frontiers in Immunology

REVIEW
published: 16 May 2019
doi: 10.3389/fimmu.2019.01114

Innate Immune Cells: A Potential and Promising Cell Population for Treating Osteosarcoma

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Specialty section:
This article was submitted to Cancer Immunity and Immunotherapy, a section of the journal Frontiers in Immunology

Received: 24 November 2018
Accepted: 01 May 2019
Published: 16 May 2019

Citation:
Wang Z, Wang Z, Li B, Wang S, Chen T and Ye Z (2019) Innate Immune Cells: A Potential and Promising Cell Population for Treating Osteosarcoma. Front. Immunol. 10:1114. doi: 10.3389/fimmu.2019.01114

Advanced, recurrent, or metastasized osteosarcomas remain challenging to cure or even alleviate. Therefore, the development of novel therapeutic strategies is urgently needed. Cancer immunotherapy has greatly improved in recent years, with options including adoptive cellular therapy, vaccination, and checkpoint inhibitors. As such, immunotherapy is becoming a potential strategy for the treatment of osteosarcoma. Innate immunocytes, the first line of defense in the immune system and the bridge to adaptive immunity, are one of the vital effector cell subpopulations in cancer immunotherapy. Innate immune cell-based therapy has shown potent antitumor activity against hematologic malignancies and some solid tumors, including osteosarcoma. Importantly, some immune checkpoints are expressed on both innate and adaptive immune cells, modulating their functions in tumor immunity. Therefore, blocking or activating immune checkpoint-mediated downstream signaling pathways can improve the therapeutic effects of innate immune cell-based therapy. In this review, we summarize the current status and future prospects of innate immune cell-based therapy for the treatment of osteosarcoma, with a focus on the potential synergistic effects of combination therapy involving innate immunotherapy and immune checkpoint inhibitors/oncolytic viruses.

Keywords: osteosarcoma, innate immune cell, adoptive cell therapy (ACT), vaccine, immmune checkpoint

INTRODUCTION

Osteosarcoma is the most common primary malignant bone tumor and it often leads to pulmonary metastasis, which is the major cause of death of osteosarcoma patients (1). Surgical resection combined with neoadjuvant and postoperative chemotherapy has increased long-term survival rates to 70% for patients with localized osteosarcomas, but <20% for patients with recurrent and/or metastasized osteosarcomas. The current standard treatment strategy has remained unchanged for decades (2). Therefore, there is urgent need to develop novel therapies to improve the overall survival rates of osteosarcoma patients, particularly those experiencing relapse and/or metastasis.

Immunotherapy is becoming an attractive therapeutic strategy for the treatment of osteosarcoma. The human immune system, which consists of innate and adaptive immunity, plays a critical role in suppressing tumor growth. The major effector cells in adaptive immunity targeting osteosarcoma are cytotoxic T cells (CTLs). A previous study demonstrated that CTLs played an important role in immune surveillance in osteosarcoma patients (3). In addition, adoptive transfer of T cells successfully resulted in tumor inhibition in mouse models of osteosarcoma (4–6).

Recently, the role of innate immune cells in the control of tumor progression has been characterized. Innate immune cells contribute to tumor suppression through direct recognition and killing, through self-activation to trigger a strong adaptive immune response, or through both mechanisms (7). The antitumor immunocompetence of innate immune cells provides a rational basis for innate immune cell-based therapy, which has shown promise for the treatment of hematopoietic malignancies and solid tumors (8). Indeed, successful treatment of osteosarcomas in preclinical studies using innate immune cells has been reported (9, 10). Our previous studies have shown that innate immune cells were effective against osteosarcoma (11–14). In this paper, we describe the anti-osteosarcoma roles of the following major classes of innate immune cells: dendritic cells (DCs), macrophages, natural killer (NK) cells, natural killer T cells (NKT) cells, and γδ T cells. We also review the current status of innate immune cell-based therapy for the treatment of osteosarcoma and potential future improvements based on the results of treatment of other types of tumors. Moreover, immune checkpoint inhibitors (ICPIs) represent a new frontier in cancer therapy and have shown a certain degree of therapeutic effects in osteosarcoma patients (15). Some immune checkpoints are not only expressed on T cells, but also on DCs, macrophages, NK cells, NKT cells, and γδ T cells; blocking these immune checkpoints reverses their anti-tumor activity in tumor immunity. Therefore, we detail the effects of immune checkpoint-inhibition on immune cells and the potential for synergy based on combining innate immune cell-based therapy with immune checkpoint manipulation for the treatment of osteosarcoma. In addition, as oncolytic virus (OV) therapy is known to induce an innate immune response, we also discuss the combinational potential of innate immune cell-based therapy and OVs.

DENDRITIC CELLS

DCs, which are professional antigen-presenting cells (APCs), take up and present antigens to naïve T cells, ultimately stimulating them to differentiate into tumor killers (16). Recently, a series of studies have shown that DCs can also activate innate immune cells with robust antitumor activity such as γδ T cells, cytokine-induced killer (CIK) cells (17–19).

However, established tumors always endeavor to reduce the availability of antigen presentation by APCs, resulting in immunosuppression, which disrupts the generation of antitumor immune responses (20, 21). In response, DC vaccines have been developed to bypass this mechanism. This procedure can be summarized as follows: DCs are isolated from peripheral blood mononuclear cells (PBMCs), matured, and loaded *ex vivo* with tumor antigens with defined cocktails, and then infused back into the patient (**Figure 1**). Theoretically, these antigen-activated DCs can successfully boost the immune response. Recent preclinical studies of osteosarcoma DC vaccines are listed in (**Table 1**). They can be classified into three major groups based on the protocols for loading various sources of antigens (33): (1) DCs co-cultured with peptides, proteins, or tumor-cell lysates; (2) DCs transfected with DNA, RNA coding for antigens, or total RNAs derived from tumor cells; and (3) fusions between DCs and devitalized tumor cells. Yu et al. (23, 24) tested the efficacy of osteosarcoma DC vaccines either fused with whole-tumor cell or transduced with total tumor RNA. Most immunized tumor-free rats acquired partial or complete protection from tumor challenge. In addition, vaccination induced tumor suppression in tumor-bearing mice (23, 24). Other studies tested the potential of combination therapy consisting of a DC vaccine and targeted drugs such as anti-transforming growth factor-β (TGF-β)/glucocorticoid-induced tumor necrosis factor receptor (GITR) antibodies (30, 32). The results of these studies showed that primary and metastatic tumor growth was inhibited. In addition, the tumor microenvironment (TME) was remodeled with reduced number of regulatory T lymphocytes (Tregs), reduced levels of immunosuppressive cytokines, and an increased number of $CD8^+$ T lymphocytes (30, 32). However, DC vaccines were less effective for the treatment of osteosarcomas in clinical trials (34–36). For instance, only two out of 12 patients exhibited a strong anti-tumor immune response, and none exhibited any clinical effects, after receiving 3 weekly DC vaccine administrations (35). However, DC vaccines were well-tolerated in all the clinical trials.

Three explanations can be proposed for the lack of clinical benefits in patients. (1) Compromised quality and quantity of the immune effector cells in patients. Osteosarcoma patients commonly receive a full course of upfront chemotherapy, which may damage the innate and adaptive immune effectors and thus limit their availability and efficacy to respond to the increased antigen presentation. (2) Poor migration of effector cells to the tumor site, probably due to down-regulation of chemokine expression. (3) Other strong immunosuppressive mechanisms, for example, immune checkpoints on immune cells. An effective cancer vaccine should be able to overcome tumor-associated immune suppression and reinstate immune surveillance (37). Therefore, increasing the ratio of active effector cells to tumor target cells, enhancing the infiltration of the effectors, or remodeling the TME in combination with administering DC vaccines may enhance antigen presentation, immune response, and clinical efficacy.

MACROPHAGES

In normal bone biology, osteoclasts, which are highly specialized macrophages, are involved in bone resorption and have central functions in bone homeostasis (1). Macrophages in the vicinity of osteosarcoma cells are identified as tumor-associated macrophages (TAMs). They consist of a large variety

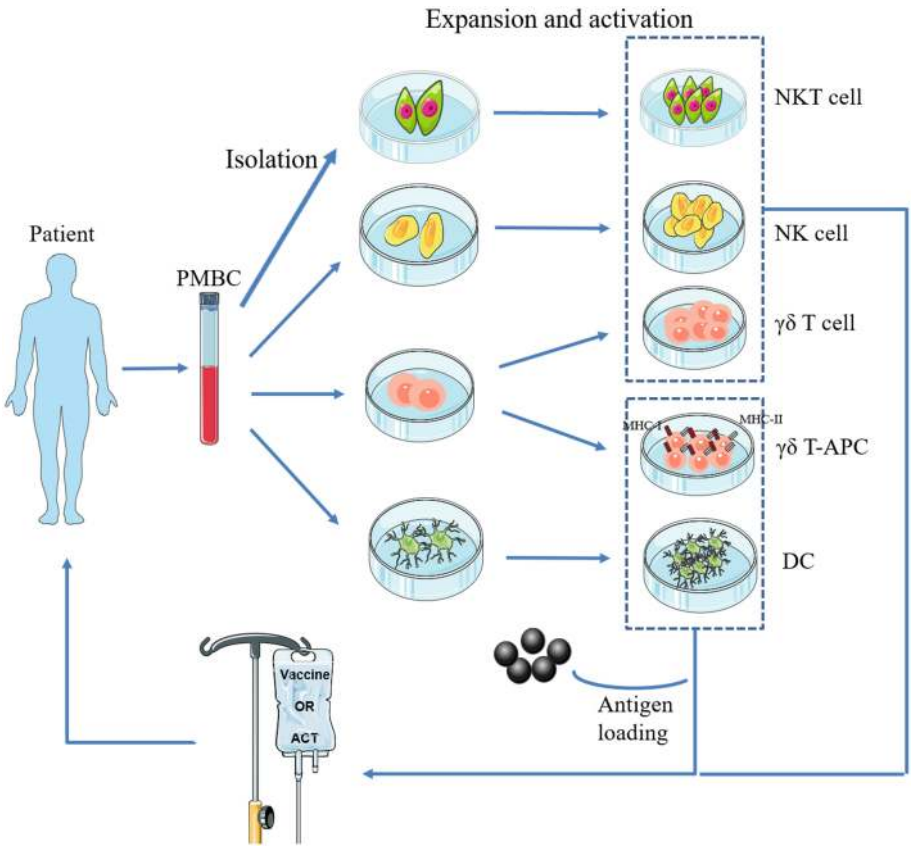

FIGURE 1 | Basic procedure of adoptive transfer of innate immune cells. NKT cells, NK cells, γδ T cells, and DCs are isolated from a patient's PBMCs, expanded and activated *ex vivo,* and then infused back into the body. In particular, γδ-APC and DCs need to be loaded with tumor antigen(s).

of subpopulations, which were initially classified as anti-tumor M1-polarized macrophages and pro-tumor M2-polarized macrophages (38). TAMs infiltrate massively into osteosarcoma tissues and contribute to tumor progression through multiple pathways. In preclinical models, macrophages recruited by interleukin (IL)-34 released by osteosarcoma cells promoted tumor progression and the metastatic process (39). Han et al. (40) found that osteosarcoma patients with detectable metastasis at diagnosis have more TAMs in the primary site. Interestingly, TAMs occurred at a higher rate in osteosarcoma lung metastases than in the corresponding primary lesions and promoted lung metastasis and induced epithelial-mesenchymal transition in osteosarcoma by activating the cyclooxygenase (COX)-2/signal transducer and activator of transcription (STAT)-3 axis (40). Additionally, Han et al. revealed that the number of M2-TAMs was correlated with the frequency of suppressive T-cell immunoglobulin and mucin-domain containing-3 (TIM-3)$^{+}$ programmed cell death 1 (PD-1)$^{+}$ T lymphocytes in osteosarcoma patients (41). TIM-3/Gal9 interactions between T cells and monocytes have been shown to resulted in an immunosuppressive response (42). These results indicate that TAMs promote tumor growth by suppressing intra-tumor T-lymphocytes. However, several studies have reached different conclusions. A study by Buddingh et al. demonstrated that TAMs were associated with metastasis inhibition in high-grade osteosarcoma patients (43). This result was recently confirmed in orthotopic osteosarcoma mouse models (44). Moreover, a biopsy study revealed that a high level of CD163 (a marker of M2-polarized macrophages) was related to longer metastasis progression-free survival (MPFS), and CD68 (a marker for macrophages) exhibited a similar association (45). The possible reason may be that the polarization/phenotype and infiltration of TAMs change dynamically during tumor growth, and the current studies do not fully represent the whole dynamic process of TAMs in the TME.

Despite the contradictory roles of TAMs in the TME, three therapeutic strategies targeting TAMs have shown potential for treating osteosarcoma. (1) Preventing polarization of M1 macrophages to M2, or directly suppressing the M2 phenotype. Pharmacological therapy for the treatment of osteosarcoma using all-trans retinoic acid (46), resveratrol (47), and dihydroxy coumarins (48) has shown favorable results involving the suppression of M2-polarized macrophages. (2) Enhancing non-TAM macrophages recruitment. A study showed that upregulation of Secreted Protein, Acidic and Rich in Cysteine-like 1 (SPARCL1) protein induced osteosarcoma

TABLE 1 | Pre-clinical studies of DC-based vaccines for osteosarcoma.

Type of DC vaccine	Study type	Ancillary therapy	Effect	References
Autologous DCs transfected with total tumor mRNA	*In vitro*	CIK cells	Effective osteosarcoma cytolysis	(19)
	In vivo	None	Induction of specific CTL responses, tumor rejection in 70% of vaccinated tumor-bearing rats, and development of long-term immunological memory to reject a subsequent tumor rechallenge	(22)
	In vivo	None	Induction of specific CTL responses, tumor rejection in 80% of vaccinated tumor-bearing rats and development of long-term immunological memory to reject a subsequent tumor rechallenge	(23)
Allogeneic DCs fused with tumor cells	*In vivo*	None	Protection from tumor challenge in 70% of pre-vaccinated rats and tumor rejection in 60% of tumor-bearing rats	(24)
	In vitro	None	Effective activation of T cells	(25)
Autologous DCs fused with tumor cells	*In vitro*	None	Effective activation of T cells	(26)
	In vivo	None	Atrophy or disappearance of tumor bodies and higher survival times and rates	(27)
Autologous DCs loaded with tumor cell lysate	*In vitro*	None	Increased induction of CTL activity	(28)
	In vivo	None	Increased number of $CD8^+$ T lymphocytes in the metastatic areas, and reduced pulmonary metastases	(29)
	In vivo	Anti-TGF-β antibody		(30)
	In vivo	Anti-CTLA-4 antibody		(31)
	In vivo	Anti-GITR antibody	Increased number of $CD8^+$ T lymphocytes in tumor tissue and serum, inhibition of primary tumor growth, and prolonged survival	(32)

cells to secrete chemokine ligand 5, resulting in macrophage recruitment. The recruited macrophages exerted anti-tumor effects and inhibited osteosarcoma metastasis (49). (3) Activating macrophages. Mifamurtide, an immunoadjuvant currently approved for osteosarcoma therapy in the European Union, can activate the tumoricidal properties of macrophages and inhibit human osteosarcoma cell growth (50, 51). A report from the international Children's Oncology Group found that the addition of mifamurtide to chemotherapy significantly improved overall survival from 70 to 78% and resulted in a trend toward improved event-free survival (EFS) among patients with no signs of metastasis (52). Similar benefits were observed in patients with metastatic osteosarcomas, although the results were not statistically significant (53).

NATURAL KILLER CELLS

NK cells express a repertoire of activating and inhibitory receptors (**Table 2**) that recognize altered expression of proteins on target cells, allowing for control of NK cell functions. After activation, they exhibit spontaneous cytolytic activity against cells undergoing malignant transformation (54). Recently, immunologists found that NK cells could stimulate DC recruitment into the TME, resulting in inhibition of tumor growth (55). Osteosarcoma patients had lower numbers of NK cells at the time of diagnosis compared to normal controls (56). After IL-2 administration and polychemotherapy, osteosarcoma patients had increased numbers, and increased activity, of NK cells in the blood, the magnitude of which strongly correlated with the clinical outcomes (57). These data indicate that NK cells have anti-tumor immune activity and play a role in immune surveillance in osteosarcoma patients. Importantly, osteosarcoma cell-surface molecules make osteosarcoma cells particularly susceptible to NK cell-mediated killing. CD54 and CD58 (both of which are adhesion molecules) are fully expressed on osteosarcoma cells, allowing for easy recognition by, and a strong association with, NK cells (58, 59). In addition, human leukocyte antigen (HLA) class I (a ligand for inhibitory receptors on NK cells) is typically downregulated (3), while

TABLE 2 | Activating and inhibitory receptors on human NK cells.

Type	Receptors	Ligands
Activating receptors	NKG2D	MICA/B, ULBP1–4
	CD94-NKG2C	HLA-E
	KIR2DL4	HLA-G
	KIR2DS1	HLA-C2
	KIR2DS2	HLA-C1
	KIR2DS3	Unknown
	KIR2DS4	HLA-A11
	KIR2DS5	Unknown
	KIR3DS1	HLA-Bw4
	NKp30	B7H6, BAT3, pp65 of HCMV, viral HA PfEMP1 of *Plasmodium falciparum*
	NKp46	Heparin, viral HA and HN
	NKp44	Viral HA and HN, PCNA, proteoglycans
	DNAM-1	CD112, CD155
Inhibitory receptors	KIR2DL1	HLA-C2
	KIR2DL2	HLA-C1
	KIR2DL3	HLA-C1
	KIR3DL1	HLA-Bw4
	KIR3DL2	HLA-A3, -A11
	NKR-P1A	LLTI
	CD94-NKG2A	HLA-E
	ILT2 (CD85j)	HLA-A, -B, -C, HLA-G1, HCMV UL18
	CD244(2B4)	CD244(2B4)

major histocompatibility complex class I chain-related protein A/B (MICA/B) and UL16-binding protein (ULBP) (ligands for activating receptors on NK cells) (60, 61) are overexpressed on osteosarcoma cells, allowing for easy activation of NK cells.

Treatment of patients with cells that have been isolated, manipulated, and expanded *ex vivo*, and then reinfused into the patient, is called adoptive cell therapy (ACT) (**Figure 1**). Infused immune cells migrate and infiltrate into the tumor site and mediate antitumor effects. There are three ancillary strategies to further improve the therapeutic effectiveness of adoptive NK cell transfer in osteosarcoma immunotherapy (**Table 3**). First, epigenetic drugs, such as histone deacetylase inhibitors (HDACi, e.g., valproic acid [VPA], entinostat) and DNA-methylation inhibitors (DNMTi, e.g., hydralazine) can increase the expression of ligands for activating receptors (MICA/B, ULBP, and CD155) or death receptors (Fas) on osteosarcoma cells, enhancing NK cell-mediated lysis (62, 63, 65). Another DNA-methylation inhibitor, decitabine, has been shown to enhance γδ T cell-mediated cytotoxicity by inducing ligands for activating receptors (natural killer group 2D, member D [NKG2D] ligands [NKG2DLs]) on osteosarcoma cells (12). Combining decitabine with the NK cells might be equally effective for treating osteosarcoma. Additionally, some traditional chemotherapeutic drugs (including doxorubicin, cisplatin, and gemcitabine) have been found to increase NK cell-activating ligand expression in tumors (71). Though similar studies in osteosarcoma are rare, chemotherapeutic drugs can modulate death receptors (DRs) on osteosarcoma cells, which may make them more sensitive to Fas-mediated NK cell cytotoxicity. For example, gemcitabine up-regulated cell-surface Fas expression and was effective in treating osteosarcoma lung metastases (72). Interestingly, treatment with cisplatin could not upregulate the cell-surface Fas antigen but it did sensitize human osteosarcoma cells to Fas-mediated apoptosis by down-regulating the expression of FLICE inhibitory protein long form (FLIP-L). Second, cytokine therapy can enhance the conjugate-forming capacity of NK cells to osteosarcoma targets by augmenting the expression of CD18 and CD2 (68) (both of which are adhesion molecules on NK cells), and intercellular adhesion molecule (ICAM)-1 (67) and fibronectin (69) (both of which are adhesion molecules on osteosarcoma cells). Interestingly, cytokine therapy can also increase the killing activity of NK cells. For instance, IL-15, the most promising NK cell-activating cytokine, can strongly enhance NK cell-mediated cytolytic activity toward chemotherapy-resistant osteosarcoma (60, 66). IL-2 can also strongly augment NK cell activity (73). It has been widely shown that, in neuroblastoma, IL-2 administration combined with immunotherapy (involving anti-GD2 antibody) enhanced NK cell proliferation and cytotoxicity (74), and showed promising results in clinical trials (75). Importantly, IL-2 aerosolization in dogs and mice with osteosarcoma lung metastasis similarly enhanced the local proliferation and cytotoxicity of NK cells and induced metastatic regression (76, 77). Third, monoclonal antibodies can target various receptors on NK cells to improve NK cell cytotoxicity. One approach is to develop a monoclonal antibody (mAb) to facilitate antibody-dependent cell-mediated cytotoxicity (ADCC) against osteosarcoma cells. Cetuximab, a mAb that targets epidermal growth factor receptor (EGFR) on target cells, with an Fc region that binds to CD16 on NK cells, increases NK-dependent lysis of EGFR-expressing osteosarcoma cell lines by enhancing ADCC (70). Another approach is to block the inhibitory NK cell receptors (such as NKG2A or KIR2DL-1,−2, and−3) using mAbs (78, 79). However, this approach has not been evaluated for treating osteosarcoma. Emerging evidence has shown promising strategies for osteosarcoma treatment, and carefully designed clinical trials may demonstrate the effectiveness of these therapies.

Genetic engineering of immune cells can endow them with additional antitumor specificity. For instance, transduction of precise and functionally active chimeric antigen receptors (CARs) into NK cells has led to stronger cytotoxicity toward osteosarcomas. A receptor designated NKG2D-DAP10-CD3ζ (comprising the NK cell- activating receptor NKG2D and two key signaling molecules, DAP10 and CD3ζ) was recently developed. Transduction with this chimeric receptor markedly increased NKG2D surface expression on NK cells and the transmission of activating signals. In a xenograft model of osteosarcoma, adoptive transfer of these CAR-NK cells significantly decreased the overall tumor burden (80). However, there are technical challenges to overcome to obtain sufficient numbers of functionally active NK cells from a patient's blood. The emergence of the human NK92 cell line consisting of activated NK cells may resolve the challenges faced by CAR-NK cell-based therapy, as NK92

TABLE 3 | Classification of immunomodulatory strategies for improving the killing effectiveness of adoptive NK cell transfer therapy against osteosarcoma.

Immunomodulatory strategy		Mechanism	Study type	Comment
Epigenetic drug	VPA	Augmented expression of MICA/B on tumor cells	*Ex vivo*	VPA sensitized human osteosarcoma cells to cytotoxicity of NK cells (62)
	Entinostat	Augmented expression of MICA/B, ULBP, and CD155 on tumor cells	*In vivo*	Entinostat failed to augment the efficacy of NK cell therapy in a nude mouse model of human osteosarcoma lung metastasis (63)
	Entinostat	Downregulation of the anti-apoptotic protein, c-FLIP, and increased levels of Fas within the membrane lipid rafts on tumor cells	*Ex vivo*	Entinostat sensitized osteosarcoma cells to NK cell-mediated apoptosis (64)
	VPA+ hydralazine	Augmented expression of MICA/B and Fas on tumor cells	*Ex vivo*	VPA combined with hydralazine enhanced the susceptibility of osteosarcoma cells to Fas- and NK cell-mediated cell death (65)
Cytokine	IL-15	Enhanced DNAM-1 and NKG2D signaling pathways	*Ex vivo*	IL-15 enhanced cytolytic activity against chemotherapy-resistant osteosarcoma cells (60)
	IL-15	Prevention of down-regulation of NKG2D on NK cells	*Ex vivo*	IL-15 reversed inhibition of NK cell-mediated cytolytic activity against osteosarcoma (66)
	IL-12+IFN-γ+IL-18	Enhanced expression of ICAM-I on HOS cells	*Ex vivo*	IL-12 enhanced NK-mediated cytolysis of HOS cells in the presence of IFN-γ and with IL-18 (67)
	IL-12+IL-2	Increased density of CD18 and CD2 molecules on NK cells	*Ex vivo*	A combination of IL-12 and IL-2 increased lytic activity against and binding to osteosarcoma cells (68)
	IL-17	Increased expression of fibronectin on U2 OS cells	*Ex vivo*	IL-17 enhanced NK cell-mediated adhesion and cell lysis activity against osteosarcoma (69)
Monoclonal antibody	Cetuximab	ADCC	*Ex vivo*	Cetuximab augmented cytolytic activity of resting NK cells, which was specifically directed toward osteosarcoma cells (70)

cell line is relative ease in *ex vivo* large-scale expansion and effective receptor transfection (81). Adoptive transfer of NK-92 cells transduced to express various CARs was shown to cause tumor regression in various tumor xenografts (82, 83). CAR-NK-92 cell-based therapy is currently being evaluated in clinical trials for $CD33^+$ acute myeloid leukemia (AML; NCT02944162) and $CD7^+$ leukemia and lymphoma (NCT02742727). Therefore, utilizing NK-92 cell line for producing sufficient CAR-NK cells (e.g., NKG2D-DAP10-CD3ζ-transduced NK92 cells) to effectively target and eliminate osteosarcoma is a promising strategy that requires further evaluation. However, NK92 cell line must be irradiated before being infused into patients (81), which limits the survival and proliferation of NK cells—two key factors that are known to influence the efficacy of NK cell-based immunotherapy (84). In contrast, large-scale differentiation of human induced pluripotent stem cells (iPSCs) into NK cells (with phenotypic and functional similarities to NK cells isolated from peripheral blood) is relatively easy (85). After CAR transduction, the efficiency of NK cell production from iPSCs is similar to the efficiency of NK cell production from non-CAR-expressing iPSCs (86). Moreover, NK cells derived from human iPSCs that express CARs (CAR-iPSC-NK cells) have a typical NK cell phenotype. In a mouse xenograft model of ovarian cancer, CAR-PSC-NK cells (with a CAR comprising the NK cell-activating receptor NKG2D, the co-stimulatory domain 2B4 and the key signaling molecule CD3ζ) showed increased *in vivo* expansion and improved activity with less toxicity (87). CAR-iPSC-NK cells mediate their activity without requiring HLA matching; therefore, theoretically, they can also be used to treat other solid tumors including osteosarcoma. Recently, clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated protein 9 (Cas9) technology has been used to edit CAR T cells (88). For example, knocking out immune checkpoints may protect CAR T cells from being exhausted (89). Knocking out αβ T-cell receptors (TCR) (88) or β2-microglobulin (β2M) (90) minimized the risks associated with "off-the-shelf" CAR T cells. Delivering a CAR gene to a specific locus, TCR α constant (TRAC), yielded therapeutic CAR T cells that were more potent (91). To achieve a robust anti-tumor effect, applying CRISPR/Cas9 technology to edit CAR-NK cells (e.g., by knocking out immune checkpoints) should be further investigated.

NATURAL KILLER T CELLS

NKT cells express molecular markers of both NK cells (e.g., NK1.1, Ly49, NKRs, and KIRs) and T cells (e.g., αβ TCR, CD44, CD69, and CD122). In tumor immunity, activated NKT cells are able to kill tumors via different NK and T cell-associated mechanisms (92, 93). In addition, high numbers of tumor-infiltrating NKT cells correlated with good clinical outcomes in cancer patients (94, 95). However, in some tumor types, the number of NKT cells was higher compared to the number in normal tissue (94, 96). Further studies focusing on function and phenotype of tumor-infiltrating NKT cells showed that they expressed fewer activating receptors and produced lower

amounts of pro-inflammatory cytokines compared with para-carcinoma tissues (97, 98).

A similar contradictory function of NKT cells in osteosarcoma immunity was observed. One research group found that NKT cells purified from human PBMCs and expanded *ex vivo* enhanced osteosarcoma cell death induced by standard chemotherapy (doxorubicin, cisplatin, and methotrexate) (99). In contrast, other researchers found that tumor-infiltrating NKT cells had a negative regulatory role, involving suppression of CTL function (100). A hypothetical model of NKT cell functional transformation in osteosarcoma is as follows: during the early tumor stage, the NKT cell subpopulation exerts effective antitumor immune responses against tumors. However, during tumor progression, NKT cells become overstimulated and anergic, and they finally transform, contributing to tumor immune escape (101).

Two major aspects of current NKT cell therapeutic strategies should be carefully considered in light of this hypothetical model. (1) *in situ* expansion and activation of NKT cells in early tumor stages or adoptive transfer of *ex vivo* expanded and activated autologous NKT cells into patients (**Figure 1**). α-galactosylceramide (GalCer) or α-GalCer-pulsed autologous DCs is a common strategy to activate NKT cells *in vivo* or *ex vivo* (102, 103). Recent studies found that iPSCs might be more effective at amplifying the numbers of autologous NKT cells (104, 105). (2) Skewing of pro-tumor NKT cells toward anti-tumor subtypes in advanced tumor stages. The addition of IL-12 (106) or chemical modification of α-GalCer (107) skewed the conventional α-GalCer-produced TH1- and TH2-associated cytokines toward only TH1-associated cytokine production. These data indicate that pro-tumor NKT cells were transformed to anti-tumor subtypes following this intervention.

γδ T CELLS

It has been found that γδ T cells can mediate effective antitumor immune responses. In a methylcholanthrene (MCA)-induced sarcoma model, γδ T cell-deficient mice had an increased incidence of tumor development (108). Preclinical studies found that γδ T cells could directly kill malignant cells through the generation of cytokines (tumor necrosis factor [TNF]-α and interferon [IFN]-γ), upregulation of activating receptors or their ligands (Fas-L, NKG2D, TRAIL, and TNF), expression of CD16 for ADCC, and release of granzymes and perforin (109). Recent studies indicated that, in the short-term, γδ T cells possess phenotypic characteristics of DCs after activation by phosphoantigens (110). The effect of priming a strong $CD8^+$ T cell-mediated anti-tumor response using peptide-pulsed γδ T cells was even more powerful than the effect induced by DCs (111, 112).

The main advantages of adoptive γδ T cell transfer immunotherapy (**Figure 1**) are as follows: (1) γδ T cells can infiltrate the TME (113, 114); (2) they exert cytotoxic activity against cancer cells in an HLA-independent manner; and (3) they can be expanded and activated *ex vivo* by simple yet effective protocols (115). Kato et al. (116) initially reported the ability of γδ T cells to directly recognize and kill osteosarcoma cell lines NY, SAOS2, and OST. However, these cell lines were only moderately susceptible to γδ T cell cytotoxicity. Therefore, later studies have focused on adjuvant therapies to potentiate the immunosensitivity of osteosarcoma cells to γδ T cells (**Table 4**). Zoledronate (ZOL) significantly reduces skeletal complications in patients with bone metastases from solid tumors (120) and inhibits osteosarcoma growth (121). Our group and other researchers demonstrated that ZOL could also enhance the anti-osteosarcoma activity of γδ T cells (14, 117). However, the specific mechanisms have not been elucidated and a high dose of ZOL is necessary to achieve this effect, while the ZOL concentration in the blood declines rapidly (122). Recently, a study by our group found that a ZOL-related mechanism was associated with increased accumulation of mevalonate pathway intermediates (11). We also found that VPA (the HDACi) and ZOL had a synergistic effect on the enhancement of γδ T cell-mediated cytotoxicity against osteosarcoma cells by facilitating the accumulation of mevalonate pathway intermediates (11). More usefully, this combination therapy reduced the ZOL dose required in adoptive γδ T cell transfer immunotherapy, facilitating its clinical application (11). In addition, the expression of human epidermal growth factor receptor 2 (Her-2) was associated with tumor progression and poor prognosis in osteosarcoma patients (123). However, no therapeutic effectiveness was observed pre-clinically or clinically for trastuzumab (an anti-Her-2 monoclonal antibody)-driven osteosarcoma therapy (124). However, Liu et al. reported that trastuzumab aided γδ T cell-mediated lysis of osteosarcoma cells by enhancing ADCC (13), suggesting a promising novel combination regimen to treat osteosarcoma. Additionally, it was reported that bispecific antibodies could enhance the cytotoxicity of γδ T cells. For example, a research group designed a bispecific antibody, Her2/Vγ9, that binds to Vγ9 on γδ T cells and Her-2 on pancreatic tumor cells (125). Infusion of this novel bispecific antibody improved recognition and binding between adoptively transferred γδ T cells and tumor cells, significantly reducing pancreatic tumor growth in mouse models. This result suggests that Her2/Vγ9 antibody might promote the capacity of γδ T cells to lyse osteosarcoma cells to a greater extent than Her2 antibody. Furthermore, IFN-γ and decitabine (a DNA demethylation drug) increased γδ T cell cytotoxicity against osteosarcoma cells by increasing the expression of Fas and NKG2DLs on tumor cell surfaces (12, 118).

Recent achievements in cell engineering and further studies of γδ T cell physiology have provided an improved foundation for improving γδ T cell-based immunotherapies. Three potential perspectives related to potentiating the cytotoxicity of γδ T cells are as follows. (1) T cells transduced with TCRs that specifically target the NY-ESO-1 antigen on tumors are called NY-ESO-1-specific TCR-engineered T cells. These cells can be activated upon encountering NY-ESO-1 antigens presented by HLA molecules and they then specifically target and kill tumor cells. Adoptive transfer of NY-ESO-1-specific TCR-engineered T cells represents a potentially effective therapeutic approach for the treatment of osteosarcoma (126). However, introduction

TABLE 4 | Chronological summary of studies on γδ T cell therapy against osteosarcoma.

References	Ancillary therapy	Study type	Cell type and source	Mechanism	Result
Muraro et al. (117)	ZOL + IL-2	*In vitro*	γδ T cells from HD	Unknown	Potent anti-tumor activity of γδ T cells against osteosarcoma cell lines
Li et al. (118)	IFN-γ	*In vitro*	γδ T cells from HD	Up-regulated expression of Fas on osteosarcoma cell lines	Enhanced cytotoxic effect of γδ T cells against osteosarcoma cell lines
Li et al. (14)	ZOL	*In vitro*	Vγ9Vδ2 T cells from OP and HD	TCR-mediated and partly NKG2D-mediated granule exocytose and TRAIL pathways	Potent anti-tumor activity of Vγ9Vδ2 T cells
Liu et al. (13)	Trastuzumab + ZOL	*In vitro*	Vγ9Vδ2 T cells from HD	ADCC	More efficient ability of Vγ9Vδ2 T cells to recognize and lyse osteosarcoma cell lines.
Li et al. (119)	Celastrol	*In vitro*	γδ T cells from OP and HD	Up-regulation of death receptors 4/5 on osteosarcoma cell lines	Increased osteosarcoma cell lysis by γδ T cells
Wang et al. (11)	ZOL+VPA	*In vivo*	γδ T cells from OP and HD	Increased accumulation of the mevalonate pathway intermediates in osteosarcoma primary cells and cell lines	Enhanced γδ T cell migration and antitumor effect.
Wang et al. (12)	Decitabine	*In vivo*	γδ T cells from OP	Increased expression of NKG2DLs on osteosarcoma cell lines	Enhanced antitumor effect of combination therapy of γδ T cell infusion and decitabine administration

HD, healthy donors; OP, osteosarcoma patients.

of α/β chains has the potential to result in mispairing with endogenous α/β TCR chains, resulting in mixed TCR dimers with unknown specificities, which can lead to adverse complications such as autoimmune responses and toxicity. However, previous studies showed that α and β TCR chains could not form heterodimers with γ and δ TCR chains when transduced into γδ T cells (127). Meanwhile, αβ TCR-transduced γδ T cells exhibited high levels of cytokine release and cytotoxic activity (127, 128). Therefore, using NY-ESO-1-specific αβ TCR-transduced γδ T cells to treat osteosarcoma may be a safe and effective strategy. (2) γδ T cells may be ideal candidates for cell vaccine manufacturing (**Figure 1**). The advantages of γδ T cell vaccines compared to DC vaccines are as follows (129): first, obtaining and expanding γδ T cells to create an unlimited number is easy, economical, and highly selective; second, γδ T cell vaccines display excellent survival during *ex vivo* preparation, allowing for possible freezing for storage and shipment to cancer clinics in large quantities; third, the status of γδ T cells is uniform (effector-memory), while DCs remain heterogeneous (immature-mature-exhausted); finally, γδ T cells have functional uniformity with stable induction of primarily pro-inflammatory responses. (3) Mechanistic target of rapamycin (mTOR) is important for regulating T cell metabolism and function. Recent studies have demonstrated the important role of mTOR in γδ T cells. Rapamycin (the US Food and Drug Administration [FDA]-approved mTOR inhibitor) increased the yield and durability of the elicited γδ T cell response (130). Later studies demonstrated that the immune stimulatory effects of rapamycin are mediated by boosting perforin release, enhancing tumor core infiltration, and upregulating NKG2D and TNF-α (131, 132). Therefore, it is conceivable that inhibition of mTOR receptors could contribute to γδ T cell-mediated osteosarcoma cell killing.

COMBINATION THERAPY WITH IMMUNE CHECKPOINT INHIBITORS

Immune checkpoint molecules are key modulators of the anti-tumor T cell immune response by a narrow definition. Actually, multiple immune checkpoint molecules are also expressed on innate immune cells, which function as immunomodulators. Their interactions activate either inhibitory or activating immune signaling pathways. Indeed, metabolic pathways play a critical role in the functional modulation of immune cells and could, by extension, be considered as immune checkpoints. Here, we focus on the inhibitory immune checkpoints that influence adaptive and innate immune cells. Blocking inhibitory checkpoints can reverse the exhaustion state of immune cells and inhibit tumor growth. Importantly, one clinical trial demonstrated the immune response to ICPIs in osteosarcoma patients (15) and rational combinations of immunotherapies, particularly those involving ICPIs, have demonstrated increased efficacy in cancer patients (133). Therefore, ICPIs have the potential to improve efficacy of innate immune cell-based therapy for osteosarcoma.

Programmed Cell Death 1

Programmed cell death 1 (PD-1) is a receptor expressed on the surface of T lymphocytes, and innate immune cells. PD-1 binds a specific ligand, programmed cell death ligand 1 (PD-L1), which is expressed on several types of malignant cells and APCs in tumor foci. It is widely accepted that PD-1 is an exhaustion marker for CTL (134), which is the main anti-tumor effector cell during checkpoint blockade therapy. A study aiming to find predictors of DC vaccine responses showed that glioblastoma patients with tumor-infiltrating lymphocytes (TILs) with a higher $PD\text{-}1^{+}/CD8^{+}$ ratio had worse prognosis (135). These data indicated that DC vaccine-primed $CD8^{+}$ T cells

became exhausted via the PD-1-PD-L1 axis, which is one of the reasons that DC vaccines have showed unsatisfactory results in osteosarcoma patients. This obstacle might be overcome by ICPIs. On the other hand, evidence indicates that a mechanism of acquired resistance to ICPIs involved alterations in antigen presentation (136). This problem can be solved by growing DC vaccines *ex vivo*. Therefore, PD-1 inhibitors and DC vaccines have complementary roles regarding antitumor efficacy (37, 137, 138). For instance, an *ex vivo* study demonstrated that anti- PD-1 treatment enhanced T-cell responses induced by DC vaccines fused with myeloma cells (137). Furthermore, in melanoma-bearing mice, anti-PD-1 treatment increased the function and infiltration of TILs induced by DC vaccines, and augmented anti-tumor activity (138). Currently, there are ongoing phase I/II clinical trials studying the effects of different types of DC vaccines combined with nivolumab (a mAb that blocks PD-1) for the treatment of glioma (NCT02529072), glioblastoma multiforme (NCT03014804, NCT02529072), and solid tumors (NCT02775292).

Interestingly, some cancer types exhibit low MHC I expression and/or neoantigen burden, which renders them resistant to recognition by $CD8^+$ T cells, but sensitive to PD-1/PD-L1 axis blockade (139). This suggests that other immune cell types might also be suppressed by this axis. PD-1 expression on NK cells has been detected in cancer patients, including those with Kaposi sarcoma and ovarian carcinoma (140, 141). Preclinical observations showed that PD-L1 upregulation on several types of tumor cells or DCs suppressed NK cell-mediated tumor cell lysis, and blockade of PD-1 restored NK cell anti-tumor activity and inhibited tumor growth (141, 142). Importantly, a recent clinical study demonstrated that blocking PD-1 and PD-L1 elicited a strong NK cell response that was indispensable for the full therapeutic effects of immunotherapy (139). These data suggested the importance of the PD-1/PD-L1 axis in inhibiting NK cell responses *in vivo* and revealed that NK cells mediate the effect of PD-1/PD-L1 blockade immunotherapy. In addition, combination therapy consisting of NK cell transfusion and PD-1 blockade resulted in more potent cytolytic activity against tumor cells *in vitro* (142, 143). Unfortunately, a phase II clinical trial evaluating the effects of pembrolizumab, an anti-PD1 mAb, on the NK cell exhaustion phenotype in patients with unresectable stage III/IV melanoma (NCT03241927) has just been terminated because of difficult participant enrollment. Otherwise, this trial can aid in understanding how NK cell activity and exhaustion interplay with PD-1 expression and function, and it can lead to the development of more effective combination therapies.

$PD\text{-}1^+$ TAMs, which exhibited an M2-like surface profile and M2-like functional characteristics and suppressed $CD8^+$ (144) and $CD4^+$ (145) T cell function, were detected in human cancers. In a human LM7 osteosarcoma mouse model, macrophages in lung metastases highly expressed PD-1 (146). PD-1 blockade significantly decreased the number of osteosarcoma lung nodules by increasing the macrophage tumor infiltration and polarization from M2 to M1 (146). Other research showed that PD-1 levels on tumor-infiltrating DCs were increased during tumor progression, and these DCs responded poorly to tumor antigens, and suppressed T cell activity and infiltration (147). In a murine model of ovarian cancer, targeting PD-1 on DCs significantly enhanced antigen-specific T cell responses and slowed tumor growth (147).

PD-1/PD-L1 expression was increased in osteosarcoma patients and correlated with poor prognosis (148, 149). In preclinical trials, PD-1 blockade resulted in anti-metastatic effects in osteosarcoma murine models (150, 151). However, PD-1 blockade was ineffective in an orthotopic osteosarcoma model (152). In addition, data from a multicenter, two-cohort, single-arm, open-label, phase II trial revealed that the effect of pembrolizumab (a PD-1 inhibitor) on osteosarcoma patients was poor (only one [5%] of 22 patients showed a partial response) (15). Therefore, it was urgent to improve the therapeutic effects of PD1/PDL-1 inhibitors. Recently, oncologists defined tumors lacking various inflammatory immune cell infiltration as "cold tumors," and the opposite as "hot tumors" (153). Hot tumors are more susceptive to ICPIs. However, osteosarcomas are relatively "cold tumors." A potential approach for reducing acquired resistance to ICPIs is turning a cold tumor into a hot tumor, resulting in enhanced infiltration of inflammatory immune cells (both adaptive and innate immune cells) into the tumor (154, 155). Therefore, further investigation of combination therapy involving an ICPI with an innate immune cell-based therapy (such as ACT and vaccines) for the treatment of osteosarcoma may be of value.

Cytotoxic T-Lymphocyte-Associated Protein 4

Cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) is another major immune checkpoint molecule on T cells induced by activation. CTLA-4 negatively regulates T cell function (156), and blocking CTLA-4 can reactivate T cells and enhance the efficacy of osteosarcoma vaccines. For example, in a C3H murine osteosarcoma model, tumor lysate-pulsed DCs with CTLA-4 blockade prevented lung tumor metastasis (31). Furthermore, a clinical study on the combined effects of a synthetic mRNA-electroporated DC vaccine and ipilimumab (an anti-CTLA-4 mAb) for patients with pretreated advanced melanoma showed a 6-month disease control rate of 51% and a promising overall response rate of 38% (eight complete and seven partial responses) (157). These results greatly increased interest in combination therapies involving vaccines and ICPIs. However, studies focusing on CTLA-4 expression on NK cells are scarce. CTLA-4 was detected on tumor-infiltrating NK cells in tumor-bearing mice and was closely associated with the inhibition of DC-induced IFN-γ production by NK cells (158). No studies have evaluated the expression of CTLA-4 on human NK cells. However, CTLA-4 may exist on human NK cells and may modulate their effector functions in cancer immunity.

CTLA-4 is significantly associated with carcinogenesis of osteosarcomas, which provides a potential therapeutic target (159). In a preclinical study, co-inhibition of CTLA-4 and PD-L1 resulted in complete control of metastatic osteosarcoma (151). Combined therapy involving anti-CTLA-4 antibody and a DC vaccine led to a similar outcome (31). Future studies should explore the possibility of combining anti-CTLA-4 mAb and NK cell-based therapy.

T-cell Immunoglobulin and Mucin-Domain Containing-3

T-cell immunoglobulin and mucin-domain containing-3 (TIM-3) is expressed by innate and adaptive immune cells. Importantly, all TIM-3$^+$ T cells in cancer patients co-express PD-1 (160). The current view is that CTLs with TIM-3-PD-1 co-expression are functionally more "exhausted" than those that express PD-1 alone (161, 162). Therefore, a DC vaccine combined with co-inhibition of TIM-3 and PD-1 may further prime T cells and maintain their cytotoxicity against malignant cells.

The inhibitory function of TIM-3 on innate immune cells (including NK cells, NKT cells, DCs, and macrophages) is consistent with its function on T cells (163). TIM-3 expression on peripheral NK cells correlated with their exhausted phenotype and predicted poor prognosis of patients with advanced melanoma and lung adenocarcinoma (164–166). Blockade of TIM-3 on NK cells from these patients increased NK cell-mediated cytotoxicity and IFN-γ production. Interestingly, researchers found that co-expression of TIM-3 and PD-1 is a marker of functionally exhausted NK cells in advanced tumors, as is the case for T cells (167). TIM-3 expression on macrophages is associated with inhibitory function in inflammatory diseases and cancers (168–170). For instance, in hepatocellular carcinoma, TIM-3 expression on TAMs was significantly enhanced by tumor-derived signals, which caused the macrophages to undergo alternative activation and inhibited CTL activation. Subsequent interference with TIM-3 on the TAMs successfully suppressed hepatocellular carcinoma growth (170). Recent studies showed that M1 macrophages had low expression of TIM-3, providing further evidence of its negative regulatory function in macrophages. In DCs, TIM-3 inhibits DC activation and maturation via the Btk-c-Src signaling pathway (171). In the TME, the interaction between TIM-3 and high-mobility group box 1 (HMGB1) prevented activation of tumor associated DCs by impeding sense of immunogenic nucleic acids, thereby suppressing anti-tumor responses (172). In γδ T cells, TIM-3 served as an exhaustion marker and protected the human body from inflammatory attack in different diseases (173, 174). Its role in tumor infiltrating γδ T cells has not been characterized.

Co-blocking CTLA-4 and PD-1 led to synergistic anti-tumor effects (175, 176). Interestingly, anti-CTLA-4 antibody showed a unique curative effect in anti-PD-1-resistant cancer (177). These results indicate that TIM-3 plays an essential role in tumor immunity. Therefore, TIM-3 is a candidate target for improving the effect of innate immune cell-based therapy.

CD39/CD73 and Adenosine Receptors

In the TME, ATP conversion to ADP and/or AMP occurs in the presence of CD39 (also known as NTPDase 1), while CD73 (also known as 5′-NT) dephosphorylates AMP to adenosine. Accumulated extracellular adenosine exerts regulatory functions by binding to one of four adenosine receptors (ARs), A1R, A2AR, A2BR, and A3R (**Figure 2**).

A2AR activation increased cell-surface expression of PD-1 and CTLA-4 on T cells and inhibited proliferation and pro-inflammatory cytokine production (178). Similarly, a recent study showed that tumor-infiltrating CD8$^+$ T cells expressed high levels of CD39 and exhibited an exhausted phenotype with impaired production of cytokines and high expression of inhibitory receptors (179). These observations suggested that CD39 was an immune checkpoint that could be targeted to restore the T cell immune response against tumors. In addition, genetic ablation or therapeutic inhibition of CD73 or AR improved the effector functions and infiltration of CTLs, and significantly reduced tumor growth (180–182). Importantly, these interventions augmented the efficacy of adoptive T cell anticancer therapy against ACT-resistant tumors (183, 184). These results indicated the potential to improve the efficacy of vaccines by inhibiting the adenosinergic pathway. Intravenous administration of CD73-specific small interfering RNA (siRNA)-loaded chitosan-lactate nanoparticles (ChLa NPs) potentiated the antitumor effects of a DC vaccine in 4T1 breast cancer-bearing mice, with augmented CTL effector function, improved T cell proliferations, and increased production of inflammatory cytokines (185). Similarly, another study demonstrated that co-targeting of A2AR and CD73 in conjunction with a DC vaccine successfully reduced tumor growth, prolonged survival, and enhanced specific antitumor immune responses in the same mouse model of breast cancer (186).

Notably, A2AR is abundantly expressed on NK cells (at a 5-fold higher level, at the mRNA level, compared to that in T cells), and A2AR activation inhibited NK cell cytotoxicity and proliferation in several tumors (187–189). A recent study found that co-inhibition of A2AR and PD-1 in a B16F10 lung metastasis model resulted in a therapeutic effect that was more dependent on infiltrating NK cells than T cells (190). These findings indicate an important role of A2AR regarding NK cell function in tumor immunity. In addition, antagonism of A2AR reduced the percentage of CD56^{bright} NK cells in favor of accumulation of mature CD56^{dim} NK cells with high cytotoxic activity (191). This suggested that A2AR antagonism could enhance adoptive NK cell immunotherapy. Adenosine-differentiated DCs displayed high levels of tolerogenic molecules (VEGF and indoleamine 2,3-dioxygenase [IDO]) and anti- inflammatory cytokines (IL-10), which impaired the DC antigen presenting function and subsequent T cell priming, resulting in accelerated tumor growth in mice (192, 193). Selective inhibition of A2BR improved DC activation and chemokine release, and subsequently increased T cell infiltration and adaptive responses in mice, resulting in reduced growth of carcinomas (194). Moreover, activation of the A2AR pathway in DCs increased the expression of programmed cell death 1 ligand 2 (PDL2, a ligand for the inhibitory receptor PD1), which directly inactivated effector T cells (195). Similarly, A2BR plays a prominent role in M2 polarization of macrophages (196). Macrophages differentiated in the presence of adenosine expressed arginase, IDO, and TGF-β, and had limited T cell stimulatory activity (196). Additionally, TAMs expressing CD39 and CD73 contributed to tumor growth through the production of adenosine (197, 198). Studies of the effects of adenosine-related molecules on γδ T cells are sparse. Upregulation of CD39 on human Vγ9Vδ2 T cells directly abrogated the γδ TCR agonistic activity of phosphoantigens (199). Through this

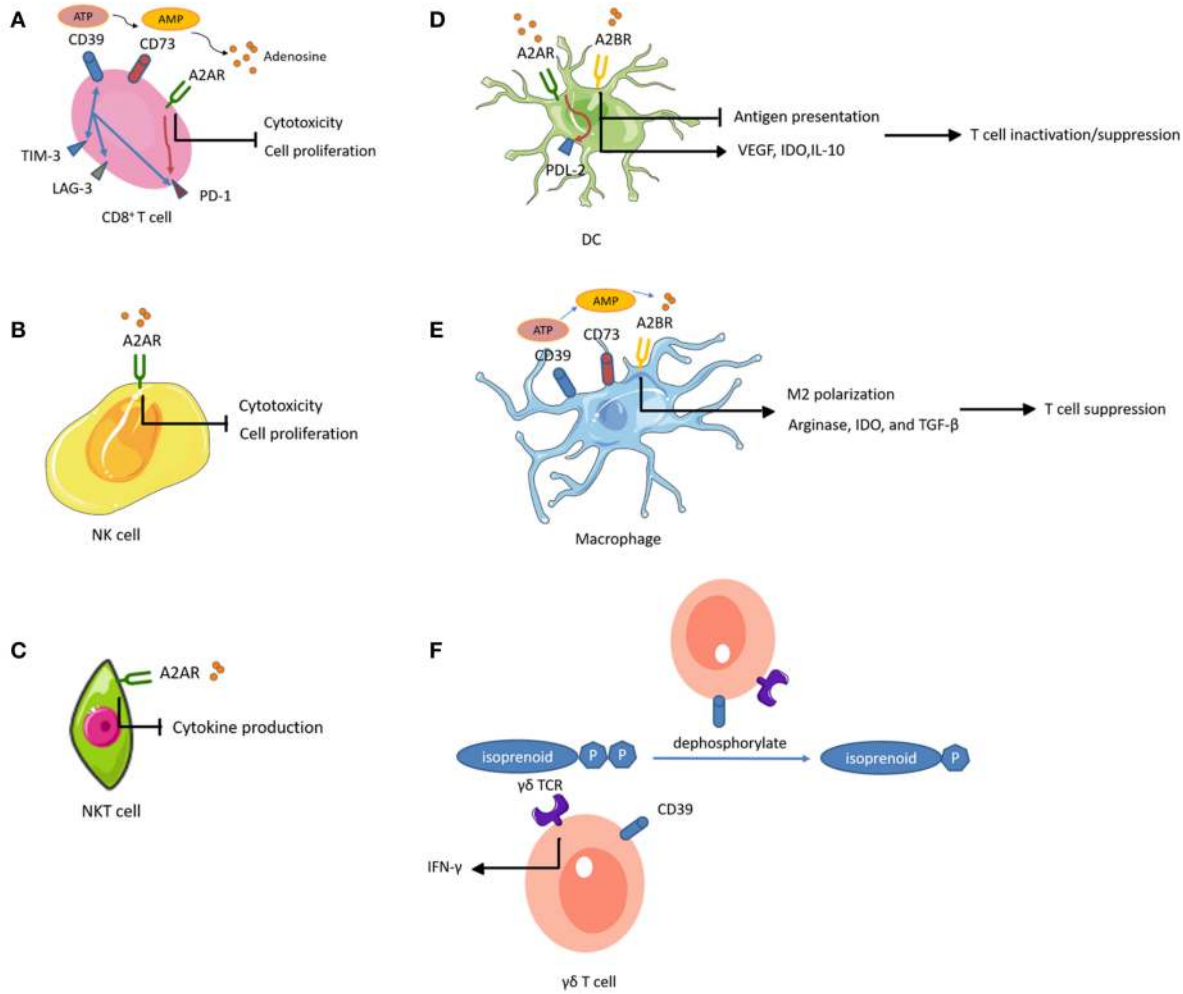

FIGURE 2 | Adenosine-mediated immunosuppression of immune cells. Expression of CD39 and CD73 generates adenosine, an immunosuppressive metabolite. Activation of adenosine receptors (ARs) suppresses the proliferation and effector functions of cytotoxic lymphocytes, and promotes polarization toward exhausted or immunosuppressive function. **(A)** $CD39^+$ $CD8^+$ T cells highly express other inhibitory immune checkpoints such as PD-1, TIM-3, and lymphocyte activating 3 (LAG-3). **(A,B)** On $CD8^+$ T cells and NK cells, A2AR activation inhibits their proliferation. **(A–C)** On $CD8^+$ T cells, NK cells, and NKT cells, A2AR activation impairs their cytotoxic potential. **(A,D)** A2AR signal path on $CD8^+$ T cells and DCs promotes the expression of other inhibitory immune checkpoints. **(A,E)** CD39 and CD73 expression on $CD8^+$ T cells and macrophages contributes to adenosine accumulation. **(D)** On DCs, A2BR stimulation impairs DC antigen presentation and subsequent T cell priming while inducing VEGF, IDO, and IL-10 secretion and subsequent T cell suppression. **(E)** Activation of A2BR on macrophages favors M2 phenotype polarization and induces arginase, IDO, and TGF-β, mediating T cell suppression. **(F)** The ecto-ATPase CD39 inactivates isoprenoid-derived Vγ9Vδ2 T cell phosphoantigens.

pathway, CD39 reduced Vγ9Vδ2 T cell activation and IFN-γ production. This study revealed a previously unrecognized immunoregulatory function of CD39, which is independent of the adenosinergic pathway. A2AR activation also increased anti-inflammatory cytokine production in NKT cells, indicating that A2AR played a negative immune regulatory role in NKT cells (200).

Recent studies showed that intratumoral hypoxia and hypoxia inducible factor-1α (HIF-1α)-dependent pathways up-regulated the tandem activities of CD39 and CD73, leading to adenosine accumulation in the TME and tumor immune escape (201, 202). Adenosinergic pathways have not been characterized in osteosarcoma. However, studies have shown that hypoxia contributed to human osteosarcoma progression (203). It is conceivable that hypoxia-mediated tumor protection is dependent on adenosinergic pathway-mediated immunosuppression. Therefore, targeting CD39, CD73, and ARs has the potential to reinstate osteosarcoma immunity and improve immunosensitivity to innate immune cell-based immunotherapy.

Clinical Studies of Innate Immune Cell-Based Immunotherapy and Immune Checkpoint Inhibitors

In this section, we mainly discuss the results of major clinical studies and ongoing clinical trials for treatment of osteosarcoma

TABLE 5 | Clinical trials of DC vaccination, cell infusion, and ICPIs for treating osteosarcoma.

Intervention	Ancillary therapy	Trial phase	Status	References
Autologous DCs loaded with tumor cell lysates	None	I	Unknown	(35)*
	None	I/II	Unknown	(36)*
	Gemcitabine	I	Recruiting	NCT01803152
Autologous DCs loaded with TAAs or TAA-derived peptides (MAGE-A1, MAGE-A3, NY-ESO-1)	Decitabine	I/II	Completed	NCT01241162
Pembrolizumab (targeting PD-1)	None	II	Active, not recruiting	NCT02301039
SHR1020 (targeting PD-1)	Apatinib	II	Active, not recruiting	NCT03359018
Nivolumab (targeting PD-1) with ipilimumab (targeting CTLA-4)	None	II	Not yet recruiting	NCT02982486
Nivolumab with or without ipilimumab	None	I/II	Recruiting	NCT02304458
	None	II	Suspended	NCT02500797
NK cell infusion	None	I/II	Unknown	NCT02409576
	Haploidentical stem cell transplantation	II	Active, not recruiting	NCT01807468
	Hematopoietic cell transplantation	II	Recruiting	NCT02100891

TAA, tumor-associated antigen.
**These studies were not found in ClinicalTrials.gov.*

on innate immune cell-based immunotherapy and ICPIs for the treatment of osteosarcoma. As discussed above, the results of the initial clinical trials of DC vaccines were unsatisfactory (34–36), possibly due to tumor-associated immune suppression. A recent clinical trial (NCT01803152) has reported some improvements. The DC vaccine used was similar to the previous study (34–36), but the vaccine was combined with gemcitabine, which inhibits myeloid-derived suppressor cells (MDSCs) that play a vital role in tumor-associated immune suppression. In the field of innate cell infusion, NK cells are at the forefront. In an early clinical study, NK92 cells were infused into a patient with advanced osteosarcoma, though no treatment response was observed (204). More trial participants are required. We found several ongoing studies of expanded, activated haploidentical NK cell infusions for the treatment of sarcomas (these studies are summarized in **Table 5**), which should provide information on the effectiveness and safety of this approach. Only one clinical study published results regarding the curative effects of ICPIs for the treatment of osteosarcoma, which showed a 5% response rate to pembrolizumab (a PD-1 inhibitor) (15). Multiple clinical trials targeting PD-1 and/or CTLA-4 are ongoing (**Table 5**), and we expect an improved curative effect, which will provide a foundation for combination regimens involving targeting PD-1 and/or CTLA-4 along with innate immune cell-based immunotherapy.

COMBINATION THERAPY WITH ONCOLYTIC VIRUSES

Oncolytic viruses (OVs) are emerging as a novel therapeutic class, which selectively replicate in and lyse cancer cells without harming normal cells. Like chemotherapy and radiotherapy, the therapeutic outcomes of OVs are determined not only by direct cancer cell lysis, but also by immune activation (205). Here, we mainly discuss the innate immune responses induced by OVs.

Virus-infected cancer cells tend to down-regulate their MHC-I molecules making themselves more sensitive to NK cells (206). In this regard, several studies have been conducted to examine the anti-tumor effect of the combination of NK cells with OVs. As expected, combination therapy showed an additive or synergistic anti-tumor effect (207, 208). In addition, OV infection can lead to increased tumor infiltration of M1 type macrophages and NK cells (209, 210). Furthermore, infected cells can trigger a Toll-like receptor response due to the expression of pathogen-associated molecular patterns (PAMPs) on the cell surface or due to detection by intracellular components of Toll-like receptors (211). Additionally, OV infection can cause the exposure of calreticulin, HMGB-1, nucleic acids, and type I IFNs (212), and the induction of immunogenic cell death (213), which are essential ligands and innate immune sensing pathways for activation of DCs and macrophages (7). Oncolysis by OVs could also cause the release of tumor associated/specific antigens that are then cross-presented by DCs, ultimately eliciting an adaptive immune response against the tumor (214, 215). Some OVs, such as reovirus (216) and M protein mutant vesicular stomatitis virus (DeltaM51-VSV) (217), can directly activate DCs and facilitate their antigen presentation function.

CONCLUSION

In view of the recent insights into the biology and immunology of osteosarcoma, immunotherapy is becoming an increasingly attractive treatment strategy. It is generally assumed that

adaptive immune cells, especially CTLs, have the greatest potential to eliminate tumors, due to their professional antigen recognition activity and specific killing of tumors (218). However, the characteristics of osteosarcomas (e.g., low expression of MHC-I molecules, absence of specific tumor antigens, and impaired antigen presentation) impede the anti-tumor capacity of CTLs (3, 20). Innate immune cells have unique advantages related to eliminating osteosarcoma due to their roles in antigen presentation, antigen-specific T cell priming, and MHC-independent direct cell killing. Efficacy can be further improved by using auxiliary strategies such as epigenetic modification, gene engineering, and mAb therapy. However, existing immunosuppressive mechanisms, especially the immune checkpoints imposed on immune cells, act as major obstacles to efficacy of innate immune cell-based therapy. Considering the role of OVs in induction of innate immune response, it is reasonable to combine innate immune cell-based therapy with ICPIs or OVs to treat osteosarcoma.

AUTHOR CONTRIBUTIONS

This review paper was written by ZeW, revised by ZhW, and BL, suggested by SW and TC, and edited and guided by ZY. All authors read and approved the final version of the manuscript.

ACKNOWLEDGMENTS

This study was supported by the National Natural Science Foundation of China (grant no. 81872173).

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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.

The reviewer BT and handling Editor declared their shared affiliation.