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.

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. The 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.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 STAT3 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-γHiCD8HiFOXP3LowCD33Low 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 PI3Kδ/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 identification markers is needed. The mechanisms of accumulation, migration, and interaction with other immune and non-immune cells of MDSCs are also a mystery and require more effort.

Mast Cells

MCs rank among the top five infiltrating cells in osteosarcoma and can be classified into resting MCs and activated MCs. The level of activated MCs in the osteosarcoma microenvironment is significantly higher than that in the normal set. The infiltration of MCs (CD117+ and tryptase+) was lower in the center of the tumor mass and more distributed at the normal-tumor interface where osteolysis occurs. The special distribution of MCs may be related to their function. Heymann et al. found that under the influence of osteosarcoma cells, MCs could maintain viability and activity and produce receptor activator NF-κB ligand, 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 high abundance of activated MCs indicated unsatisfactory outcomes. Le et al. found that the proportion of MCs in patients who died was higher than that in living patients, implying a negative association between MCs and prognosis. In general, because of the unclear role of MCs in the immune microenvironment, related therapies are just beginning and urgently require further development.

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 al. tested the correlation between immune cell infiltration and 13 autophagy-related long noncoding RNAs, one of which was named RUSC1-AS1 and was negatively associated with the proportion of infiltrating immature DCs, macrophages, and mast cells. The study also illustrated that the level of plasmacytoid DCs was higher in the osteosarcoma microenvironment of the high-risk group than in the low-risk group, whereas the levels of total DCs and immature DCs were lower and associated with poor prognosis. However, the subgroup and quantity of DCs in the tumor microenvironment of the same patient are not static but dynamic in the trend of first increasing and then decreasing in quantity. DCs can trigger further immune responses by detecting tumor antigens and presenting them to helper and cytotoxic T cells, during which time they transform from immature DCs to mature DCs. Therefore, in the early stages, DCs proliferate actively and mature to activate helper and cytotoxic T cells. As the tumor grows, osteosarcoma cells develop variants resistant to DCs and phagocytes, leading to less stimulation of DC activation and eventual immune escape.

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 lesions. Although several lines of evidence have drawn a beneficial portrait of DCs in osteosarcoma, some studies have reported contradictory results. Koirala et al. examined the role of PD-L1 and explored its prognostic value. They concluded that PD-L1 is significantly associated with DCs, T cells, and NK cells. Furthermore, DCs (28.3% vs. 83.9%) and TAMs (45.5% vs. 84.4%) were significantly associated with worse 5-year event-free survival. Another study investigating the immune classification in osteosarcoma also showed a negative association between DCs and prognosis. Their analysis suggested that the number of DCs in live patients was less than that in dead patients, in contrast to NK cells and CD8+ T cells.

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-transforming growth factor-β (TGF-β) antibodies, agonist anti-glucocorticoid-induced tumor necrosis factor receptor antibodies, and doxorubicin. For example, Kawano et al. combined DCs and anti-TGF-β antibodies to treat osteosarcoma and detected enhanced systematic immune responses in vivo. Existing attempts to utilize DCs aim to maximize tumor killing by virtue of upregulating the immunocompetence of lymphocytes. Nevertheless, the studies mentioned above also focused on the pro-tumor activity of DCs, which is a vital risk when using DC-associated therapy. Making full use of the advantages and avoiding the disadvantages with further precision therapy is the key to DCs application in the future.

Lymphoid Cells

T Cells

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 αβT and γδT. 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 leukocyte antigen Class I, whereas CD4+ and CD8+ T cells are mainly clustered at the interface between pulmonary metastases and normal tissues. The number of T cells in metastatic lesions is significantly higher than that in primary and recurrent lesions in situ. In metastatic lesions, checkpoint and immunoregulatory molecules were calculated to be higher than those in primary lesions, including PD-1, lymphocyte activation gene-3, T cell immunoglobulin and

mucin domain-containing protein-3 (TIM-3), indoleamine 2,3-dioxygenase 1, and IFN-γ. Han et al. analyzed the biopsy tissue and peripheral blood of 16 patients with primary osteosarcoma and concluded that there were more TIM-3+PD-1− T and TIM-3+PD-1+ T cells in biopsy tissue than in peripheral blood, suggesting that the immune microenvironment in tumor lesions was inhibitory. They also reported that immune cells could interact with each other to form a vicious cycle, in which the immune activity of T cells could be inhibited by pro-tumor macrophages, and depletion of CD163+ macrophages could increase T cell growth and pro-inflammatory factor production in vitro. Another interesting case report from Japanese Hiroshima University was the case of extraosseous osteosarcoma with partial spontaneous regression and CD8+T cells, T cell-restricted intracellular 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.

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 osteosarcoma patients has been found to be higher in men than in women. A study claimed that CD8+ T cells might be associated with a good prognosis, whereas γδT cells have a poor prognosis. Autoimmune regulator expression is an indispensable transcription factor for T cells, resulting in peripheral immune tolerance, which develops and obtains central tolerance in the thymus. Matsuda et al. analyzed 43 biopsy samples of conventional osteosarcoma and found that autoimmune regulator expression was expressed in 58.1% of the samples and was related to increased Tregs (FOXP3+), lung metastasis, and reduced overall survival. The results suggest that autoimmune regulator expression might be an ideal prognostic indicator and a satisfying target for drug design. Based on the above studies, it is not difficult to conclude that if the infiltration of T cells in the surgically removed tumor biopsy can be carefully analyzed, it may provide guidance for the next step of treatment and predict the prognosis.

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 improved metastasis-free survival. Currently, popular immune

checkpoint inhibitors are being explored in the field of osteosarcoma treatment. Yoshida et al. validated that anti-PD-1 antibody (4H2) could decrease Treg infiltration in subcutaneously implanted models of murine osteosarcoma cell line LM8, and finally, tumor volume decreased in size and overall survival was prolonged. Cascio et al. reported that PD-1/PD-L1 was not the only immune checkpoint axis in human osteosarcoma lesions; herpesvirus entry mediator (HVEM/CD270) and indeterminate receptor to B7H3/CD276 were also expressed. The expression of these three immune checkpoints was significantly higher in metastatic lesions than in the primary lesions. The levels of the three ligands were positively correlated with each other and with peritumoral T-cell infiltration. Therefore, owing to low intratumor effector T cell infiltration in osteosarcoma, combined therapies of immune checkpoint inhibitors may be used to magnify immune infiltration, improve the immune microenvironment, and finally repress tumors in the future. Some researchers have combined immune checkpoint inhibitors with other therapies to further optimize osteosarcoma treatment. He et al. observed that PD-1/PD-L1 therapy combined with L-arginine improved the therapeutic effect in immunocompetent BALB/c mouse models. L-arginine significantly increased the infiltration of CD8+ T cells, splenic CD8+ T cells, serum IFN-γ, and anti-α-PD-L1 antibody to prevent the exhaustion of CD8+ T cells and promote the expression of IFN-γ, granzyme B, and perforin. The combination of L-arginine and PD-1/PD-L1 immunotherapy significantly increased overall survival in mice; therefore, the 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 flourishing in osteosarcoma. Adoptive T cell transfer is one of the current research hotspots, which involves the introduction of specific T cells amplified in vitro into patients to supplement and enhance T cell-related immunity. Common applicable T cells include CTL, γδT, and gene-engineered tumor-specific T cells. In particular, gene engineering is moving to the center stage of osteosarcoma. Osteosarcoma-associated antigens are numerous and complicated, mainly represented by activated leukocyte cell adhesion molecules (CD166), B7H3, and epidermal growth factor receptor. T cells can be loaded with specific osteosarcoma-associated antigens by gene engineering, among which αβT cell receptor-modified T cells, chimeric antigen receptor T cells (CAR-T cells), especially HER-2 CAR-T cells, disialoganglioside GD2 CAR-T cells, and B7H3 CAR-T cells have been discussed the most. CAR-T cell therapy has progressed to the third

generation with preliminary advances in hematologic tumors and neuroblastoma, but is still in the exploratory stage in osteosarcoma. Fernandez et al. verified the safety and effectiveness of installing natural killer group 2 member D (NKG2D) CARs containing 4-1BB and 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 combination of targeting angiogenesis L19 tumor necrosis factor alpha (L19mTNF-α; L), melphalan, and gemcitabine. Atti et al. showed increased T cell infiltration in the microenvironment of an osteosarcoma murine model treated with the alkylating agent trabectedin. However, CD8+ T cells were exhausted in no time, which may be due to the high expression of PD-1. Therefore, the team suggested the addition of PD-1/PD-L1 blockers to compensate for this failure to achieve better anti-tumor effects. Belisario et al. found that the ratio of ATP-binding cassette transporter (ABC)-A1, an activator of anti-tumor Vγ9Vδ2 T cells, to ABC-B1, an inducer of chemotherapy resistance, could indicate chemo-immune resistance. In addition, zoledronic acid increased the sensitivity of drug-resistant osteosarcoma cells by enhancing intratumor apoptosis and the ratio of ABC-A1 to ABC-B1 and Vγ9Vδ2 T-cell infiltration. Workenhe et al. reported that the combination of HSV-1 ICP0 null oncolytic virus KM100 and mitoxantrone, an immunogenic cell death-inducing chemotherapeutic drug, could significantly increase the survival benefit by increasing the infiltration of CD8+ T 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,

and several clinical trials of sarcoma are underway, which is expected in the field of osteosarcoma. In summary, the function of T cells in the immune microenvironment of osteosarcoma and their interactions with other components have not been fully recognized, and relevant therapies remain in the preliminary stage of exploration. Further research is required to expand this field and inspire applications.

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 is not the predominant mechanism of the anti-tumor immune responses, it plays an 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 still very limited, and new therapies based on B cells lack satisfactory results.

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 the Gene Expression Omnibus database and compared the differentially expressed genes, differentially methylated regions, copy number, and functional enrichment of the two groups. The results showed that hypermethylation in the fragment crystallizable region of immunoglobulin E, high-affinity I, receptor for γ polypeptide was significantly associated with osteosarcoma development. IgE elevation is often seen in allergic diseases; therefore, the specific role of IgE epigenetic alterations in osteosarcoma immunity needs to be further explored.

Therapeutic Strategies and Clinical Applications

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 for the design of anti-tumor drugs. Receptor tyrosine kinase-like orphan receptor 2 has been found to be a highly expressed osteosarcoma-associated antigen, and its antibodies are potential drugs. Hellmann et al. fabricated a clinically used monoclonal antibody with high affinity to receptor tyrosine kinase-like orphan receptor 2 using human Ig transgenic animals, providing a new idea for osteosarcoma immunotherapy. Autoantibodies can also be used for etiological studies. Mazzoni et al. discovered that IgG reacting with Simian virus 40 mimotopes was significantly higher in the sera of osteosarcoma patients than in those of breast cancer patients, undifferentiated nasopharyngeal carcinoma patients, and healthy people, demonstrating an association between Simian virus 40 and osteosarcoma pathogenesis. The Ig superfamily may conceal the secrets to osteosarcoma progression and metastasis. Leukocyte-associated immunoglobulin-like receptor-1 is a collagen receptor of the Ig superfamily, and the related

pathways in lymphocytes and monocytes have received increasing attention. Leukocyte-associated immunoglobulin-like receptor-1 overexpression decreased Glut1 and epithelial-mesenchymal transition-related molecules, thereby inhibiting osteosarcoma cell metabolism and metastasis and providing a new target for slowing osteosarcoma progression. Although studies on antibodies and immunoglobulins in osteosarcoma are scarce, they may exert considerable influence on the diagnosis, treatment, and scientific research of osteosarcoma in the future.

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 not only kill tumor cells directly but also control tumor progression and metastasis. They 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 by expressing FasL. The PD-1/PD-L1 axis can regulate the anti-tumor effects of NK cells. Blocking the PD-1/PD-L1 axis with a PD-L1 antibody, which inhibits NK cell toxicity by secreting granzyme B, can enhance the killing effect of NK cells on human osteosarcoma cells. Male patients have been found to have more NK cells than female patients in osteosarcoma lesions. In addition, NK cells were suppressed in the osteosarcoma microenvironment, while TGF-β expression increased. TGF-β can promote angiogenesis, bone remodeling, and cell migration by inhibiting the expression of activation receptor NKG2D and reducing the release of perforin by NK cells. To overcome these negative effects and induce resistance of NK cells to TGF-β, researchers have continuously exposed NK cells to low-dose TGF-β and IL-2 in vitro, thereby alleviating immunosuppression in the osteosarcoma microenvironment. This strategy might be applicable to human NK cell-based therapies in the future.

Therapeutic Strategies

For cancer cells that evade adaptive immune surveillance through antigen shedding, MHC-I downregulation, or T cell inhibition, NK cells—alone or in combination—have great therapeutic potential. Current strategies are focused in three directions:

1. **Adoptive NK cells**,
2. **Cytokine-based therapies** to enhance NK cell immune activity, and
3. **CAR-NK cells**.

Adoptive NK cell therapy has shown promise in osteosarcoma treatment. This approach aims to compensate for immune deficiencies and reactivate suppressed NK cell-mediated anti-tumor immunity. NK cells used for treatment can be obtained from autologous or allogeneic peripheral blood, umbilical cord blood, hematopoietic progenitors, and pluripotent stem cells. These therapies are generally safe and do not cause graft-versus-host disease, unlike CAR-T cells or immune checkpoint inhibitors. Interestingly, mismatched allogeneic donors may provide greater anti-tumor effects than matched or autologous NK cells in osteosarcoma.

In one approach, researchers combined N-803 (an IL-15 superagonist) with dinutuximab (a monoclonal antibody targeting GD2) to treat ex vivo expanded peripheral blood NK cells. This combination enhanced NK cell cytotoxicity and increased survival in osteosarcoma mouse models. Other studies using IL-15 showed that this cytokine can restore the sensitivity of chemotherapy-resistant osteosarcoma to NK cells through DNAX accessory molecule-1 and NKG2D pathways. These findings highlight IL-15's potential as a valuable NK cell-activating agent.

CAR-NK cells are another innovative therapy where NK cells are engineered with specific antibodies. This strategy has already shown success in treating Ewing’s sarcoma and B-cell leukemia. However, research on CAR-NK cells in osteosarcoma is still lacking. Continued studies on NK cell biology, checkpoint inhibition, CAR engineering, and NK cell expansion are crucial and urgently needed.

Non-Immune Cells

Mesenchymal Stem Cells

Cancer stem cells (CSCs) are competitive clones that drive tumorigenesis. They are a key reason for the heterogeneity of osteosarcoma and are involved in recurrence, metastasis, and drug resistance. Osteosarcoma patients can be divided into two clusters based on CSC-related gene expression. Cluster 1 shows higher immune infiltration and better prognosis than Cluster 2, with more CD8+ T cells and fewer follicular helper T cells and M0 macrophages. These findings suggest that CSCs are closely related to the immune landscape of osteosarcoma, with different subtypes influencing immune infiltrates and prognosis.

Many studies suggest that osteosarcoma originates from mesenchymal stem cells (MSCs). MSCs are adult multipotent stem cells found in bone, adipose tissue, and dental pulp. They play a significant role in osteosarcoma development by regulating immune responses and inducing cell fusion and differentiation. Naive MSCs and tumor-derived MSCs may have different effects on tumor progression. While naive MSCs may show both anti- and pro-tumor effects,

tumor-derived MSCs tend to promote tumor cell proliferation, increase CSC proportion, and drive epithelial-mesenchymal transition with strong immunosuppressive activity.

There are two non-immune mechanisms by which MSCs promote osteosarcoma proliferation and metastasis:

1. Interaction with osteosarcoma cells via IL-8 and aquaporin 1

2. Genetic mutations (e.g., Rb, C-MYC, TP53, K-Ras, IHH) that lead to MSC transformation into osteosarcoma cells

MSCs can also become cancer-associated fibroblasts when exposed to osteosarcoma cells, which enhances tumor cell proliferation, migration, and invasion through molecules like monocyte chemotactic protein 1, growth-related oncogene-alpha, TGF-β, and intercellular adhesion molecule 1. These fibroblasts secrete extracellular matrix components that promote cell communication, adhesion, and tumor heterogeneity.

MSCs also contribute to angiogenesis, stimulating endothelial cell migration and invasion under osteosarcoma influence. In terms of immunomodulation, MSCs can secrete anti-inflammatory substances and inhibit pro-inflammatory ones, helping osteosarcoma cells evade immune detection. This is often mediated by extracellular vesicles (EVs), especially exosomes.

EVs from MSCs contain miRNAs, RNA, and proteins that inhibit T cell proliferation and immune response. MSC-EVs containing TGF-β and IFN-γ can promote the transformation of mononuclear cells into Tregs. MSCs also suppress B cell immune functions by increasing CXCL8 and MVB1 RNA levels, which reduce IgM production. Furthermore, MSCs promote M2 polarization of tumor-associated macrophages by secreting IL-6. Other cytokines involved in MSC-EV migration and inflammation suppression include IL-10, hepatocyte growth factor, leukemia inhibitory factor, CCL2, VEGF-C, and CCL20.

Therapeutic Strategies

Therapeutic applications of MSCs focus on two main areas:

1. Regulating their signaling and secretion

2. Using them as drug delivery vehicles

For example, researchers have engineered MSCs to deliver an oncolytic virus and granulocyte-colony stimulating factor to enhance immune infiltration and slow tumor growth. However, more studies are needed to clarify the pharmacokinetics and safety of MSC-based delivery systems.

Some studies suggest that MSCs can suppress osteosarcoma proliferation, metastasis, and recurrence. Direct injection of MSCs into tumors has been shown to reduce local tumor growth and recurrence in mouse models, whereas intravenous MSC injection led to increased lung metastasis. The anti-tumor effects may arise from promoting apoptosis, inhibiting angiogenesis, and regulating immune responses.

This contradictory evidence likely stems from our incomplete understanding of the diverse sources and functions of MSCs. In osteosarcoma, targeting pro-tumor MSC subtypes or amplifying anti-tumor MSC subtypes remains experimental. More research into MSC-derived exosomes and other EVs is needed for developing precise and effective therapies.

Circulating Tumor Cells (CTCs)

Osteosarcoma cells not only reside in tumor masses but can also circulate in the bloodstream as circulating tumor cells (CTCs). These cells can evade localized treatments (surgery or radiotherapy) and survive systemic therapies in small numbers, leading to recurrence and metastasis.

CTCs have been linked to the immune microenvironment of osteosarcoma. Inhibition of IL-6 has been shown to suppress CTC levels and osteosarcoma cell proliferation. Mechanistically, recombinant human IL-6 activates the JAK/STAT3 and MAPK/ERK1/2 pathways. While both promote proliferation, only JAK/STAT3 enhances cell migration. This has been confirmed in both in vitro experiments and mouse models. Given that activated STAT3 signaling promotes immunosuppression, CTCs likely contribute to an immunosuppressive tumor microenvironment in osteosarcoma.