

Osteosarcoma is a primary malignant tumor that tends to threaten children and adolescents, and the 5-year event-free survival rate has not improved significantly in the past three decades, bringing grief and economic burden to patients and society. To date, the genetic background and oncogenesis mechanisms of osteosarcoma remain unclear, impeding further research. The tumor immune microenvironment has become a recent research hot spot, providing novel but valuable insight into tumor heterogeneity and multifaceted mechanisms of tumor progression and metastasis. However, the immune microenvironment in osteosarcoma has been vigorously discussed, and the landscape of immune and non-immune component infiltration has been intensively investigated. Here, we summarize the current knowledge of the classification, features, and functions of the main infiltrating cells, complement system, and exosomes in the osteosarcoma immune microenvironment. In each section, we also highlight the complex crosstalk network among them and the corresponding potential therapeutic strategies and clinical applications to deepen our understanding of osteosarcoma and provide a reference for imminent effective therapies with reduced adverse effects. Keywords: osteosarcoma, immune microenvironment, therapeutic strategies, clinical applications, immune cells, non-immune cells, complement, exosomes

1 Introduction

Osteosarcoma is a rare primary cancer, characterized by the production of an abnormal and immature osteoid matrix. Despite its rarity in the whole spectrum of diseases, with an annual incidence rate of 4.7 per million, osteosarcoma ranks first among malignant bone tumors in young people (0-19 years) and has complex heterogeneity. In certain circumstances, osteosarcoma is associated with or secondary to other diseases, such as Paget's disease, retinoblastoma, Li-Fraumeni syndrome, Rothmund-Thomson syndrome, and Bloom syndrome, which may be rooted in genetic risks, adding to the complexity of the condition.

primary clinical manifestations of osteosarcoma are bone pain, swelling, and functional impairment. As the onset is usually insidious, it may not be taken seriously in the early stages. Another terrible situation is misdiagnosis as osteomyelitis, benign tumors, or metastatic bone tumors, which consequently leads to improper treatment. Osteosarcoma treatment is based on its classification and staging. A combination of surgery and chemotherapy is the first choice of treatment for high-grade osteosarcoma. Chemotherapy is considered to be applied preoperatively or postoperatively, according to specific conditions. For low-grade osteosarcoma, surgery alone is no worse than surgery plus chemotherapy. Surgery is also the preferred option for resectable metastases and pathological fractures. The MAP regimen, comprising doxorubicin, cisplatin, and high-dose methotrexate, is the cornerstone of chemotherapy. It is worth noting that the impact of methotrexate on older adult patients is unpredictable and lacks positive evidence. Therefore, replacing methotrexate with ifosfamide is recommended for patients over 40 years of age. Second-line chemotherapy includes ifosfamide, cyclophosphamide, etoposide, carboplatin, gemcitabine, docetaxel, sorafenib, regorafenib, and samarium. Muramyl tripeptide is an innate immunomodulatory drug that has already been approved in Europe for the treatment of patients under the age of 30 years with resected osteosarcoma. Despite such exploration, the 5-year event-free survival rate of 70% for patients with osteosarcoma has not improved significantly over the last three decades, which indicates

that existing regimens remain insufficient and limited. There is great variation among different individuals in response to the same regimen of therapeutic management. Therefore, there is still a long way to go for osteosarcoma treatment research. Currently, the focus on tumors has expanded from the tumor cell itself to the tumor environment, in which tumor cells are promoted to uncontrollably proliferate, migrate, and resist apoptosis and drugs. An increasing number of studies have shown that changes in the tumor microenvironment are important. The immune microenvironment is a novel perspective to view and interpret, and its overall feature is immune suppression to help tumor cells escape immune surveillance. Components of the immune microenvironment of osteosarcoma are

mainly divided into two categories: cellular and acellular substances. The former includes immunocytes, such as tumor-associated macrophages (TAMs), tumor-associated neutrophils (TANs), myeloid-derived suppressor cells (MDSCs), mast cells (MCs), T cells, B cells, natural killer cells (NK cells), and dendritic cells (DCs). Non-immune cells, including mesenchymal stem cells (MSCs) and circulating tumor cells (CTCs), can actively interact with the immune system and promote the formation of inhibitory immune networks. The complement system and exosomes with special immune effects are also hot spots in the field of microenvironment research. Surgery and stereotactic radiotherapy can largely remove localized tumors at early stages. However, both approaches are limited by space and cannot eradicate all osteosarcoma cells in the body, especially metastatic and circulating osteosarcoma cells, which may lead to relapse and progression. Studies have shown that tumor cells may be in constant confrontation with the immune system, and the balance can be disrupted at a certain time point. Once tumors are generated, they are difficult to completely remove. Immunity is promising for eliminating tumor cells from the body at the cellular level. Drugs that target the immune microenvironment are gradually stepping onto the stage with great application potential. Few reviews have focused on the panorama of the immune microenvironment in osteosarcoma specially. Instead of rigidly borrowing conclusions from other studies on the immune microenvironment in other solid tumors, this review systematically summarizes the main components in the immune microenvironment of osteosarcoma and their functional characteristics, as is shown in Figure 1. In each column, we also list relevant therapeutic strategies and clinical applications in progress. The ultimate aim is to provide more information and insight into the understanding and treatment of osteosarcoma.

Figure 1. MDSCs TANs NETs CD11b ROS, NO L-arginine, IL8 MCs RANKL TAMs CSF-1R T cells PD-1 CD47R EGFR IL13, IL14 TLR CSF-1R PD-1 CTLA-4 BTLA CD276R AIRE hSFRP2 B cells A IL10, IL35 TGF- β FasL CD1d TNF- α , FasL perforin granzyme NK cells IL6 TGF- β , IFN- γ CXCL8, MVB1 CTCS T C1 TGF- β AFP HSP Complement TIM-3 OAA PD-L1 GRM4 IL23, IL12 CCR7 MSCs dependent IL6, IL8 cytotoxicity pro-tumor DOX Exosomes anti-tumor DCs Open in a new tab

Immune and non-immune components in the immune microenvironment of osteosarcoma and mechanisms of their pro-tumor/anti-tumor effects. CSF-1R, colony-stimulating factor 1 receptor; PD-1, programmed cell death protein-1; EGFR, epidermal growth factor receptor; IL, interleukin; NETs, neutrophil extracellular traps; ROS, reactive oxygen species; NO, nitric oxide; RANKL, receptor activator NF- κ B ligand; TGF- β , transforming growth factor-beta; IFN- γ ,

interferon-gamma; CXCL8, C-X-C motif chemokine ligand 8; AFP, a-fetoprotein; HSP, heat shock protein; TIM-3, T cell immunoglobulin and mucin domain-containing protein-3; OAA, osteosarcoma-associated antigens; PD-L1, programmed cell death protein ligand-1; GRM4, glutamate metabotropic receptor 4; CCR7, chemokine receptor 7; TNF- α , tumor necrosis factor-alpha; CTLA-4, cytotoxic T-lymphocyte-associated protein-4; BTLA, B And T-lymphocyte attenuator; AIRE, autoimmune regulator expression; hSFRP2, humanized secreted frizzled-related protein 2; TLR, toll-like receptor; TAMs, tumor-associated macrophages; TANs, tumor-associated neutrophils; MDSCs, myeloid-derived suppressor cells; MCs, mast cells; MSCs, mesenchymal stem cells; CTCs, circulating tumor cells; C, complement; DCs, dendritic cells; NK cells, natural killer cells.

2 Cells

2.1 Myeloid Cells

2.1.1 Tumor-Associated Macrophages

TAMs are the most abundant tumor cells in the immune microenvironment of osteosarcoma and account for approximately 50% of the total tumor volume. TAMs play important roles in matrix remodeling, inflammation, vascularization,

immune defense, and regulation. There are two TAM phenotypes: M1 which is classically activated and M2 which is alternatively activated. Generally, the M1 phenotype participates in inflammation with a reversed impact on metastasis, whereas the M2 phenotype is involved in wound healing and immune regulation with an accelerative impact on metastasis. The exact mechanisms of the complex impact include gene alteration, Notch pathway abnormality, macrophage polarization, and helper T cells (Th) 1/Th2 cytokine disturbance. Therefore, as the understanding of TAMs has deepened, researchers have attempted to inhibit M2 polarization in various ways to prevent tumor progression.

Therapeutic Strategies

All-trans retinoic acid prevents the migration of osteosarcoma cells both in vitro and in vivo by inhibiting interleukin (IL) 13- or IL14-induced M2 polarization. Another study indicated that all-trans retinoic acid could decrease cancer stem cell (CSC) properties strengthened by the M2 phenotype, increasing the number of CD117 Stro-1 cells and the overexpression of CD133, CXCR4, Nanog, and Oct4. Therefore, all-trans retinoic acid is expected to be added to the existing standard regimens. Paradoxically, studies have suggested that the M2 phenotype might be anti-tumor and anti-metastatic in osteosarcoma. Classifying TAMs into M1 and M2 subgroups to analyze their influence on osteosarcoma pathogenesis, metastasis, and drug resistance may be crude and one-sided. Researchers are also attempting other approaches targeting TAMs to treat osteosarcoma. In human osteosarcoma implantation mice, scientists utilized a specific macrophage-eliminating liposome to ablate TAMs, which led to decreased

tumor growth. Moreover, when epidermal growth factor receptor was silenced by short hairpin RNA in implanted osteosarcoma cells, tumor growth stimulated by recruited and polarized

macrophages was suppressed. TAM-specific surface molecules are ideal targets for drug development. CD47R and programmed cell death protein-1 (PD-1) are two popular surface molecules that can be used to generate immune checkpoint inhibitors. Mifamotide and camrelizumab are being tested for their pharmacological effects on blood and lymphatic vessel formation, immunosuppression, and drug resistance. Pexidartinib (PLX3397), an inhibitor of colony-stimulating factor 1 receptor, has the potential to reprogram TAMs and activate T cells infiltration in osteosarcoma, resulting in decreased tumor growth and lung metastasis. In summary, TAMs are potential target candidates for new therapies.

2.1.2 Tumor-Associated Neutrophils

Most research on neutrophils in patients with osteosarcoma focuses on the neutrophil-lymphocyte ratio or circulating neutrophils. Increased pre-treatment or preoperative neutrophil-lymphocyte ratio might be correlated with poor outcomes, which means that neutrophil-lymphocyte ratio can be investigated as a prognostic biomarker. However, there has been insufficient research on neutrophil infiltration in osteosarcoma microenvironment. Neutrophils in the tumor immune environment, known as TANs, exhibit phenotypic heterogeneity and functional versatility. In osteosarcoma, research on TANs is still in its early stages. The lifespan of TANs may be longer than that of circulating neutrophils under stimulation by pro-inflammatory factors such as interferon-gamma (IFN- γ). Neutrophil extracellular traps are web-like chromatin structures formed by granule proteins and chromatin secreted by neutrophils. Unlike traditional

phagocytosis and killing factor secretion, neutrophil extracellular traps can promote metastasis via the DNA receptor coiled-coil domain containing protein 25. Leshner et al. also found that peptidylarginine deiminase 4, which is vital for extensive chromatin decondensation to form neutrophil extracellular traps, is overexpressed in osteosarcoma. Similar to the M1 and M2 subtypes of TAMs, Fridlender et al. reported that TANs could also be polarized to the anti-tumor N1 phenotype and pro-tumor N2 phenotype. Yang et al. found that the number of neutrophils in non-metastatic tissues (detected by the neutrophil-specific marker CD11b) was higher than that in metastatic tissues. The infiltrated neutrophils exerted anti-tumor effects by coordinating the recruitment of immune cells, but also mediating antibody-dependent cellular cytotoxicity. In addition, neutrophil infiltration has been suggested to correlate with hypoxia-associated genes. Emerging evidence indicates that the hypoxic microenvironment plays a pivotal role in tumor progression, and a retrospective study assessed the validation of

hypoxia-associated risk score as a prognostic factor of metastasis. The results of this study indicated that TANs were downregulated in the high hypoxia-risk group. The authors concluded that hypoxia might downregulate anti-tumor immune cells, which contribute to immune escape and metastasis. Unfortunately, both studies mentioned above did not take functional differences between subtypes into consideration, but merely counted the total number of TANs. This may be because of the difficulty in identifying the ideal markers. More detailed research is needed to uncover the complex role of TANs in the immune microenvironment of osteosarcoma. Development of therapeutic strategies associated with TANs in osteosarcoma is still ongoing and require creative ideas based on basic scientific research.

2.1.3 Myeloid-Derived Suppressor Cells

MDSCs are a population of heterogeneous immunosuppressive immature myeloid cells that can differentiate into TAMs, TANs, and tumor-associated DCs. MDSCs not only interact with immune substances but also closely interact with osteoclasts, osteoblasts, chondrocytes, and other stromal cells in the bone and joint microenvironment to promote the pathogenesis and metastasis of osteosarcoma. MDSCs are classified as granulocytic MDSCs/polymorphonuclear MDSCs (G-MDSCs/PMN-MDSCs) and monocytic MDSCs (M-MDSCs). Recent studies have identified early bone marrow mesenchymal stem cells (e-MDSCs) that act as precursors of both PMN-MDSCs and M-MDSCs. Among all immune cells, MDSCs interact with T cells most closely, which exerts the effect of inhibiting proliferation of T cells, reducing T cell-mediated immune responses and promoting T cell apoptosis by consuming L-arginine and producing reactive oxygen species in the microenvironment. Different MDSC subpopulations undergo different pathways to inhibit T cell function. PMN-MDSCs produce reactive oxygen species mainly by activating signal transducer and activator of transcription (STAT) 3 and upregulating nicotinamide adenine dinucleotide phosphate oxidase, whereas M-MDSCs produce nitric oxide mainly by activating STAT1 and upregulating inducible nitric oxide synthase to inhibit the effect of T cells. MDSCs suppress not only acquired anti-tumor immunity but also innate anti-tumor immunity. In addition to T cells, MDSCs inhibit the function of NK cells and DCs. Interestingly, stimulated by the hypoxic microenvironment, MDSCs express high levels of vascular endothelial growth factor, vascular endothelial growth factor analog Bv8, basic fibroblast growth factor, and matrix

metalloprotease 9 to facilitate angiogenesis and the formation of a pre-metastatic niche, which has a strong relationship with osteosarcoma metastasis.

Therapeutic Strategies and Clinical Applications

Because MDSCs extensively infiltrate osteosarcoma lesions and inhibit anti-tumor immunity, researchers have been inspired to develop related therapies. The process of obliterating osteosarcoma cells with some existing drugs involves modulation of MDSCs immune responses. Studies have shown that current neoadjuvant chemotherapeutic drugs (doxorubicin, cisplatin, ifosfamide) could reduce the number of MDSCs in osteosarcoma patients, boost local immune states, and increase immune sensitivity. All-trans retinoic acid has been found to affect not only TAMs but also MDSCs by reducing the number of M-MDSCs and the potency of PMN-MDSCs. Metformin has been shown to modulate the metabolism of MDSCs to play an anti-tumor role in osteosarcoma by downregulating oxidative phosphorylation and upregulating glycolysis, which is also related to the enhancement of T cell immunity. MDSCs can also be targets of the drugs themselves. Tumor cell surface vimentin-targeted interleukin 12 alters the immune profile (IFN- γ HiCD8HiFOXP3LowCD33Low) in mice transplanted with osteosarcoma and lowers the number of MDSCs, thereby controlling tumor recurrence and metastasis. Because infiltrating MDSCs in the osteosarcoma microenvironment express the chemokine receptor CXCR4, Jiang et al. designed an antagonist of CXCR4, AMD3100, and tested its synergistic effect in combination with an anti-PD-1 antibody in an osteosarcoma murine model. In addition, Shi et al. combined a functional inhibitor of

PMN-MDSCs via selectively suppressing PI3K δ/γ , (S)-(-)-N-[2-(3-Hydroxy-1H-indol-3-yl)-methyl]-acetamide (SNA), with an anti-PD-1 antibody to treat mice bearing osteosarcoma, and they validated that tumor growth was restrained and survival time was prolonged. Other studies have attempted to inhibit osteosarcoma progression by preventing the migration of MDSCs to the tumor microenvironment. Guan et al. found that in an osteosarcoma murine model, anti-IL18 therapy significantly reduced the abnormal upregulation of MDSCs in peripheral blood, thus effectively curbing chemotaxis and infiltration, and finally inhibiting tumor progression. In addition to serving as a drug target, the number of MDSCs in the peripheral blood or tumor microenvironment of osteosarcoma is also a promising candidate as a prognostic biomarker. However, owing to the lack of highly specific markers for MDSCs, MDSCs-related therapy of osteosarcoma has not been sufficiently safe, and further research on its id

a key molecular triad controlling bone remodeling. The dissolution and reconstruction of bone can help immunosuppressive cells further infiltrate the tumor microenvironment and shield the immune escape of tumor cells. Therefore, Inagaki et al. proposed that MCs could function as biomarkers for osteolysis.

Clinical Applications

The most popular application of MCs in osteosarcoma is as a prognostic marker. MCs have been found to have the potential to predict metastasis and survival. Fan et al. reported that the abundance of activated MCs in osteosarcoma microenvironment is associated with negative outcomes, which might indicate the prognosis of patients. Wei et al. detected a correlation between immune-related genes and long noncoding RNAs to compare the different landscapes of immune-related long noncoding RNA pairs in localized and metastatic osteosarcoma. A significant difference in immune infiltration was observed between localized and metastatic osteosarcoma, and the

2.1.5 Dendritic Cells

DCs are derived from the bone marrow and can be divided into three major subgroups: plasmacytoid DC, myeloid/conventional DC1, and myeloid/conventional DC2. DCs act as a bridge between innate and adaptive immunity and are the most important antigen-presenting cells. Inflammatory infiltration varies markedly among different types of sarcomas, and DCs do not differ. There are more infiltrating cells represented by (DC-SIGN/CD11c+) DCs, CD14+/CD68+ TAMs, and CD3+ T cells in conventional high-grade osteosarcoma, undifferentiated pleomorphic sarcoma, and giant cell tumor of the bone than in Ewing's sarcoma, chordoma, and chondrosarcoma. Another study found that the quantity of resting DCs was significantly higher in tissues with high immune scores in contrast to the low immune score group, and the degree of DC activation was positively correlated with outcomes. Furthermore, the infiltration of DCs into osteosarcoma tissues was found to be related to autophagy. Zhang et

DCs are known to drive the pathogenesis of osteosarcoma through oncogenes and the tumor suppressor glutamate metabotropic receptor 4. Glutamate metabotropic receptor 4-/- DCs

secrete more IL23 and IL12 than wild-type DCs, leading to rapid tumor growth and accelerated progression in mouse models. DCs cultivated with osteosarcoma cells express increased IL23 and decreased IL12, and the higher ratio of IL23/IL12 can be reduced by augmented glutamate metabotropic receptor 4 signaling. Agonists of glutamate metabotropic receptor 4 or an antibody against IL23 may be promising treatment candidates. DCs may also be associated with metastasis in patients with advanced osteosarcoma. A study on the single-cell RNA landscape revealed that CCR7 participates in the deformation, chemotaxis, migration, and survival of DCs, which are crucial to tumor metastasis. The study also demonstrated that compared with primary and recurrent lesions, the proportion of CD1c+ DCs is large in lung metastatic

Scientific research has shed light on the therapeutic potential of DCs, and scientists have achieved some inspiring success. Some agents or partial components of the agents enhance the impact of DCs. For example, capsaicin was reported to enhance the phagocytosis of osteosarcoma cells (MG-63) by DCs in vitro. The most popular treatment approach for DCs is vaccination. Several vaccines have shown encouraging efficacy, such as the CD1c+ DC vaccine and vaccination with polyinosinic:polycytidylic acid (poly I:C) activated and tumor antigen-loaded CD103+ myeloid/conventional DC1s. In addition to vaccines, liposomal-muramyl tripeptide phosphatidylethanolamine has a good chance of extending overall survival and survival without metastasis by charging DCs or producing T cells, not only when used alone but also in combination with other approaches. Scientists have already investigated the effect of DCs to explore the possibility of their application in combination with anti-

T cells are thymus-derived lymphocytes that mature and reside in thymus-dependent areas of peripheral immune organs. T cells play a vital role in both cellular and humoral immunities. The classification of T cells according to different criteria is very complex. In the activation stage, T cells can be divided into naive, effector, and memory T cells. According to the T cell receptor characteristics, including distribution and major histocompatibility complex restriction, T cells can be divided into $\alpha\beta$ T and $\gamma\delta$ T. On the principle of function, T cells can be divided into Th, including Th1, Th2, Th9, Th17, Th22, and follicular helper T cells, cytotoxic T lymphocytes (CTLs), and regulatory T cells (Tregs), including natural Tregs, inducible Tregs, and other Tregs. T cell infiltration plays a critical role in osteosarcoma anti-tumor immunity, and its classification is highly heterogeneous. In osteosarcoma, tumor-infiltrating lymphocytes are mainly distributed in the region expressing human I

antigen-1+ T cells, and granzyme B+ T cells in the tumor mass. These studies suggest that sophisticated T cell infiltration occurs in osteosarcoma in terms of regions, subtypes, and molecules.

Therapeutic Strategies and Clinical Applications

Given that T cells play a significant role in the immune microenvironment of osteosarcoma, T cell-related applications show vigorous vitality, the mechanisms of which can mainly be divided into the following aspects: 1) T cell infiltration profile used as an auxiliary indicator of diagnosis, such as disease staging, patient clustering, prediction of metastasis, drug resistance, and survival outcomes; 2) immunotherapies targeting T cell-related immune responses, including

strengthening the function of effector T cells and weakening the inhibitory effect of Tregs; 3) adoptive T cell therapy, including CTL, $\gamma\delta$ T, and gene-engineered tumor-specific T cells; and 4) non-immunotherapies involving T cell-related pathways. All four aspects are discussed

T cell-related therapeutic strategies and clinical applications in osteosarcoma. T cells are widely explored to predict outcomes and promote anti-tumor treatment. The left column shows that T cell infiltration profile may be associated with different prognosis. The right column illustrates that therapies involving T cells are mainly divided into three categories: 1) regulation of T cell-related immune responses by modulating receptors and blocking checkpoints, 2) adoptive T cell therapy based on CTLs, $\gamma\delta$ T cells and gene-engineered tumor-specific T cells, 3) non-immunotherapies containing T

cell-related mechanisms. TLR, toll-like receptor; CSF-1R, colony-stimulating factor 1 receptor; M2, tumor associated macrophages M2 phenotype; PD-1, programmed cell death protein-1; PD-L1, programmed cell death protein ligand-1; BTLA, B And T-lymphocyte attenuator; HVEM, herpesvirus entry mediator; CTLA-4, cytotoxic T-lymphocyte-associated protein-4; CTLs, cytotoxic T lymphocytes; TCR, T cell receptor; CAR-T cells, chimeric antigen receptor T cells; HER-2, human epidermal growth factor receptor-2; GD2, disialoganglioside; NKG2D, natural killer group 2 member D; TNF- α , tumor necrosis factor-alpha; MDSCs, myeloid-derived suppressor cells; HSV, herpes simplex virus. First, T cells are potential prognostic predictors and assistant indicators for clinical diagnosis. Weak immunosuppressive signals and strong T-cell immune responses have been found to be significantly associated with improved outcomes in osteosarcoma patients. The number of activated CD8⁺ T cells in tumor lesions of osteo

Second, T cell and T cell-induced immune responses are common targets in immunotherapies. Toll-like receptor is a key molecule involved in innate immunity, and acts as a bridge between nonspecific and specific immunity. Yahiro et al. found that activation of the Toll-like receptor 4 signaling pathway could further stimulate CD8⁺ T cells in murine models, thereby inhibiting osteosarcoma progression. Fujiwara et al. reported that the colony-stimulating factor 1 receptor inhibitor PLX3397 could consume TAMs and Tregs (FOXP3⁺) and enhance CD8⁺ T cell infiltration in primary and metastatic lesions. This phenomenon has been observed both in vitro and in vivo. In vitro, PLX3397 inhibited colony-stimulating factor-1 or tumor-conditioned media stimulation of pERK1/2 and reduced the pro-tumor M2 polarization of TAMs. In an osteosarcoma orthotopic xenograft model, systemic administration of PLX3397 significantly inhibited primary tumor growth and lung metastasis, contributing to improve

addition of L-arginine may be a future direction. Takahashi et al. found that a combination of dual checkpoint blockade therapy (anti-PD-L1 and anti-cytotoxic T-lymphocyte-associated protein-4) and X-ray irradiation could control primary osteosarcoma and diminish metastasis in vivo, which was associated with increased recruitment of CD8⁺ T cells and decreased infiltration of Tregs. Immune checkpoint inhibitors have not been shown to be significantly effective in all the studies. Nasarre et al. investigated the role of a monoclonal antibody targeting humanized

secreted frizzled-related protein 2, a protein that promotes angiogenesis and metastasis, in metastatic osteosarcoma resistance to PD-1/PD-L1 inhibitors, and its impact on T cells. They found that humanized secreted frizzled-related protein 2 monoclonal antibody inhibited T cell proliferation and osteosarcoma metastasis by lowering the expression of NFATc3, CD38, and PD-1. Third, adoptive T-cell therapy is popular and is

CD3z signaling domains in CD45RA⁺ T cells through lentiviral transduction in vivo and in vitro. They found that the anti-tumor activity of NKG2D-CAR memory T cells was enhanced by strengthening the interactions between NKG2D ligands and receptors in osteosarcoma. However, it is not enough to increase the adaptation of effector T cells to osteosarcoma; it is also crucial to facilitate their proliferation, prolong their lifespan, enhance their resistance to the inhibitory immune microenvironment, and promote their susceptibility to tumor cells. Gene-engineered T cells can be used in treatment in the future, with more precise targets, flexible controllability, and richer functions. Fourth, many non-immunotherapies are relevant to the regulation of immune microenvironmental pathways. Mortara et al. found a significant increase in CD4⁺ and CD8⁺ T cells and a decrease in MDSCs and Tregs in the microenvironment of osteosarcoma syngeneic mouse tumor models, which responded well to the com

cells and neutrophils in the osteosarcoma microenvironment of BALB/c mice bearing HER-2/neu TUBO-derived tumors. The oncolytic HSV-1 did not reverse tumor immune tolerance in vitro, indicating that the two drugs might share some overlap in pharmacological mechanisms to achieve the effect that one plus one was greater than two. Furthermore, some T cell-associated therapies have not yet achieved initial success, but they can inspire researchers. The addition of immunomodulatory cytokines such as IL2, IL15, and liposomal-muramyl tripeptide phosphatidylethanolamine might induce T cell proliferation and differentiation to improve the survival outcomes of osteosarcoma patients, but the evidence is insufficient. Studies on specific monoclonal antibodies and bispecific antibodies targeting osteosarcoma cells are also ongoing, but there is abundant evidence. Anti-tumor vaccines are thought to be able to clear small residual lesions in the body by inducing active or passive specific immunity, an

Clinical trials of anti-tumor vaccines in sarcoma.

NCT number Phases Diseases Vaccines

NCT00923 351 I, II Ewing's sarcoma, undifferentiated/embryonal sarcoma, desmoplastic small round cell tumor, synovial cell sarcoma, rhabdomyosarcoma Tumor lysate/CD25 depleted lymphocytes with tumor lysate/KLH pulsed dendritic cell vaccine

NCT00405 327 II Sarcoma, neuroblastoma, Wilm's tumor Tumor lysate-pulsed dendritic cell vaccine

NCT00001 566 II Ewing's sarcoma, rhabdomyosarcoma Autologous dendritic cell vaccine

NCT01141 491 II Sarcoma Trivalent ganglioside vaccine

NCT01241 162 | Ewing's sarcoma, osteogenic sarcoma, rhabdomyo sarcoma, synovial sarcoma, neuroblastoma Autologous dendritic cell vaccine

NCT00069 940 | Sarcoma, central nervous system tumors, gastrointestinal stromal tumor Telomerase 540-548 peptide vaccine

NCT00003 023 | Sarcoma, neuroblastoma BCG vaccine, monoclonal antibody A1G4 anti-idiotypic vaccine

NCT00199 849 | Sarcoma, prostate cancer, bladder cancer, NY-ESO-1 plasmid DNA cancer vaccin

non-small cell lung cancer, esophageal cancer [Open in a new tab](#)

2.2.2 B Cells and Antibodies

B cells are derived from lymphoid stem cells in the bone marrow and reside in the lymphoid follicles of the peripheral lymphoid organs when they mature. B cells are not only the protagonists of humoral immunity by producing antibodies but are also a type of antigen-presenting cells involved in immunoregulation. According to the activation stage, B cells can be divided into three categories: initial B cells, memory B cells, and effector B cells/plasma cells, among which the latter is the main source of antibodies. Regulatory B cells are a type of B cells with immunosuppressive effects. Regulatory B cells inhibit CD4⁺ T cells, CTLs, macrophages, and DCs by secreting inhibitory cytokines such as IL10, TGF- β , IL35, and expressing membrane surface regulatory molecules such as FasL and CD1d, and promote the transformation of T cells into Tregs, thus weakening anti-tumor immune responses. Although humoral immunity

indispensable role. Overall, B cells and humoral immunity are receiving increasing attention in anti-tumor immunity, and breakthroughs are expected in this area.

Therapeutic Strategies and Clinical Applications

The infiltration of B cells in osteosarcoma is complex, with differences in cell subtypes and patient sex. Yang et al. obtained data on osteosarcoma cases from The Cancer Genome Atlas and performed a comprehensive assessment of the infiltration of immune cells. They found more memory B cells and activated B cells in osteosarcoma lesions in men than in women. Li et al. analyzed the immune cells in the microenvironment of osteosarcoma, Ewing's sarcoma, multiple myeloma, and cancer bone metastases and found that osteosarcoma patients with high infiltration of B cells had a better prognosis and activated B cells were positively correlated with survival. Therefore, infiltration of effector B cells may be a good prognostic indicator. Research on B cells in osteosarcoma is st

Antibodies produced by B cells and plasma cells proliferate and differentiate from memory B cells and mainly exist in serum, tissue fluids, secretory fluids, and on the surface of certain cells. Antibodies regulate tumor growth and metastasis through antibody-dependent cell-mediated

cytotoxicity, modulatory effects, activation of complement, closure of tumor cell receptors, and alteration of tumor cell adhesion. Contrary to common sense, some antibodies can bind to antigens on the surface of tumor cells and block killing. Immunoglobulins are globulins with antibody activity or similar chemical structure domain antibodies that are mainly distributed in the serum or on B-cell membranes. Studies on antibodies and immunoglobulins in osteosarcoma are relatively few and need to be further investigated. IgE has been found to be associated with osteosarcoma development. Zhang et al. conducted bioinformatic analysis on 19 osteosarcoma cases and six normal samples obtained from th

Immunoglobulins, their receptors, and their transporters can be used as predictors and diagnostic factors. Wang et al. found that positive expression of polymeric immunoglobulin receptor, the transporter of dimeric IgA, and pentameric IgM, was significantly associated with poor prognosis in patients with osteosarcoma, indicating that polymeric immunoglobulin receptor might be a good prognostic biomarker. Guerra et al. analyzed biochemical and immunological parameters in the saliva of healthy children and children with cancer before and after antineoplastic treatment, including osteosarcoma. The total concentration of IgA in the saliva of children with cancer was significantly lower than that in healthy children, independent of antineoplastic treatment. This noninvasive test provides a new clue for the diagnosis and treatment of childhood cancer. In addition, antibodies specific for osteosarcoma-associated antigens are the mainstay of humoral immunity and the raw material fo

2.2.3 Natural Killer Cells

NK cells are a class of innate lymphoid cells that express the intracellular transcription factors E4BP4+ and CD3-CD19-CD56+CD16+ on their surface. NK cells are widely distributed in the blood, peripheral lymphoid tissues, liver, and spleen. NK cells have been found to not only kill tumor cells directly but also control tumor progression and metastasis. NK cells express a variety of cytokine receptors related to chemotaxis and activation and can be recruited to the tumor microenvironment to kill tumor cells by releasing perforin, granzyme, and tumor necrosis factor- α and expressing FasL. The PD-1/PD-L1 axis can regulate the anti-tumor effects of NK cells. Zhang et al. found that blocking the PD-1/PD-L1 axis with a PD-L1 antibody, which inhibited NK cell toxicity by secreting granzyme B, could enhance the killing effect of NK cells on human osteosarcoma cells. Yang et al. conducted a comprehensive analysis of immune infiltration in the osteosarcoma microenviron

Therapeutic Strategies

For cancer cells evading adaptive immune surveillance by antigen shedding, lowering major histocompatibility complex-I, and inhibiting T cells, NK cells alone or in combination show great application prospects. Attempts have been focused in three directions: adoptive NK cells, cytokine-based targeting therapies that enhance the immune activity of NK cells, and chimeric antigen receptor-NK cells (CAR-NK). Adoptive NK cells have achieved initial success in the treatment of osteosarcoma. The basic principle is to compensate for the hypothetically existing immune deficiency and reactivate the suppressed NK anti-tumor immunity. NK cells used for treatment can be obtained from sources such as autologous or alien peripheral blood, umbilical

cord blood, hematopoietic progenitors, and pluripotent stem cells. The advantage is that it is safe, and there is no graft vs. host disease, such as CAR-T cells and immune checkpoint inhibitors. Mismatched allogeneic donors may

checkpoint inhibition, CAR technology, and the expansion of autologous and allogeneic NK cells is of great value and urgently needed.

2.3 Non-Immune Cells

2.3.1 Mesenchymal Stem Cells

CSCs are competitive clones that drive tumorigenesis. CSCs are considered the key reason for the huge heterogeneity of osteosarcoma and cause recurrence, metastasis, and drug resistance. Xu et al. divided osteosarcoma patients into two clusters based on osteosarcoma tumor stem cell-related genes. Cluster 1 had a higher immune infiltration score and a better prognosis than Cluster 2. The Cluster 1 immune microenvironment is characterized by fewer follicular helper T cells, M0 macrophages, and more CD8⁺ T cells. These results suggest that CSCs are potentially associated with the immune microenvironment landscape in osteosarcoma, which means that different CSCs may develop different immune infiltrates and have different prognoses. There is a lot of research indicating that osteosarcoma originates from MSCs. MSCs are

MSCs play an important role in osteosarcoma tumorigenesis by regulating immune responses and by inducing cell fusion and differentiation. A study on osteosarcoma histogenesis suggested that naive MSCs and tumor-derived MSCs may exert different effects on osteosarcoma development. Naive MSCs were found to have both anti-tumor and pro-tumor effects. Tumor-derived MSCs have been shown to promote tumor cell proliferation, increase CSCs proportion, facilitate epithelial-mesenchymal transition, and exhibit strong immunosuppressive activity. There are two non-immune mechanisms by which MSCs promote osteosarcoma cell proliferation and metastasis: first, the interaction between osteosarcoma cells and MSCs involving IL8 and aquaporin 1; second, abnormal gene expression, such as Rb, C-MYC, TP53, K-Ras, and IHH promote the transformation of MSCs into osteosarcoma cells. MSCs have also been found to transform into cancer-associated fibroblasts when exposed to osteosarcoma cell

chemokine ligand 20 are also very important in the increased migration of MSC-EVs to the tumor microenvironment and inflammation inhibition.

Therapeutic Strategies

The application of MSCs has focused on two aspects: 1) regulation of the signaling pathways and secretion behavior of MSCs and 2) delivery of drugs by MSCs. For example, Alvaro et al. loaded MSCs with an oncolytic virus and granulocyte-colony stimulating factor to increase immune infiltration and alleviate tumor growth. However, it should be noted that the pharmacokinetic characteristics of MSCs vectors require extensive studies to ensure both efficacy and safety. However, a few studies have shown that MSCs can effectively inhibit osteosarcoma recurrence, proliferation, and metastasis. Aanstoos et al. injected MSCs directly

into the tumor mass of osteosarcoma mice and found that tumor expansion and local recurrence were controlled. In contrast, the intravenous administration of MSCs to osteosarcoma mi

2.3.2 Circulating Tumor Cells

Osteosarcoma cells exist not only in the tumor mass, but also in circulation, and are called CTCs. CTCs can escape local treatment, such as surgical resection and radiotherapy and survive in small quantities under systemic treatment, which results in the metastasis and recurrence of osteosarcoma. Several studies have shown that CTCs play a specific role in the immune microenvironment of osteosarcoma. Zhang et al. found that the inhibition of IL6 could suppress osteosarcoma cell proliferation and reduced CTCs. The phenomenon observed in in vitro experiments was that recombinant human interleukin 6 activated Janus-activated kinase/signal transducers and activators of transcription 3 and mitogen-activated protein kinase/extracellular signal regulated kinase1/2 pathways. Both pathways promote osteosarcoma cell proliferation, but only the former promotes cell migration. This effect was also confirmed in a nude mouse model of human osteosarcoma. Given that activated Janus-

mechanisms of the interaction between CTCs and tumor immunity are not clear, CTCs are potential predictive markers and drug targets. Elimination of CTCs is expected to be a symbol of a more thorough efficacy of anti-tumor therapies.

3 Complement System

The complement system is a protein reaction system consisting of more than 30 components with precise regulation and is widely present in the serum, tissue fluid, and cell membrane surface. In addition to innate immunity to pathogens, the complement system participates in the immune regulation of inflammation and tumors. The majority of current studies suggest that complement system activation has anti-tumor effects by killing tumor cells through complement-dependent cytotoxicity, whereas other studies suggest that complement system activation has pro-tumor potential in a special immune microenvironment.

Clinical Applications

Chen et al. found that the expression level of C1q (C1qA, C1qB, and C1qC) was positively correlated with th

metastasis and prognosis. Huang et al. found that C1qA, C1qB, and C1qC levels were positively correlated with the number of follicular helper T cells, CD8⁺ T cells, and memory B cells. Moreover, the expression of these three key genes was significantly lower in metastatic osteosarcoma cell lines than in non-metastatic osteosarcoma cell lines. This finding also indicates that C1q is a positive prognostic factor. However, Jeon et al. found that after the addition of normal human serum, human bone osteosarcoma epithelial cells (U2-OS) activated the alternative pathway of the complement system, resulting in the generation of more vascular endothelial growth factor-A and fibroblast growth factor 1 and promotion of angiogenesis in models in vitro, regulated by the phospho-ERK signaling pathway. Moreover, the expression of

negative complement regulatory proteins, such as CD46, CD55, and CD59 and endogenously expressed properdin or C3, was reduced in normal human serum-treated U2-O

NKG2D on the surface of CD8⁺ T cells and NK cells, inhibits lymphocyte activation, and further impedes tumor cell recognition. Exosomes containing TGF- β 1 in mesothelioma have also been found to inhibit IL2-mediated lymphocyte proliferation more strongly than does soluble TGF- β 1, thereby increasing immune escape. In breast cancer, TGF- β in exosomes induces monocyte differentiation and accumulation of immature MDSCs. Exosomes produced by osteosarcoma cells have been reported to have a bidirectional effect on tumors; however, there are only a few studies on their immune effects. Exosomes have tumor-associated antigens on their surfaces, which can interact with antigen-presenting cells and induce tumor-specific toxic T-cell immune responses. Similar to exosomes secreted by osteoblasts, exosomes secreted by osteosarcoma cells also have immunosuppressive effects, even at a much higher degree. Exosomes containing TGF- β , α -fetoprotein, and heat shock proteins slow T cell

delivery vectors in cancer, packaging drugs and modifying exosomes creatively to obtain more controlled and stable release and fewer adverse effects is one of the directions for future research. Table 2 lists clinical trials on exosomes in other malignant tumors, indicating that exosomes may also be versatile in osteosarcoma research.

Table 2.

Clinical trials on exosomes in other malignant tumors.

NCT number	Diseases	Exosomes	Functions
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NCT02702 856	Prostate cancer	Urinary exosome gene signature	Diagnosis
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NCT03830 619	Lung cancer	Serum exosomal long noncoding RNAs	Diagnosis
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NCT03032 913	Pancreatic ductal adenocarcinoma	CTCs and onco-exosomes	Diagnosis
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NCT04720 599	Urologic cancer	ExoDx Prostate Intelliscore	Diagnosis
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NCT02662 621	Malignant solid tumors	HSP70-exosomes in the blood and urine	Diagnosis
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NCT03911 999	Prostate cancer	Urinary exosomal microRNA	Prediction
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NCT03031 418	Prostate cancer	ExoDx Prostate Intelliscore	Prediction
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NCT03895 216	Bone metastases	Deregulated miRNAs in the circulating exosomes	Prediction
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NCT02862 470	Thyroid cancer	Urinary exosomes	Prediction
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NCT01159 288	Non-small cell lung cancer	Vaccination with tumor antigen-loaded dendritic cell-derived exosomes	Treatment
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5 Conclusions, Limitations, and Perspectives

Osteosarcoma is a primary malignant bone tumor that mainly occurs in children, adolescents, and the elderly, with a poor prognosis. The immune microenvironment of osteosarcoma is a complex system with high heterogeneity, which is closely related to the escape of tumor cells from immune surveillance, uncontrolled proliferation, and metastasis. Amplifying the effect of anti-tumor immunity and inhibiting pro-tumor immunity may be a promising approach to eliminate microscopic tumor lesions and CTCs. This review systematically summarizes the roles of cellular and non-cellular components in the immune microenvironment of osteosarcoma and the progress in related therapeutic strategies and cli

osteosarcoma is still at the stage of borrowing analogous findings from other solid tumor types, which may be vague and one-sided. 2) Second, most studies remain in the preclinical stage, and evidence of translational medicine is insufficient. 3) Finally, the immune system influences the entire body, and immune-related therapies should be both safe and effective. If not employed properly, they can lead to new and serious diseases; therefore, adverse effects should be considered seriously. Based on this, some future directions are proposed: 1) Diagnosis: Explore the multi-factor predictive model, diagnosis model, and grading scores of osteosarcoma that contain immune components to promote precision treatment. 2) Treatment: Develop new approaches that are more effective than existing treatment schemes under the premise of safety, try to combine immunotherapy with other therapies, and pursue cheaper and faster manufacturing. 3) Research: Find more ideal identification markers of immune ce