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Genetically Modified T-Cell Therapy for Osteosarcoma: Into the Roaring 2020s

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Abstract

T-cell immunotherapy may offer an approach to improve outcomes for patients with osteosarcoma who fail current therapies. In addition, it has the potential to reduce treatment-related complications for all patients. Generating tumor-specific T cells with conventional antigen-presenting cells ex vivo is time-consuming and often results in T-cell products with a low frequency of tumor-specific T cells. Furthermore, the generated T cells remain sensitive to the immunosuppressive tumor microenvironment. Genetic modification of T cells is one strategy to overcome these limitations. For example, T cells can be genetically modified to render them antigen specific, resistant to inhibitory factors, or increase their ability to home to tumor sites. Most genetic modification strategies have only been evaluated in preclinical models; however, early clinical phase trials are in progress. In this chapter, we will review the current status of gene-modified T-cell therapy with special focus on osteosarcoma, highlighting potential antigenic targets, preclinical and clinical studies, and strategies to improve current T-cell therapy approaches.

Keywords

Pediatric cancer; Osteosarcoma; Cancer immunotherapy; T-cell therapy; Gene therapy; Chimeric antigen receptor; Tumor antigens

Introduction

Adoptive T-cell therapy refers to the isolation of allogeneic or autologous T cells, followed by ex vivo manipulation, and subsequent infusion into patients for therapeutic gain [162]. Channeling the cytotoxic killing and specific targeting ability of T cells through adoptive transfer has the potential to improve outcomes for patients with osteosarcoma. An early example of adoptive T-cell therapy for osteosarcoma was reported by Sutherland and colleagues [178]. A 14-year-old girl who had the same human leukocyte antigen (HLA) type as her mother received unmanipulated maternal lymphocytes. Lymphocytes isolated from the patient post-infusion killed osteosarcoma cells in vitro, but the patient had only a

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minimal clinical response prior to disease progression. Since Sutherland's report, significant advances in immunotherapeutic techniques have taken place.

Cell therapy with conventional T cells has shown promise in several clinical settings [16, 82, 162]. Examples include donor lymphocyte infusions (DLI) after stem cell transplantation to treat CML relapse [93], infusion of Epstein-Barr virus (EBV)-specific T lymphocytes to treat EBV-related lymphomas and nasopharyngeal carcinoma [10–12, 34, 110, 174], infusion of tumor-infiltrating lymphocytes (TILs) to treat melanoma [45, 124, 162], and the infusion of virus-specific T cells to prevent and treat viral-associated disease in immunocompromised patients [65, 87, 98, 101, 185].

Since the ex vivo generation of T cells specific for tumor-associated antigens (TAAs) is often cumbersome, investigators have developed genetic modification strategies to render T cells TAA specific [19, 44, 83, 167, 193]. For example, infusion of T cells genetically modified with chimeric antigen receptors (CAR) specific for CD19 (CD19-CAR) has shown remarkable success resulting in FDA approval of two CD19-CAR T-cell products [54, 58, 119, 120, 139, 144, 145]. While CAR T-cell therapy shows promise for some patients with solid tumors [2, 6, 62, 63, 111, 137, 150, 195, 207], responses have been considerably less impressive compared to CD19-CARs. Besides rendering T cells tumor-specific, genetic modifications enable the generation of T cells with enhanced effector functions (Table 10.1). While these approaches have been mainly evaluated in preclinical models, some are already being actively explored in the clinic. In this chapter, we will review the current status of gene-modified T-cell therapy for patients with osteosarcoma, highlighting potential antigenic targets, preclinical and clinical studies, and strategies to improve T-cell therapeutic approaches.

T-Cell Therapy Targets for Osteosarcoma

Developing successful antigen-specific T-cell therapy depends on the availability of specific TAA. Once a TAA is identified, TAA-specific T cells can be either generated using conventional antigen-presenting cells or by gene transfer to recognize and induce killing of TAA-positive osteosarcoma.

TAA are potential candidates for immunotherapy, including T-cell therapy, if they are (1) expressed at higher than normal levels on tumor cells compared to nonmalignant host cells; (2) are normally only expressed during fetal development or at immunoprivileged sites, such as the testes; (3) contain novel peptide sequences created by gene mutation; (4) are viral antigens; (5) are antigens produced by epigenetic changes, or (6) are antigens on non-transformed cells in the tumor microenvironment [21, 158, 188]. Unaltered tissue-differentiation antigens on tumors can also be targets for T-cell immunotherapy, but only if the associated tissues are not essential for life and/or their products can be replaced [188]. For example, CD19-CAR T-cell therapy induces regression of CD19-positive malignancies but also leads to long-term depletion of normal, CD19+ B cells, which can be remedied by the infusion of intra-venous immunoglobulin (IVIG) [18, 58, 67, 85, 92, 144, 169].

For osteosarcoma, numerous TAA have been described that are summarized in Table 10.2. These include activated leukocyte cell adhesion molecule (ALCAM, CD166) [196], B7-H3 [115, 125], epidermal growth factor receptor (EGFR) [136], ephrin type-A receptor 2 (EphA2) [146], fibroblast activation protein (FAP) [204], G melanoma antigen (GAGE) family members [78], GD2 (a disialoganglioside; not a protein tumor associated antigen) [41, 108, 203], GD3 [41], human epidermal growth factor receptor 2 (HER2) [2, 57], interleukin 11 receptor alpha (IL11R α) [72], insulin-like growth factor 1 receptor (IGF-1R) [73, 113], melanoma-associated antigen (MAGE) [176], melanoma cell adhesion molecule (MCAM, also called MUC18) [123], NKG2D ligands (MICA, MICB, ULBP1, 2, 3) [47], New York esophageal squamous cell carcinoma 1 (NY-ESO-1) [78, 105], papillomavirus binding factor [184], tumor endothelial marker 1 (TEM1, also called endosialin or CD248) [166], and receptor tyrosine kinase-like orphan receptor 1 (ROR1) [73]. Other TAA for osteosarcoma-targeted T-cell therapy are being elucidated and should help inform future clinical trials.

Genetic Approaches to Render T Cells Specific for Osteosarcoma

Since the ex vivo generation of conventional antigen-specific T cells is often cumbersome and unreliable, investigators have developed genetic approaches to rapidly generate antigen-specific T cells. These include the forced expression of α/β T-cell receptors (TCRs), CARs, and bispecific T-cell engagers (BiTEs) [13, 29, 77, 191, 192]. Here we will focus our discussion on α/β TCRs and CARs.

α/β TCR Modified T Cells

Conventional TCRs are composed of α and β chains that form heterodimers. TCRs recognize peptides, which are derived from proteins and are presented on major histocompatibility complex (MHC) molecules on the cell surface. Isolating TCRs for adoptive T-cell therapy requires the generation of TAA-specific T-cell clones and subsequent isolation and cloning of the TCR α and β chains [187]. In general, a large number of T-cell clones need to be screened, and isolated TCRs often are of low affinity requiring additional affinity maturation. Following isolation, genes encoding the α and β chains are cloned into retroviral or lentiviral vectors and then used to transduce T cells [158]. Since T cells express endogenous α/β TCRs, mispairing between endogenous α/β and transgenic α/β TCR chains is a common problem. Several approaches have been developed to overcome this limitation, including the introduction of disulfide bonds or use of murine sequences to favor dimerization of transgenic α/β TCR chains [33, 55]. Silencing the expression of endogenous α/β TCRs by shRNAs, zinc-finger nucleases, or CRISPR/CAS9 gene editing are other options [103, 134, 148, 165, 182].

α/β TCRs have been isolated for several TAA including CEA, GP100, MAGEA3, MART1, and NY-ESO-1 [74, 81, 127, 129, 140, 157, 159, 180]. So far the safety and efficacy of α/β TCR T-cell therapy has been evaluated mainly for patients with melanoma, but studies have also been conducted for patients with sarcoma, colon cancer, and multiple myeloma. One of the first studies in humans with transgenic α/β TCR T cells was conducted by Morgan et al. and demonstrated that the infusion of autologous polyclonal T cells expressing MART1-

specific α/β TCRs was safe and induced objective tumor responses in 2 out of 15 lymphodepleted patients with melanoma [127]. To increase the response rates, the same group infused T cells expressing high affinity MART1- and gp100-specific α/β TCRs. While response rates increased, several patients developed toxicities, including skin rash, uveitis, and/or hearing loss, which were not associated with antitumor responses [81]. Recognition of normal tissues expressing low levels of CEA has also been reported for the adoptive transfer of CEA-specific α/β TCR T cells [140]. In contrast, infusion of NY-ESO-1-specific α/β TCR T cells was well tolerated with objective responses for 11/18 patients with synovial sarcoma and 11/20 patients with melanoma [160]. In addition, clinical studies indicate that NY-ESO-1-specific α/β TCR T cells induce clinical responses in patients with multiple myeloma without off-target effects [157]. As mentioned above, affinity maturation is frequently used to increase the activity of α/β TCRs. However, this can lead to recognition of related antigens resulting in severe adverse events. For example, infusion of MAGE A3-specific α/β TCR T cells caused fatal neurotoxicity due to recognition of MAGE A12 as well as fatal cardiac toxicities due to recognition of titin [121, 129].

Thus clinical studies so far have not only demonstrated the potency of adoptively transferred α/β TCR-modified T cells but also their clinical limitations. Nevertheless, active exploration of α/β TCR-modified T-cell therapy is warranted for patients with osteosarcoma.

CAR-Modified T Cells

Antigen-specific T cells can also be generated by the transfer of genes encoding CARs [46, 114, 167]. CARs consist of an ectodomain that confers antigen specificity, a hinge, a transmembrane domain, and an endodomain that contains signaling domains derived from the T-cell receptor CD3- ζ chain and costimulatory molecules such as CD28 or 41BB. Depending on the number of costimulatory domains, CARs are referred to as first generation (no), second generation (one), or third generation (two) CARs. CARs targeting multiple pediatric malignancies have been developed [1, 2, 23, 51, 54, 56, 58, 62, 69, 72, 115, 116, 120, 131, 137, 150, 164, 168]. CAR ectodomains are most commonly generated by joining the heavy and light chain variable regions of a monoclonal antibody (MAb), expressed as a single-chain Fv (scFv) molecule. CARs recognize unprocessed antigen on tumor cell surfaces and do not require peptide presentation on MHC molecules.

CAR T-cell therapy has several advantages compared to α/β T-cell therapy. Because CARs do not require antigen presentation on MHC molecules, generation of CAR T cells for patients does not require HLA matching. This property also renders CAR T cells resistant to tumor escape mechanisms, such as downregulation of HLA molecules and defects in the MHC class I processing pathway. A second advantage is that MAbs already exist for numerous surface antigens, obviating the need of cumbersome α/β TCR isolation. Additionally, CAR T cells recognize carbohydrate and glycolipid antigens, in addition to protein antigens [41, 114, 167]. Furthermore, CARs confer T-cell specificity in a single molecule unlike artificial α/β TCRs, which require the expression of two molecules that are prone to heterodimerization with the endogenously expressed α/β TCR chains. A potential drawback of CARs is that, in general, only cell surface molecules are recognized. However,

the isolation of scFvs that recognize HLA-molecule/peptide complexes has allowed the generation of CARs that recognize peptides derived from intracellular proteins [112, 122, 153, 170, 201].

Multiple osteosarcoma TAAs have been evaluated for gene-modified T-cell targeting in preclinical animal models and/or clinical trials, including those specific for HER2, GD-2, B7-H3, IL11R α , ALCAM (CD166), IGF-1R, NKG2D ligands (MICA, MICB, ULBP1/2/3), and ROR1 (Table 10.2). Of these approaches, HER2-CAR T cells have been comprehensively evaluated preclinically and in early phase clinical trials. While HER2 is not gene amplified in osteosarcoma, 60–70% of osteosarcoma are HER2+, and HER2-positivity is associated with poor outcomes [57, 130]. Preclinically, T cells expressing a second-generation CAR, derived from the monoclonal antibody FRP5, with a CD28. ζ -endodomain showed promising antitumor activity in both local and lung metastatic osteosarcoma models [1]. In addition, HER2-CAR T cells had potent antitumor activity against osteosarcoma sarco-spheres, which are enriched in osteosarcoma-initiating cells [155]. However, safety concerns were raised in regards to targeting HER2 with CAR T cells in humans. One patient, who received high dose chemotherapy followed by the infusion of 1×10^{10} high affinity third-generation HER2-CAR T cells plus IL2, developed respiratory failure within 12 hours of T-cell infusion and died [128]. Subsequently, up to $1 \times 10^8/\text{m}^2$ T cells expressing a second-generation HER2-CAR were given to pediatric and adolescent patients with sarcoma. While the infusions were safe, infused T cells did not expand significantly, and antitumor activity was limited [2]. Of 17 patients treated, 4 had stable disease for up to 14 months. Three patients had tumor removed after treatment. HER2-CAR T cells were present in two of these three tumors, and one tumor had 90% necrosis on pathologic examination. Given the safety data generated from this study, lymphodepletion was subsequently added to enhance HER2-CAR T-cell expansion and persistence. An early report demonstrated CAR T-cell expansion in 9 of 11 patients treated with lymphodepleting chemotherapy followed by HER2-CAR T cells [132]. Eight patients developed low-grade cytokine release syndrome (CRS) that resolved with supportive care, and thus far, treatment was felt to be safe. One patient had a complete response to treatment, three had stable disease, and five had progressive disease [132]. In addition, one patient, who was in complete remission with very aggressive, recurrent disease prior to lymphodepletion and HER2-CAR T-cell infusion, remains in complete remission with a follow-up of >3 years. Given these promising results, HER2-CAR T cells could provide additional benefits to patients earlier in treatment, for example, as consolidative therapy for patients with HER2+ tumors that are metastatic at diagnosis.

GD2 is another osteosarcoma TAA that has been extensively evaluated, mainly for patients with neuroblastoma. Pule et al. expressed a first-generation GD2-specific CAR on Epstein-Barr virus (EBV)-specific T cells and treated 11 children with advanced neuroblastoma [111, 150]. Three patients had complete responses (sustained in 2), while an additional two with bulky tumors showed substantial tumor necrosis. Heczey et al. used a combinatorial approach with anti-PD-1 antibody, lymphodepleting chemotherapy, and third-generation GD2-CAR T cells with CD28 and OX40 costim domains for patients with relapsed/refractory high-risk neuroblastoma [62]. Results demonstrated the therapy was safe, albeit with limited clinical response. For patients with osteosarcoma, a clinical trial using varicella

zoster virus (VZV)-specific GD2-CAR T cells in combination with VZV vaccine and lymphodepleting chemotherapy is underway ([NCT01953900](#)). Results from this and other studies should provide insight into the risks and benefits of using GD2-specific T cells for treating patients with osteosarcoma. If multiple CAR T-cell therapies are deemed safe, we envision future trials combining CARs targeting multiple osteosarcoma TAAs to limit antigen escape.

B7-H3, also called CD276, is another promising TAA found on a high percent of osteosarcoma samples [125]. B7-H3 functions to inhibit T-cell activation [97, 104] and is associated with osteosarcoma invasiveness and increased metastatic potential [194]. Majzner and colleagues reported that second-generation B7-H3-CAR T cells with a 41BB costim domain have anti-osteosarcoma activity in both local and lung metastatic models [115]. Clinically, B7-H3 antibodies have been systemically infused on early phase trials, including one treating pediatric patients with osteosarcoma ([NCT02982941](#)). B7-H3-specific T cells are not currently in clinical trials. However, a bispecific B7-H3xCD3 antibody (MGD009), which activates host T cells to target B7-H3+ tumors, is being evaluated as monotherapy ([NCT02628535](#)) or in combination with PD-1 blockade ([NCT03406949](#)), demonstrating that T-cell targeting of B7-H3 on solid tumors is an active area of research. Given these data, B7-H3-CAR T-cell trials are expected soon.

In summary, CAR T cells have shown promising antitumor activity in preclinical animal models, and initial clinical experiences are encouraging. However, several challenges remain including in vivo T-cell expansion and persistence, the inhibitory tumor microenvironment, T-cell trafficking to tumor sites, and safety. As reviewed in the next section, we and others believe that additional genetic modifications of T cells have the potential to overcome these obstacles.

Genetic Approaches to Enhance the Effector Function of Osteosarcoma-Specific T Cells

Enhancing T-Cell Expansion and Persistence In Vivo

Dramatic T-cell expansion and long-term persistence post infusion of adoptively transferred T cells has been observed in lymphodepleted patients post hematopoietic stem cell transplantation or in patients that have been lymphodepleted with chemotherapy and/or radiation prior to T-cell transfer [45, 54, 58, 65]. Since T-cell expansion post antigen recognition requires costimulation, investigators have most commonly included CAR endodomains derived from costimulatory molecules CD28 or 4-1BB, discussed in a recent review [189]. The optimal costimulatory domain to include in new CAR T cell constructs is largely unknown because direct comparisons are rarely performed in humans. Numerous preclinical studies have documented the benefit of added costimulation [17, 20, 149, 173]; however, only two studies in humans have done “head-to-head” comparisons to date [156, 169]. Savoldo et al. compared first-generation CD19-CARs with a ζ -domain to second-generation CD19-CARs with a CD28. ζ -domain [169]. While CD28 costimulation enhanced expansion of adoptively transferred CAR.CD28. ζ T cells compared to CAR. ζ T cells, the effect was limited. Ramos and colleagues reported outcomes for patients with non-

Hodgkin's lymphoma, who received simultaneous infusion of second-generation CD19-CD28.ζ- and third-generation CD19-CD28.41BB.ζ-CAR T cells [156]. In this study, third-generation CD19-CARs had improved expansion and longer persistence compared to second-generation CARs. These findings were most pronounced for patients with low disease burden and low circulating CD19+ B cells, indicating that third-generation CARs may have superior effector function for patients with low CD19 antigen load. While these studies provide insight into commonly used costimulatory domains for CD19+ malignancies, comparison of costimulatory domains on a broad scale for patients with osteosarcoma is not currently feasible, and preclinical evaluation remains critical to guide the choice of costimulatory domain(s) for genetically modified T cells in clinical trials.

While CD28 and 41BB are the most commonly used costimulatory domains, development of noncanonical costimulatory domains or strategies to provide costimulation with a second molecule expressed in CAR T cells are actively being explored. A recent study demonstrated that mesothelin-specific CD4- and CD8-CAR T cells require different costimulatory signals for optimal persistence against solid tumors in vivo [60]. Intriguingly, CD4-CARs persisted best with ICOS costimulation and CD8-CARs best with 41BB. Given that persistence of both CD4- and CD8-CAR T cells are likely important for long-term antitumor activity, these findings could prove critical insight for developing the next generation of genetically modified T-cell therapies. While intriguing and important, applicability of these findings for designing new CARs is challenging. Optimal costimulation cannot be predicted without preclinical empiric evaluation, making widespread use of this strategy unlikely with current technologies. Conceivably, new techniques for predicting optimal CAR T-cell costimulation could be developed in the years to come. This would relieve a large burden imposed by current methods of carefully evaluating multiple costimulatory domains for each new CAR product developed.

Providing costimulation via an inducible system is another technique for enhancing CAR T-cell expansion and persistence against solid tumors. Two separate groups developed CAR T cells with an inducible MyD88 and CD40 costimulatory domain (iMC). For this method, MyD88 and CD40 are part of a single construct that contains dimerization domains, which can be activated by rimiducid, also known as chemical inducer of dimerization or CID. At baseline, iMC domains are inactive and signal only when treated with CID [49, 118]. Importantly, Mata and colleagues incorporated iMC costim into first-generation HER2-CAR (HER2iMC-CAR) T cells and compared effector function to second-generation HER2-CARs against osteosarcoma in vitro and in vivo. Notably, the second-generation CAR used here was the same CAR used in clinical trials discussed above (HER2. CD28-CAR). In the presence of CID, HER2iMC-CAR T cells had significantly enhanced: (i) proliferation, (ii) cytokine production, and (iii) anti-osteosarcoma activity compared to second-generation HER2-CAR T cells. This "remote control" system is now being evaluated in PSCA-specific CAR T cells for adults with solid tumors (NCT02744287). Given that iMC or other inducible constructs could be incorporated into nearly any genetically modified T-cell product, results from this study and others should inform decisions on using it for patients with osteosarcoma.

Costimulatory ligands also show promise for enhancing CAR T-cell expansion and persistence. When activated, T cells upregulate costimulatory receptors such as 41BB. In this regard, investigators showed that second-generation CAR T cells modified to constitutively express 41BB ligand (41BBL) on the cell surface demonstrate significantly enhanced function compared to T cells containing a standard third-generation CAR, with CD28 and 41BB incorporated into the endodomain [208]. This benefit extends beyond 41BBL, as other tumor necrosis factor superfamily ligands, such as CD40 ligand showed a similar benefit [38]. Importantly, CD19.CD28-CARs with 41BBL as a second costimulatory molecule have been used to treat patients on a phase I clinical trial ([NCT03085173](#)). An early report from this study describes a positive safety profile [138]. Twenty-five adult patients with lymphomas were treated. Sixteen patients experienced low-grade CRS (grade 1 or 2), and none had severe CRS. Eight patients had neurotoxicity with two cases reported as grade 3. Twenty-one patients were evaluable for response at the time of the report, and 12 achieved a complete response [138]. With promising results coming out of this trial, similar methods are likely to be adopted for other CAR T-cell targets.

Other options to enhance expansion and persistence in vivo include transgenic expression of cytokines (discussed below) and vaccination post-infusion to boost T-cell expansion. Lastly, most studies have been conducted with unselected T cells. Some studies indicate that it might be advantageous to express CARs in T cells that are specific for viruses, so that infused cells could be boosted by vaccination (e.g., influenza) [35] or by viruses, which are present latently in humans (e.g., EBV) [150]. In addition, expressing CARs in T-cell subsets, such as central memory T cells, has the potential to enhance T-cell persistence [7, 179].

Genetic Modifications to Overcome Tumor-Mediated Immunosuppression

Malignant cells including osteosarcoma and their supporting stroma develop an intricate environment to suppress the immune system [8, 50, 53, 66, 152, 186]. They (1) secrete immunosuppressive cytokines such as transforming growth factor β (TGF β) or IL10, (2) attract immunosuppressive cells such regulatory T cells (Tregs) or myeloid-derived suppressor cells (MDSCs), (3) inhibit dendritic cell maturation, (4) express molecules on the cell surface that suppress immune cells including FAS ligand (FAS-L) and PD-L1, and (5) create a metabolic environment (e.g., high lactate, low tryptophan) that is immunosuppressive.

Three broad approaches have been developed to overcome tumor immune suppression: (1) increasing CAR T-cell activation, for example, by enhanced costimulation (discussed above) or by local production of transgenic cytokines, (2) engineering CAR T cells to be resistant to immune evasion strategies used by the tumor, and (3) targeting cellular components of the tumor stroma. Any one may affect more than one mechanism of tumor immunosuppression [39, 99].

CAR T cells can be engineered to produce immunostimulatory cytokines by transgenic expression of cytokines such as IL-15 [70, 75, 94, 151], which improves CAR T-cell expansion and persistence in vivo. In addition, it renders T cells resistant to the inhibitory effects of Tregs by activation of the phosphoinositide 3-kinase (PI3K) pathway [143]. Results from a clinical trial using GD2-CAR invariant natural killer T cells modified to

secrete IL-15 for patients with neuroblastoma ([NCT03294954](#)) should provide important insight into adapting this strategy for patients with osteosarcoma. Alternatively, transgenic expression of IL-12 in CAR T cells acts directly to enhance T-cell activity [24, 26, 28, 205]. In addition, IL-12 reverses the immunosuppressive tumor environment by triggering apoptosis of inhibitory tumor-infiltrating macrophages, dendritic cells, and MDSCs through a FAS-dependent pathway [88], resulting in enhanced antitumor activity of adoptively transferred T cells in several preclinical animal models. While there are safety concerns in regard to constitutive IL-12 expression [206], CAR T cells secreting IL-12 are actively being explored via compartmental injection to treat patients with advanced stage solid tumors ([NCT02498912](#)). Additionally, CAR T cells modified to secrete IL-18 show promise in preclinical solid tumor models [3, 27, 71]. Another approach to provide cytokine signaling to gene modified T cells without the presence of cytokine is through a constitutively active IL-7 receptor [171].

Conversely, instead of themselves being engineered to produce cytokines, CAR T cells can be engineered to be resistant to cytokines such as IL-4 and TGF β that inhibit their cytolytic function. TGF β is widely used by tumors as an immune evasion strategy [202], since it promotes tumor growth, limits effector T-cell function, and activates Tregs. These detrimental effects of TGF β can be negated by modifying T cells to express a dominant-negative TGF β receptor type II (DNR), which lacks most of the cytoplasmic kinase domain [9, 12, 48]. DNR expression interferes with TGF β -signaling and restores T-cell effector function in the presence of TGF β , and long-term results describing benefits of this strategy for patients with EBV-positive lymphomas were recently published [12].

Engineering T cells to actively benefit from inhibitory signals generated by the tumor environment is also possible, by converting inhibitory signals into stimulatory signals [4, 100, 107, 126, 197, 199]. For example, linking the extra-cellular domain of the TGF β RII to the endodomain of toll-like receptor (TLR) 4 results in a chimeric receptor that not only renders T cells resistant to TGF β but also induces T-cell activation and expansion [197]. Chimeric IL-4 receptors are another example of these “switch receptors.” Many tumors secrete IL-4 to create a TH2-polarized environment. Multiple reports have shown that expression of chimeric IL-4 switch receptors, consisting of the ectodomain of the IL-4 receptor and the endodomain of the IL-7R α or the IL-2R β chain, enable T cells to proliferate in the presence of IL-4 and retain effector function including TH1-polarization [4, 102, 126, 199].

Silencing genes that render T cells susceptible to inhibitory signals in the tumor microenvironment may also improve T-cell function. For example, many tumor cells express FAS ligand, and silencing FAS in T cells prevents FAS-induced apoptosis [43]. Besides silencing genes, expression of a constitutively active form of serine/threonine AKT (caAKT), which is a major component of the phosphatidylinositol 3-kinase (PI3K) pathway in T cells, has also been shown to improve T-cell function [177]. caAKT-expressing T cells sustained higher levels of NF- κ B and had elevated levels of antiapoptotic genes such as Bcl2, resulting in resistance to Tregs and TGF β .

Lastly, most solid tumors have a stromal compartment that supports tumor growth directly through paracrine secretion of cytokines, growth factors, and provision of nutrients, and contributes to tumor-induced immune suppression [32, 61]. For example, we have shown in preclinical studies that T cells expressing CARs specific for fibroblast activation protein (FAP) expressed on cancer-associated fibroblasts (CAFs) have potent antitumor effects [84]. In addition, combining tumor-specific CAR T cells with FAP-specific CAR T cells enhanced antitumor activity. While some concerns have been raised in regard to targeting FAP [161, 183], our findings indicate that targeting FAP on CAFs has the potential to improve antitumor effects of adoptively transferred CAR T cells. Targeting the tumor vasculature with CARs to enhance T-cell therapy for solid tumors has also been explored [25, 133]. Targeting the tumor vasculature with vasculature endothelial growth factor receptor 2 (VEGFR2)-specific CAR T cells combined with providing tumor-specific T cells synergized in inducing tumor regression in several syngeneic, preclinical tumor models [25]. In addition, transgenic expression of VEGFR2-specific CARs and IL-12 in T cells was sufficient to eradicate tumors, indicating that combining countermeasures might potentiate effects [24].

While many of the discussed genetic modification strategies have not been explored in osteosarcoma models, these strategies could be readily integrated in current T-cell therapy approaches for osteosarcoma.

Genetic Modification of T Cells to Improve Homing to Tumor Sites

T-cell homing to solid tumor sites might be limited. For example, Kershaw et al. evaluated the safety and efficacy of first-generation folate receptor (FR)- α CAR T cells in patients with ovarian cancer [90]. Infused T cells persisted less than 3 weeks in all but one patient and did not specifically home to tumor sites as judged by ^{111}In scintigraphy. No antitumor activity was observed. Since then, several investigators have shown in preclinical models that the expression of chemokine receptors in CAR T cells that recognize chemokines secreted by solid tumors can enhance T-cell homing. For example, transgenic expression of chemokine receptors CCR2b or CXCR2 in T cells enhances trafficking to CCL2- or CXCL1-secreting solid tumors including melanoma and neuroblastoma [36, 89]. Another recent report demonstrates that CAR T cells modified to express CXCR1 or CXCR2 have enhanced homing to brain tumors via recognition of IL-8. Interestingly, tumors only secreted IL-8 after local radiation therapy, making this combinatorial strategy an intriguing method for enhanced CAR T-cell homing [79]. While these specific genetic modification techniques have not been implemented in clinical trials using CAR T cells, one study evaluating if CXCR2 gene modification can improve homing and antitumor activity of tumor-infiltrating lymphocytes is underway ([NCT01740557](#)).

Improving Safety of T-Cell Therapy

Toxicities can be divided into four categories: (1) toxicities due to genetic modification, which have not been observed with genetically modified T cells in humans so far [5, 14, 117], (2) “on target organ” toxicities (e.g., depletion of normal B cells post CD19-CAR T cells) [85], (3) “on target, off organ” toxicities (e.g., liver toxicity of carbonic anhydrase IX

CAR T cells to target renal cell carcinoma) [95], and (4) systemic inflammatory syndromes [58, 85, 144].

Genetic safety switches have been developed to selectively destroy genetically modified T cells once adverse events occur. The most widely used suicide gene strategy for T-cell therapy is to introduce the herpes simplex virus thymidine kinase (HSV-tk) gene into T cells. HSV-tk phosphorylates acyclovir, valacyclovir, and ganciclovir to toxic nucleosides [31]. T cells transduced with HSV-tk are robustly killed in the presence of these medications and clinical studies demonstrate effectiveness of the strategy. A drawback to utilizing HSV-tk as a safety switch for T-cell therapy is the immunogenicity of HSV-tk, and that some patients require acyclovir, valacyclovir, or ganciclovir to treat herpetic diseases. Therefore, genetic safety switches using non-immunogenic human components have been developed, such as inducible caspase 9 (iC9) [40, 175]. As opposed to using CID to activate costimulatory domains, the drug can also be used to activate caspase-induced cell death. Once exposed to CID, T cells genetically modified with iC9 rapidly undergo apoptosis. Furthermore, repeated doses of CID can remove remaining populations of genetically modified cells expressing low levels of iC9 [209], demonstrating that administration of CID is safe and functional in clinical settings. Another approach includes the transgenic expression of CD20 or truncated EGFR (tEGFR), rendering T cells sensitive to the clinically approved MAbs rituximab or cetuximab, respectively [76, 141]. Multiple clinical trials are open using CAR T cells modified to express tEGFR as a safety mechanism ([NCT03085173](#), [NCT03618381](#), [NCT03244306](#), [NCT03710421](#), [NCT02153580](#), [NCT02159495](#), [NCT02051257](#), [NCT02028455](#), [NCT03070327](#), [NCT02028455](#), [NCT02706405](#), [NCT01865617](#), [NCT02146924](#), [NCT03389230](#)). While suicide gene switches can selectively kill infused cells, systemic inflammatory syndromes might be difficult to control with this approach since resident immune cells, which are activated by the infused T cells, most likely contribute. Studies indicate that IL6 plays a critical role in these syndromes, and the infusion of the IL6 receptor MAb (tocilizumab) alone or in combination with steroids proved to be effective [58, 85, 144].

While suicide switches are one strategy to prevent “on target, off organ” toxicities, other strategies include the generation of T cells that are only fully activated if they encounter a unique “antigen address” at the tumor site. Examples include the development of T cells expressing two CARs in which one TAA-specific CAR has an endodomain with a ζ -signaling domain and a second CAR, specific for another TAA, provides costimulation [91, 96, 200]. For this type of approach, success depends on targeting two antigens that are unlikely to be found on a given normal tissue, making antigen selection critical for translating this approach to target osteosarcoma.

Combinatorial T-Cell Therapy

As for other cancer therapies, combinatorial therapies hold promise for improving T-cell therapy for cancer [190]. These can be divided into approaches that (1) kill tumor cells without affecting T cells, (2) enhancing the expression of TAA, (3) improving T-cell expansion and persistence, and (4) reversing the inhibitory tumor microenvironment. For example, the BRAF inhibitor vemurafenib has no adverse effects on T-cell function, and

combining vemurafenib with adoptive transfer of T cells enhanced antitumor effects in preclinical animal models of melanoma [42, 106]. Increasing the expression of TAA in cancer cells can be achieved with epigenetic modifiers such decitabine [30, 37].

Combining T-cell therapy with blocking antibodies specific for negative regulators of T-cell responses such as the cytotoxic T-lymphocyte-associated protein (CTLA-4) and programmed cell death 1 (PD-1) is one strategy to increase their function [86, 142, 181, 198]. The role of CTLA-4 as a negative regulator of T-cell responses has been well demonstrated in CTLA-4-deficient mice and preclinical tumor models. Based on these studies, an antibody to block human CTLA-4 (ipilimumab) was developed, and a phase III randomized clinical trial showed that 23% of patients with metastatic melanoma survived more than 4 years following ipilimumab treatment, leading to FDA approval [68].

Similarly, combining T-cell therapy with MAbs that block PD-1 and/or its ligands (PD-L1 and PD-L2) is another promising approach. Clinical trials evaluating the safety and efficacy of PD-L1 antibodies reported encouraging objective clinical response rates for patients with advanced solid tumors [15, 147]. In addition, multiple reports have demonstrated benefits of blocking the PD-1/PD-L1 axis to enhance adoptive cell transfer in preclinical models [22, 80, 154].

As mentioned in section “Enhancing T-cell Expansion and Persistence In Vivo,” the administration of vaccines is an attractive strategy to boost adoptively transferred T cells. Several groups have shown that vaccines augment the effectiveness of adoptive T-cell therapy in preclinical animal models [109, 135, 172]. Besides provision of antigen, providing potent costimulation and/or cytokines was critical for the observed effects. However, limited experience is available in humans except for an ongoing clinical trial in which patients are vaccinated with an autologous DC vaccine post α/β TCR T-cell transfer.

Lastly, reversing the immunosuppressive tumor microenvironment with small molecule inhibitors is another approach to enhance the antitumor activity of adoptively transferred T cells. For example, blocking STAT3 in combination with the adoptive transfer of T cells resulted in enhanced antitumor effects [52, 64]. In addition, several preclinical studies have highlighted the benefit of combining oncolytic viruses with the adoptive transfer of CAR T cells [59, 163].

Conclusions

T-cell therapy has shown promising results in early phase clinical studies especially for patients with hematological malignancies. For solid tumors including osteosarcoma, T-cell therapy has shown promise in preclinical studies but formidable challenges remain in developing safe and effective T-cell therapies for treating patients with osteosarcoma. These include target antigen selection, limited in vivo T-cell expansion and persistence, T-cell trafficking to tumor sites, and the hostile tumor microenvironment. Genetic modification of T cells and combining T-cell transfer with other therapies are promising strategies to overcome these obstacles.

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Table 10.1

Genetic modifications for T-cell therapy for osteosarcoma

Goal	Introduced gene class	Example
Antigen-specificity	Receptors	$\alpha\beta$ TCR, CAR, BiTE
T-cell expansion	Costim molecules	CD40L, CD80, 41BBL
	Domains of costim molecules	CD27, CD28, 41BB, OX40, ICOS, MyD88/ CD40, DAP12
	Cytokines	IL12, IL15, IL18
Resistance to inhibitory tumor environment	Costim molecules	CD40L, CD80, 41BBL
	Domains of costim molecules	CD27, CD28, 41BB, OX40
	Cytokines	IL7, IL12, IL15, IL18
	Dominant negative receptors	DN TGF β receptor
	Chimeric cytokine receptors	IL4/IL2, IL4/IL7, TGF β /41BBL
	Constitutive active cytokine receptors	C7R
	shRNAs, TALENs, CRISPR/Cas9	FAS, PD-1, CTLA-4, TIM-3
	Constitutive activated kinases	AKT
Improve T-cell homing to tumor sites	Chemokine receptors	CCR2b, CCR4, CXCR1, CXCR2
Safety	Inducible suicide genes	HSV-tk; caspase 9
	Cell surface markers	CD20, tEGFR

BiTE Bispecific T-cell engager, *DN* dominant negative, *HSV-tk* Herpes simplex virus thymidine kinase, *IL* interleukin, *TGF β* transforming growth factor β , *tEGFR* truncated epidermal growth factor receptor

Table 10.2

Tumor-associated antigens expressed in osteosarcoma

Target antigen	Cell surface expression	Preclinical in vivo studies ^a	T-cell clinical studies ^b
ALCAM (CD166)	+	+	–
B7-H3	+	+	–
EGFR	+	–	+
EphA2	+	–	–
FAP	+	–	–
GAGE 1,2,8	–	–	–
GD2	+	+	+
GD3	+	–	–
HER2	+	+	+
IL-11R α	+	+	–
IFG-1R	+	+	–
MAGE A1–6,10, 12; C2	–	–	–
MCAM (MUC18)	+	–	–
NKG2D ligands	+	+	–
NY-ESO-1	–	–	–
Papillomavirus binding factor	–	–	–
ROR1	+	+	–
TEM1	+	–	–

^a using T cells vs. osteosarcoma;^b including patients with osteosarcoma

ALCAM activated leukocyte cell adhesion molecule, *CLUAP1* clusterin-associated protein 1, *EGFR* epidermal growth factor receptor, *EphA2* ephrin type-A receptor 2, *FAP* fibroblast activation protein, *GAGE* G melanoma antigen, *GD2* disialoganglioside, *HER2* human epidermal growth factor receptor 2, *IL11R α* interleukin 11 receptor α , *IFG-1R* insulin-like growth factor 1 receptor, *MAGE* melanoma-associated antigen, *MCAM* melanoma cell adhesion molecule, *NY-ESO-1* New York esophageal squamous cell carcinoma 1, *ROR1* receptor tyrosine kinase-like orphan receptor 1, *TEM1* tumor endothelial marker 1