastic leukemia (B-ALL) [8]. The most common methodological treatment approach in the aforementioned clinical trials consists of isolation of T cells from the patient, in which stably expressed CAR constructs are introduced via retroviral or lentiviral transduction followed by CAR T-cell expansion and infusion (Figure 2). The improved CRR, mainly in B-
cell malignancies, have increased the interest in the development of CAR therapy also for various solid cancer types, in particular, those where immune checkpoint blockade has not so far demonstrated the desired clinical benefit.

Osteosarcoma (OS) is a rare form of cancer, despite being the most common bone sarcoma in adolescents and young adults [9]. This defines OS as an orphan disease. There has been a lack of improvement in survival rates during the past decades. Today still more than half of the patients within an unselected OS population eventually succumb to the disease despite the current multimodal primary treatments as well as second-line chemotherapy and surgical metastasectomy(ies). Patients with primary metastatic OS still carries a dismal prognosis with expected 5-year survival rates around 10% [10] and new treatment strategies are sorely needed. Recently, several potential targets for CAR therapy have been identified and tested in OS with promising results. Both previously explored and well-known, as well as novel targets are discussed in the present review. Some of these studies addressed the main challenges of using a CAR approach in solid tumour and attempted to optimize and improve the method specifically for OS. These challenges include identifying an optimal target to avoid toxicity and immune evasion of OS. The first two challenges are in fact very closely related to each other. Ideally, the best type of target would be an antigen highly expressed on tumour and with no or very low expression on healthy tissue to allow CAR T cells to selectively eradicate the OS cells. However, in many cases the selectivity in target expression between OS cells and normal tissues is not sufficient to avoid toxicity against normal tissues, as discussed below; in the case of HER2. Numerous strategies to avoid such toxicities have already been introduced. One of these is the optimization of the number of injected cells to reduce overall toxicities and another one is using on switch technologies to regulate CAR activity [11]. Another challenge is the biology of the disease itself. By its nature OS has a microenvironment that favors M2 macrophages which support tumour growth and promote immune suppression. Additionally, a subset of OS has high expression of the checkpoint inhibitor ligand, PDL-1, which plays a role in suppression of T cell activity through an inhibitory receptor called PD-1 [12]. In fact, 25% of the primary OS tumours with high PDL-1 expression have higher likelihood of containing PD-1 high T cells than PDL-1 low tumours [13]. These features of OS decrease the efficacy of treatment and facilitates disease progression, most commonly with lung metastases reducing the life expectancy drastically [14]. Any potential treatment under clinical development should ideally target both OS-metastases as well as the primary tumour. The following sections will cover individual targets in OS and their unique potential to develop CAR therapy in this disease.
Main text

**Human epidermal growth factor receptor 2 (HER2)**

The human epidermal growth factor receptor (HER) family comprises four members, HER1-4, which function as homo and heterodimers [15]. Ligands include epidermal growth factor (EGF) and transforming growth factor-α (TGF-α), and downstream signalling involve multiple pathways, such as the Ras/Raf/MEK/ERK1/2 pathway and the phospholipase C pathway. HER2, unlike the other members, does not bind any of the ligands, and normal function is achieved by forming heterodimers with other members [15]. However, overexpression of HER2 can lead to the formation of a functional homodimer which can signal in the absence of ligand binding and induce proliferation, angiogenesis, migration and survival [16]. The proto-oncogene function of HER2 in breast cancer was described already in 1987, with overexpression correlating with poor prognosis and increased incidence of metastases [17]. Analysis of HER2 gene-amplification and expression status is now part of routine breast cancer diagnosis and monoclonal antibodies targeting HER2 such as trastuzumab have significantly improved the outcomes of HER2-positive patients [18]. Overexpression of HER2 has further been reported in other solid tumours, such as medulloblastoma, gastric, endometrial and oesophageal cancers [19]. Together these reports demonstrated that HER2 was an ideal target for antibody-based therapy.

Several reports have shown that HER2 was overexpressed in OS, and that its presence was associated with poor clinical outcome [20], but were contradicted by other reports [21]. Since HER2 gene amplification was rarely observed in OS and the presence of the protein, although detectable, was low compared to other types of tumour, HER2 was previously not considered a valid immunotherapeutic target for OS treatment. Indeed OS cells were not susceptible to the therapeutic antibody anti-HER2 trastuzumab [22]. Nevertheless, Ahmed and coworkers showed that OS tissue sections were HER2-positive when analyzed by immunohistochemistry and confirmed findings by flow cytometry [22]. The same authors had previously shown that HER2-specific CAR T cells could recognize and kill HER2-low medulloblastoma cells resistant to trastuzumab [22], and suggested that a CAR T cells therapy strategy could overcome the low HER2 expression in OS cells. Indeed, HER2 CAR T cells induced responses in HER2-low OS cell lines in vitro by proliferation and production of IFN-γ and IL-2 and killed target cells in a HER2-specific manner. Furthermore, HER2 CAR T cells induced tumour regression and increased survival of mice in a xenotransplantation model with the HER2-low OS cell line.
LM7, both when injected intraperitoneally and intravenously to mimic lung colonization by metastasis [23]. The latter model is important as it suggested that the HER2 CAR T cells had the capacity to destroy lung metastases being the main cause of fatal outcome in OS. In a follow-up study, Rainusso et al. found that HER2-specific CAR T cells reduced the sarcosphere forming capacity of two OS cell lines in vitro, both upon simple co-culture and upon harvesting of fresh tumour cells from an orthotopic mouse model that had been treated with HER2-specific CAR T cells [24]. These findings indicate that HER2-specific CAR T cells target tumour initiating cells and could provide therapeutic benefit for patients with metastatic disease.

An important issue concerning the clinical use of HER2 CAR was published in 2010 with the fatal respiratory failure which occurred in a patient after receiving $1 \times 10^{10}$ HER2-specific CAR T cells [25]. The low level of HER2 expression on lung epithelial cells was hypothesized to be the trigger for high levels of cytokine release, which resulted in tissue damage and death. The HER2-CAR vector used was a third generation CAR (Figure 1), which contained the single chain variable fragment (scFv) derived from the humanized anti-HER2 Trastuzumab antibody fused to the CD8 hinge and transmembrane domains followed by the CD28, 4-1BB and CD3zeta signalling domains. The CAR T cells were given following a lymphodepleting regimen over 2 days. In 2015 a phase I/II clinical trial was conducted in which recurrent or refractory HER2-positive sarcoma (mainly OS) patients received escalating doses of HER2-specific CAR T cells ranging from $1 \times 10^4$ cells/m$^2$ to $1 \times 10^8$ cells/m$^2$ [26]. The CAR was derived from another antibody clone FRP5 with lower affinity for HER2 than Trastuzumab and, in addition, it targets a less exposed epitope [27]. Furthermore, their design was a second generation CAR (Figure 1) with the FRP5 scFv fused to a long hinge, CD28 transmembrane and signalling domains of CD28 and CD3zeta [26]. The redirected T cells were given without any lymphodepleting regimen and were well tolerated. At the higher doses ($1 \times 10^8$), HER2-CAR T cells were detectable from 3 hours after infusion and persisted for at least 6 weeks in seven out of nine patients examined. Three patients displayed stable disease for 12-15 weeks, after which they had their residual tumour removed, and remained in remission. Nevertheless, 12 patients displayed progressive disease. Importantly, no complete response could be observed at the metastatic sites, but signs of necrosis detected in one biopsy were attributed to the CAR T cells. The authors conclude that more patients should be analyzed to reach definitive conclusions [26]. Additional manipulation of the immune system seems essential to achieve further clinical benefit, however enhancing the potency of HER2-CAR T cells would most likely increase on-target/off-tumour toxicities [24]. Taken together, Ahmed and colleagues
demonstrated that HER2 was a valid CAR target which could safely be used [26]. When speculating on the reasons why no toxicity was observed with their CAR T cells, they claimed that the dose of injected T cells, the non-lymphodepleting regimen and the use of a second-generation design were probably important. However, to compare the outcome using two different antibodies, recognizing different epitopes, might not have a clear scientific value. One would need to put FRP5 scFv on a third generation construct or Trastuzumab on the second generation to be able to conclude on the main cause of this discrepancy, as recently depicted with GD2 CAR [28]. One potential strategy for improving cancer specificity and clinical response is to target not only one, but two tumour-associated antigens on a single cancer cell by a bispecific CAR T-cell molecule [29]. A TanCAR T-cell molecule targeting both HER2 and IL13Rα2 was previously described and shown to effectively induce response in a double positive target cell population [30]. TanCARs targeting both HER2 and IL13Rα2 showed improved efficiency in a xenograft glioblastoma tumour model when compared with single targeting-CAR T cells or a mix thereof [30]. This could be explained by the superactivation of TanCARs when both antigens were encountered simultaneously and could be exploited to tune the CAR T cells to respond sufficiently only to cells which express both targets [29]. It remains to be determined which tumour-associated antigens are best suited to target OS to limit the on-target/off-tumour challenges of HER2. In summary, HER2 represents at this stage a plausible target and that FRP5-based CAR is a promising new therapy.

Disialoganglioside (GD2)

The disialoganglioside GD2, a glycosphingolipid, is an important cancer antigen and a potential target for CAR T cells. It is overexpressed in many cancers, including OS which shows an even stronger expression than neuroblastoma [31]. GD2 displays restricted expression on normal tissues, such as stem cells, neurons, certain nerve fibres and basal layers of the skin. Importantly, the antigen expression persists during tumour development, suggesting that it is not down regulated upon GD2-targeting treatments. Anti GD2-based therapies can be traced back 30 years ago (monoclonal antibody clone 3F8), when used to treat neuroblastoma and melanoma [32], and different clones have been isolated and used naked or in combinations [33]. Recently, Long et al. found that 100% of the OS samples they investigated expressed GD2 when stained with the novel clone 14G2 binding to GD2. They constructed a third generation GD2-CAR from this clone and showed that redirected T cells could effectively recognize and lyse GD2+ sarcoma cell lines in vitro. However, this GD2-CAR T cells failed to control tumour
growth \textit{in vivo} in a xenograft tumour model with periosteal injections of OS cells [34]. These \textit{in vivo} data demonstrating lack of efficacy are surprising. Indeed it would have been interesting to monitor the HER2 CAR T-cell potency with the same xenograft model, or use validated model cell lines such as the SaOS derivative LM-7 [23]. Some treatment benefit was however observed when GD2-CAR T cells were combined with all-trans retinoic acid, which alleviated immune suppression mediated by monocyte-derived suppressor cells (MDSCs). This indicates that GD2 is a potentially promising target for CAR T-cell therapy, but that targeting multiple immune pathways might be required. A third generation GD2-CAR T cells combined with a safety switch is presently being tested for the treatment of solid tumours, including OS, in a phase I dose escalation clinical trial (NCT02107963), but results are not yet published.

\textbf{IL-11Ra}

IL-11R is the receptor for the IL-11 cytokine which has been defined as both a pro- and anti-inflammatory cytokine. IL-11Ra is ubiquitously expressed at low levels but overexpression has been observed in different cancer types and is believed to link tumour growth and immune response [35]. The function of IL-11Ra has been proposed to promote tumourigenicity in the hypoxic environment through autocrine stimulation, exemplified in prostate cancer [36]. It may likely have a similar function in OS. Due to its broad expression in tumours, efforts have been made to exploit it as a target. Importantly, its expression has been observed in different cancer types including OS [37]. Furthermore, IL11Ra expression has been demonstrated in lung metastasis from OS patients, whereas almost no, or poor expression was observed in healthy surrounding tissues [38]. Recently, a correlation between cancer progression and IL-11Ra activity was demonstrated using patient biopsies and metastatic versus non metastatic cell lines [39]. This suggests that IL11Ra could be essential for tumour development. The novelty in the targeting of IL-11Ra resides in the strategy that was used to discover it: A combinatorial phage library was screened and a cyclic nonapeptide was isolated and shown to specifically and selectively bind to the receptor [40]. This peptide was then synthesized in tandem to a peptidomimetic motif previously shown to trigger cell death by mitochondrial membrane disruption has followed its pre-clinical development with the name BMTP-11 for OS and other cancer types [39]. A few years after its isolation Huang and colleagues combined this peptide to a CAR scaffold construct (Figure 1) and showed that it had the ability to redirected T cells towards OS [38]. The design was innovative and showed some efficacy \textit{in vitro} and \textit{in vivo} using xenograft tumour models. However, since the first report using this construct, no other
publication on IL-11Ra CAR has been released. It would be interesting to develop antibodies against this target and compare their efficiency with the peptide-CAR constructs described above.

**Fibroblast Activation Protein**

Most solid tumours consist of a supportive stromal part, providing a fertile environment for tumour growth. These non-malignant cell populations include fibroblasts, endothelial and immune cells embedded in the extracellular matrix. Studies on the cancer associated fibroblasts (CAFs) demonstrated that CAFs supports tumour cells by creating an ideal microenvironment for their growth by both secreting growth factors and supplying a physical support [41]. Consequently, an increasing number of studies directed their attention to CAFs in addition to the direct tumour targeting approaches. One of them involved a type II transmembrane glycoprotein termed fibroblast activation protein (FAP). Studies demonstrated that FAP has collagenolytic activity, enabling it to degrade gelatin and type I collagen [42]. The role of FAP in tumourigenes is, however, somewhat controversial. Suppression of FAP expression in breast cancer cell lines caused cells to become sensitive to serum starvation [43]. Overexpressed FAP in CT26, a colon carcinoma cell line, resulted in increased tumour load in vivo [44]. In contrast, there are studies claiming that overexpressed FAP results in significant tumour growth suppression [45]. Nonetheless, targeting FAP with CAR T cells prevented stroma to support tumour growth. It was shown that when FAP-specific CAR T cells were used alongside a tumour antigen specific CAR, an enhanced anti-tumour activity in A549 lung cancer cells was observed [46]. Although the above-mentioned studies involved different cancer models, FAP might be an important biomarker also in OS patients [47]. Combination of tumour associated stroma-directed and OS specific CAR T cells might enhance the anti-tumour activity of the latter and relates to one of the most important issues in solid cancer immunotherapy; the tumour microenvironment.

**IGF1R and ROR1**

It was recently reported that two receptors previously described to be expressed in various tumours were potential targets for CAR-based OS treatment, namely Insulin-like growth factor receptor (IGF1R) and tyrosine kinase orphan-like receptor 1 (ROR1). IGF1R is an important receptor involved in tumour growth and survival, and several blocking antibodies have been tested in clinical trials for their ability to stop tumour progression. Its expression seems to
overlap with HER2-expression in clinical samples, which makes it less interesting as an alternative target [28]. To our knowledge, no IGF1R CAR construct has been tested clinically. ROR1 is another cancer marker involved in tumour behaviour such as polarity and migration. ROR1 has mainly been described in lymphoma. ROR2 is a related member of this family of receptors that has been shown to be expressed also in OS and other solid tumours. Importantly, although ROR1 was first reported to be chronic lymphocytic leukemia (CLL) specific, it could later also be detected in healthy tissues [48]. It is important to stress that detection in healthy tissue might vary depending on the antibody used, since another antibody, Cirmtuzumab, was shown not to bind normal tissue when injected in primates and is currently in clinical development [49]. A series of antibodies targeting ROR1 have been isolated from phage library and assessed for clinical use, and ROR1 2A2 CAR has been pre-clinically evaluated for the treatment of B-cell malignancies [50]. Recently, Huang and colleagues [51] tested both IGFR1 CAR and ROR1 CAR constructs against sarcoma cell lines and found that redirected T cells were indeed able to kill such cells in vitro. They confirmed the presence of ROR1 and IGF1R proteins in different cell lines, including OS. Finally, they tested the efficacy of their constructs in a mouse xenograft OS model and showed tumour regression. Importantly, they tested two routes of injection (intraperitoneal, i.p. and intravenous i.v.). With the i.v. route, which gives rise to lung metastasis, good control of tumour progression was demonstrated. Improved survival, however, did not reach significance for IGF1R CAR. When exploring the i.p. model, a greater effect of both CARs was shown. Unfortunately, biopsies were not stained to look for T-cell infiltration or continued antigen expression in this study. Nevertheless, these promising data combined with the surprising presence of ROR1 in OS open new therapeutic options for CAR-based treatment.

Conclusions

CAR-based therapies have demonstrated extraordinary clinical response rates in B-cell malignancies and give hope that other hard-to-beat cancers can be treated with a similar therapeutic approach. Due to this success, the OS research field has explored available targets with promising pre-clinical results for CAR therapy in patients. To enhance treatment efficacy and precision, the field can implement new developments combined with existing methods. In addition, and as mentioned by Saraf and colleagues [52], canine osteosarcoma is very similar
to its human counterpart and provides an excellent opportunity to validate targets and assess specificity of novel CAR constructs.

OS, as a primary bone-cell derived malignancy, has a complex and uncertain aetiology now accepted to have a transformed mesenchymal stem cell (MSC) origin. A recent review by Yang et al. covered several valuable studies consistent with this and provided evidence for osteosarcoma having stem cell-like properties with subpopulations of CD133+ cells, suggesting self-renewable properties, higher proliferative rates and spherical colony formation [53]. Considering its origin and the resistance to chemotherapy in primary metastatic and recurrent OS, this aspect seems essential when designing novel treatment approaches. In addition, the tumour microenvironment (TME) is a critical factor. Indeed, as previously mentioned, the most prevalent organ in osteosarcoma patients suffering metastatic relapse is by far the lungs. Bone relapse is, when it occurs, most often associated also with lung metastases and carries a dismal prognosis. Despite the surgical resection of these lung metastases, remaining micrometastases are present in the majority of these patients causing subsequent relapses and, ultimately death. Upon intravenous infusion, CAR T-cells go directly to the lungs and would have a unique ability to infiltrate and eliminate such micrometastases. The resection of lesions would allow for opportunities for analyses of CAR T-cell infiltration and function and give increased knowledge of how the TME affects the CAR T cell function. It was recently reported that metastatic genes were tightly and non-randomly controlled [54]. Combining CAR T-cell treatment with drugs targeting these metastatic genes might be an attractive solution to make the TME less immunosuppressive and more permissive to T-cell infiltration and persistence. The CAR T-cells themselves can also be engineered to be resistant to immunosuppressive factors in the TME such as TGF-β [55]. Another important challenge is the immune evasion potential of OS. As mentioned, a subgroup of OS demonstrates high expression of immune checkpoint inhibitor ligands, mainly PDL-1 which reduces the efficacy of transferred CAR T cells [12]. Consequently, immune checkpoint blockade found not to work as monotherapy in OS, could be valuable in combination with other treatment approaches such as CAR T-cell therapy. The final point of utmost importance is the CAR specificity, as all of the explored and potential targets for OS in the format of CAR therapy are not unique to tumour cells. Hence, treatment might yield toxicities. To improve specificity, the field can take advantage of recently introduced designs such as combinatorial CAR systems. These depend on dividing the CD3ζ tail and the co-stimulatory tail(s) by assigning them to two separate scFvs [56]. In this case, T cells can only receive both CD3ζ and co-stimulatory signals when the two antigens are bound
(AND design) increasing the precision of target recognition, hence CAR safety. It is worth mentioning that IGF2R, which has been shown to be overexpressed in OS independently of HER2, could also be exploited and used in a dual epitope targeting strategy (Figure 1) [57]. In conclusion, the OS field has explored many attractive targets for CAR therapy with variable success. We can expect that combining them or boosting the redirected cells with molecules or strategies that could overcome resistance would be the key to a sorely needed OS treatment revolution.
Author Contributions

All authors contributed to the preparation of this review, HK and SW performed the final editing.

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Conflict of Interest Statement

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.
References


Figure legends

Figure 1: Classical CAR designs found in pre- and clinical studies: three generations of CARs have been used to treat OS, as depicted the complexity of the composition increase with the number of signalling modules. We have added the peptide CAR where the scFv part has been replaced by a high affinity peptide specific for IL-11Ra. Some of the illustrations to make this figure were obtained and modified from the “Servier Medical Art” website.

Figure 2: CAR T-cell therapy production. White blood cells are collected from the patient blood through leukapheresis where the rest of the blood is reinfused to the patient. The lymphocyte fraction is then separated by elutriation 1). T cells from the lymphocyte fraction are activated 2) and then modified to express different chimeric antigen receptors (CAR) 3). Modified T cells are then expanded 4) and infused back to the patient 5). Some of the illustrations to make this figure were obtained and modified from the “Servier Medical Art” website.