Osteosarcoma, with poor survival after metastasis, is considered the most common primary bone cancer in adolescents. Notwithstanding the efforts of researchers, its five-year survival rate has only shown limited improvement, suggesting that existing therapeutic strategies are insufficient to meet clinical needs. Notably, immunotherapy has shown certain advantages over traditional tumor treatments in inhibiting metastasis. Therefore, managing the immune microenvironment in osteosarcoma can provide novel and valuable insight into the multifaceted mechanisms underlying the heterogeneity and progression of the disease. Additionally, given the advances in nanomedicine, there exist many advanced nanoplatforms for enhanced osteosarcoma immunotherapy with satisfactory physiochemical characteristics. Here, we review the classification, characteristics, and functions of the key components of the immune microenvironment in osteosarcoma. This review also emphasizes the application, progress, and prospects of osteosarcoma immunotherapy and discusses several nanomedicine-based options to enhance the efficiency of osteosarcoma treatment. Furthermore, we examine the disadvantages of standard treatments and present future perspectives for osteosarcoma immunotherapy.

Introduction

Osteosarcoma ranks first among malignant bone-related cancers in adolescents and has a complex heterogeneity and an abnormally produced immature osteoid matrix. Currently, the standard treatments for osteosarcoma are neoadjuvant chemotherapy (presurgery), surgical resection, and adjuvant chemotherapy (postsurgery). Despite the efforts of researchers, there has been no significant improvement in the 5-year survival rate of osteosarcoma patients over the past few decades, suggesting that existing therapeutic strategies are insufficient. Moreover, the above approaches cannot effectively eliminate all osteosarcoma cells due to nonspecific drug delivery, which is especially true for metastatic and circulating osteosarcoma cells, which might promote tumor recurrence and progression. Consequently, new therapeutic strategies against osteosarcoma urgently need to be explored.

Recently, evidence has shown that the body's immune system may be in a constant battle with osteosarcoma cells including three stages: immune clearance, balance, and escape. Moreover, the immune system plays an important role in the execution and exertion of antitumor immunity. Therefore, utilizing the immunity of the organism for more efficient suppression and treatment of cancer has become a focus for researchers. The concept of harnessing the immune system for this purpose originated over 100 years ago when a physician named William Coley successfully treated several of his cancer patients with a combination of live and attenuated bacteria, later known as "Coley's toxins", after observing a subset of prior patients enter remission following their diagnosis with the common bacterial infection erysipelas. Notably, the immune microenvironment, the dictator of osteosarcoma treatment response, facilitates cancer cell escape of immune surveillance. Therefore, therapeutic agents that modulate the immune microenvironment and use existing immunity to eliminate osteosarcoma cells are gradually being recognized as new options with great application prospects. Unsurprisingly, immunotherapy shows benefits in terms of potent anti-osteosarcoma effects and suppression of metastasis and recurrence in comparison with conventional intervention strategies, including

surgical resection and chemotherapy, which also show satisfactory efficacy in suppressing advanced osteosarcoma.

Along with rapid advances in immunology and biotechnology, nanoparticles have shown great promise for enhancing cancer immunotherapy. On the one hand, nanoparticles can effectively improve the pharmacokinetic parameters and reduce the side effects of therapeutic or imaging agents in cancer treatment by site-specific drug delivery. On the other hand, they can also target immune cells and organs to modulate the immune microenvironment to augment tumor immunotherapy. Therefore, versatile nanoplatforms, including biomimetic nanoparticles, inorganic nanomaterials, and organic nanomaterials, have been used to effectively modulate the immune microenvironment. Importantly, some immunomodulatory-based nanoplatforms have achieved satisfactory therapeutic effects in the preclinical study of osteosarcoma.

For these reasons, managing the osteosarcoma immune microenvironment and using nanomedicines in enhanced immunotherapy are gaining widespread attention as personalized treatment regimens. This review, therefore, focuses on the current understanding of the characteristics and functions of the main immune components in the tumor microenvironment, including dendritic cells, T lymphocytes, tumor-associated macrophages (TAMs), tumor-associated neutrophils (TANs), and natural killer (NK) cells. Moreover, we highlight that the benefits of nanomedicines in activating immune responses and reversing the immunosuppressive microenvironment hold great potential in osteosarcoma immunotherapy. Furthermore, the current challenges and future prospects of osteosarcoma immunotherapy are also discussed.

The immune microenvironment of osteosarcoma

Osteosarcoma tissue is surrounded by massive immune cell infiltration, resulting in the creation of a complex immune microenvironment that allows osteosarcoma cells to grow within the bone by creating an immunosuppressive microenvironment to maintain their survival and proliferation. A robust immunosuppressive microenvironment is positively correlated with overactivation of molecules associated with immune suppression, such as indoleamine 2,3-dioxygenase (IDO), programmed cell death protein 1 (PD-1), interleukin-10 (IL-10), transforming growth factor-β (TGF-β), vascular endothelial growth factor (VEGF), and signal transducer and activator of transcription 3 (STAT3), due to their immunosuppressive effects mediated by myeloid-derived suppressor cells (MDSCs), TAMs, and regulatory T lymphocytes (Tregs). Consequently, there is an urgent need to gain an in depth understanding of and characterize the osteosarcoma immune microenvironment to develop advanced immunotherapies by utilizing these immunologic biomarkers.

Dendritic cells (DCs) in the osteosarcoma immune microenvironment

DCs, the most common antigen-presenting cells (APCs) originating from the bone marrow, are mainly divided into DCs, DC1s, and DC2s. It should be noted that type 1 myeloid/conventional DCs (cDC1s) have an excellent profile of antigen presentation and cross-presentation and efficient T lymphocyte priming activity for initiating the immune response. There are significant differences in inflammatory infiltration among various types of osteosarcoma, but there is no

difference in DCs. For instance, (DC-SIGN/CD11c+) DCs are more common in conventional high-grade osteosarcoma than in other sarcomas. Moreover, DC infiltration has been found to be associated with autophagy in osteosarcoma. For example, a recent study using a machine learning-based autophagy-related long noncoding RNA signature showed the association between infiltration of immune cells and the expression of autophagy-related genes, among which RUSC1-AS1 was adversely connected to the numbers of infiltrating immature DCs, mast cells, and macrophages. With osteosarcoma progression, osteosarcoma cells can develop DC- and phagocytosis-tolerant variants, which reduces DC activation and ultimately causes immune escape. It is well known that DCs can express glutamate metabotropic receptor 4 (GMR-4) and carcinogens to drive osteosarcoma pathogenesis. Agonists of GMR-4 or antibodies against IL-23 may be potential options for osteosarcoma immunotherapy. Additionally, DCs may also play a significant role in the pulmonary metastasis of advanced osteosarcoma. A comprehensive study showed that CCR7 contributed to the proliferation, deformation, and migration of DCs, thereby playing an important role in pulmonary metastasis of osteosarcoma. This work also suggested that the number of CD1c+ DCs was higher in pulmonary metastases than in primary and recurrent lesions.

Several lines of evidence support the potential therapeutic value of DCs in osteosarcoma, but there are also some contradictory views. For example, PD-L1 levels were strongly related to the quantity of DCs and T lymphocytes in osteosarcoma. Additionally, DCs and TAMs were also found to be closely associated with survival time. Moreover, some studies have also suggested links between DCs and osteosarcoma prognosis based on immune classification, with fewer DCs than cytotoxic T lymphocytes and NK cells found in living patients.

T lymphocytes in the immune microenvironment of osteosarcoma

T lymphocytes, a type of thymus-derived lymphocyte, mature and reside in peripheral immune organs. They contribute to cellular and humoral immunity and can be classified according to different criteria. For example, they are usually classified into effector, naive, and memory T lymphocytes during the activation stage. Based on the features of cell receptors, such as major histocompatibility complex (MHC) restriction and biodistribution, these cells are divided into αβT and γδT lymphocytes. In addition, they can also be divided into cytotoxic T lymphocytes (CTLs), helper T lymphocytes (Th lymphocytes), and Tregs according to their function. Overall, T lymphocyte classification is highly heterogeneous, and T lymphocyte infiltration plays a significant role in osteosarcoma immunotherapy.

The majority of tumor-infiltrating lymphocytes are clustered at regions overexpressing human leukocyte antigen (HLA) class I in osteosarcomas, while effector T lymphocytes are mostly distributed at the border between healthy tissues and pulmonary metastases. Moreover, there are more T lymphocytes in metastases than in primary or recurrent lesions in situ. However, the levels of immune checkpoint and immunomodulatory molecules in metastatic lesions, such as PD-1, IDO, IFN-γ, and T lymphocyte immunoglobulin and mucin domain-containing protein-3 (TIM-3), have been found to be higher than those in primary tumors. A recent report investigated infiltrating T lymphocytes in biopsy tumor tissue and peripheral blood samples from sixteen primary osteosarcoma patients, showing that there were more TIM-3+ PD-1-negative or

-positive T lymphocytes in tumor tissue than in blood circulation, which indicated that the osteosarcoma immune microenvironment was suppressive. This study also showed that these immune infiltrating cells could promote the formation of an immunosuppressive microenvironment via crosstalk with each other in osteosarcoma, which suggested that the immune function of T lymphocytes could be suppressed by M2-type TAMs and that the consumption of CD163+ M2-type macrophages could activate the function and proliferation of T lymphocytes and the secretion of proinflammatory factors by M1-type macrophages. In conclusion, complex T-lymphocyte infiltration occurs in different regions and subgroups of osteosarcoma, involves various molecules, and plays different roles in antitumor immune responses.

Natural killer cells (NKs) in the immune microenvironment of osteosarcoma

NKs, considered nonspecific cytotoxic immune cells, are able to nonspecifically destroy infected abnormal cells (such as cancer cells) without prior activation or sensitization. They generally express suppressive surface receptors, such as killer-cell immunoglobulin-like receptors (KIRs), that can identify specific HLA class I molecules, including CD94/NK group 2 member A (NKG2A) and HLA-A, B and C. Moreover, they can be trained to lyse cancer cells with low expression of MHC class I produced from host cells. Their activated surface receptors generally include NKG2D and natural cytotoxic receptors (NCRs), which can recognize stress proteins on the surface of cancer cells, such as MHC class I peptide A/B (MICA/B), UL16-binding proteins (ULBPs), and the Fcg receptor CD16, which induces ADCC by recognizing the Fc portion of antibodies on opsonized cells. Coreceptors of NKG2D and NCRs, such as DNAM-1, can enhance the activation of NK cells for efficient immune responses. The balance between positive and negative signals received by NK cells determines their antitumor effects, which are mainly modulated by the secretion of cytotoxic granules (such as perforin and granzyme), the generation of cytokines (such as IFN-γ and TNF-α) activating antitumor immunity, and the expression of death receptor ligands on their surface.

Osteosarcoma cells have been shown to be readily eliminated by NK cells in some preclinical studies. For example, cultivation of NKs from normal donors with osteosarcoma feeder cells for one week resulted in a median killing effect of approximately 46.1%. Notably, this cytotoxicity was not associated with the expression levels of NK receptor ligands but was significantly suppressed through the exposure of NKs to anti-DNAM-1 and anti-NKG2D antibodies. Similarly, blockade of the NKG2D receptor, but not that of DNAM-1, could greatly reverse the cytotoxicity of NK cells against osteosarcoma cells in in vitro assays. KIRs also play a significant role in osteosarcoma treatment: KIR receptor-ligand mismatched NK cells have an excellent in vitro anti-osteosarcoma effect, and this effect is further augmented when the HLA class I molecule is blocked in osteosarcoma cells. Furthermore, intraperitoneal administration of IL-2 in combination with intratumoral administration of activated and expanded NK cells can effectively mitigate bone impairment, suppress osteosarcoma volume, inhibit pulmonary metastasis, and clearly prolong mouse survival time.

The tumor-associated macrophage (TAM)-mediated immunosuppressive microenvironment in osteosarcoma

TAMs are generally considered to be derived from the myelomonocytic lineage and to develop from hematopoietic stem cells (HSCs). Moreover, they are generally recruited from the blood circulation to the site of the lesion to eliminate infection, inflammation or tumor cells, such as osteosarcoma cells. However, emerging evidence has recently indicated that TAMs can develop in embryos before the emergence of HSCs and maintain self-renewal and proliferation. Macrophages can be divided based on their origin into tissue-resident macrophages, mainly derived from the yolk sac, and blood monocytes, derived from the fetal liver and bone marrow.

The quantity of TAMs may vary markedly in different solid tumors, including osteosarcoma, but they are the most abundant immune cell type in the tumor microenvironment, accounting for nearly 50% of the total tumor cells. TAMs play a significant role in matrix remodeling, inflammation and vascularization in antitumor immunity and modulation. In general, TAMs are characterized by protumor or antitumor effects based on the degree of malignancy of the tumor and their interactions with the tumor microenvironment because of their plasticity and heterogeneity. For instance, type-1 TAMs have the capability to phagocytose cancer cells and promote the secretion of inflammatory factors to improve antitumor immune responses. However, TAMs usually show an immunosuppressive type-2 phenotype in the tumor microenvironment and are prone to facilitate angiogenesis and extravascular invasion, which promote evasion of immune surveillance, eventually resulting in tumor progression, metastasis, and relapse. Moreover, CD14 and CD68 double-positive TAMs are the main immune infiltrating TAM subtype in bone-associated cancers, including osteosarcoma. An analysis of osteosarcoma patient RNA expression profiles, clinical features, and immune cell proportions showed that type 2 TAMs are the main immune cell type and are closely related to survival time. Another study using CD209 staining and gene expression analysis supported that there is accumulation of type-2-like TAMs in human osteosarcoma tissues and found that retinoic acid could modulate M2-like TAMs to suppress osteosarcoma initiation and stemness.

Recently, emerging evidence has also confirmed that the quantity of TAMs in various tumor tissues is closely associated with the quantity of tumor blood vessels, suggesting that TAMs promote tumor angiogenesis. For example, various proangiogenic substances, such as fibroblast growth factor (FGF), matrix metallopeptidase 9 (MMP-9), and vascular endothelial growth factor (VEGF), are produced by TAMs to promote tumor progression and metastasis in various cancers, including osteosarcoma. Moreover, TAMs can also interact with various immune effector cells to induce an immunosuppressive tumor microenvironment. They can suppress the activity of T lymphocytes to facilitate tumor immune escape by overexpressing PD-1 and cytotoxic T lymphocyte-associated antigen-4 (CTLA-4) receptors. Furthermore, M2-like TAMs can also increase vascular extravasation, promote the survival and growth of metastases, suppress cytotoxic T lymphocytes, and maintain immunosuppressive Tregs to enhance tumor invasion and metastasis. As a result, the premetastatic niche is formed at distant lesions in specific metastatic sites, including bone, lung, and liver, with the assistance of TAMs.

In addition to the above findings, recent reports have also shown that TAMs participate in local inflammatory regulation and drug resistance in osteosarcoma by interacting with other immune cells in the tumor microenvironment. However, distinct TAM subtypes may respond differently in

osteosarcoma, causing different degrees of malignancy. Specific targeting of TAMs (such as CD163-positive TAMs) rather than pandepletion of TAMs has been shown to enhance the cytotoxic activity of T lymphocytes functioning in tumor suppression. Such information might prompt researchers to define specific TAM features and subtypes in human biopsies for enhanced TAM-specific targeting. Indeed, specific TAM subgroups, characteristics and signals continuously evolve over immunological progression, modulating either protumor or antitumor activity. We have also highlighted the differences between human and murine TAMs. Overall, with the deepening understanding of TAMs, investigators will have the option to manipulate TAMs using various approaches to enhance osteosarcoma immunotherapy.

The tumor-associated neutrophil (TAN)-mediated immunosuppressive microenvironment in osteosarcoma

Neutrophils are important immune cells that are sensitive to pathogens and tissue impairment, accounting for nearly 50%-70% of total white blood cells in humans. Most clinical studies on neutrophils in osteosarcoma patients have focused on the ratio of neutrophils to lymphocytes or circulating neutrophils, and an increased ratio of neutrophils to lymphocytes pretreatment or presurgery might be closely associated with poor outcomes, indicating that this ratio can be applied as a prognostic marker for osteosarcoma. Neutrophils in the tumor microenvironment, also named TANs, show functional versatility and phenotypic heterogeneity similar to those of TAMs. However, there are limited reports about neutrophil infiltration in the osteosarcoma immune microenvironment.

In osteosarcoma, TANs may have a longer lifespan under the stimulation of proinflammatory factors (such as IFN-γ) than circulating neutrophils. The neutrophil extracellular trap (NET)-mediated immunosuppressive microenvironment plays a significant role in immune escape during cancer immunotherapy and consists of a reticular chromatin structure composed of chromatin and granule proteins produced from neutrophils; this microenvironment facilitates tumor metastasis instead of conventional phagocytosis and killing factor secretion. For example, PAD4, which is overexpressed in osteosarcoma, plays an important role in forming NETs via extensive chromatin decondensation. Moreover, TANs can also be polarized to anticancer N1-like or procancer N2-like neutrophils, which are similar to M1- and M2-like macrophages. In this process, TGF-β produced from tumor and tumor microenvironment-associated cells can effectively promote the aggregation of type-2 neutrophils in tumor regions, resulting in functional and phenotypic neutrophil changes. In addition, the stimulation of TANs with IFN-γ and TNF-α can polarize type-2 neutrophils into type-1 neutrophils to efficiently suppress tumor growth. This phenotypic switch is closely related to changes in protein secretome profiles, including changes in the levels of secreted granule-associated proteins, adhesion molecules, chemokines, and cytokines. Compared with patients with metastasis, patients with nonmetastatic osteosarcoma present significantly higher expression levels of the neutrophil-specific marker CD11b. Infiltrating neutrophils further exert positive antitumor effects by coordinating the recruitment of immune cells to effectively mediate specific immunity while activating antibody-dependent cellular cytotoxicity (ADCC). Furthermore, the infiltration of neutrophils has also been reported to be associated with the expression of hypoxia-related genes. Emerging studies have indicated that hypoxia in the tumor microenvironment promotes tumor progression, and thus, hypoxia could be

regarded as a prognostic factor for metastasis. There are significantly fewer N1-like TANs in groups with high hypoxia than in the groups without hypoxia, suggesting that hypoxia might contribute to evasion of immune surveillance and promotion of tumor metastasis by reducing antitumor immune cells. However, both studies described above only considered the total TAN number without taking functional differences between subtypes into account, which may be due to the difficulty in recognizing specific biomarkers. Therefore, more comprehensive studies are required to reveal the complex functions of TANs in osteosarcoma; furthermore, advanced treatments related to TANs are still being developed, and novel ideas derived from basic scientific research are required.

The myeloid-derived suppressor cell (MDSC)-mediated immunosuppressive microenvironment in osteosarcoma

MDSCs, as a group of immunosuppressive immature myeloid cells, are able to create an immunosuppressive tumor microenvironment by differentiating into tumor-associated DCs, TAMs, and TANs. They interact not only with the immune microenvironment but also with osteoblasts, osteoclasts, chondrocytes, and other stromal cells in the bone and joint microenvironment to facilitate the pathogenesis and metastasis of various tumors, including osteosarcoma. Generally, murine MDSCs are characterized by the coexpression of CD11b and Gr-1, and these cells are now further divided into granulocytic (G-MDSCs) and monocytic (M-MDSCs) based on their phenotypes and morphological characteristics. The former express have the cell surface marker phenotype CD11b+Ly6ClowLy6G+, while the latter are characterized by a CD11b+Ly6ChighLy6G- phenotype. Human MDSCs are different from murine MDSCs, which are characterized by no or low expression of HLA-DR and high expression of CD33. Unsurprisingly, the common myeloid biomarker CD11b is also a marker of human MDSCs, and the CD11b and CD33 double-positive HLA-DR-low population can be further classified as M or G MDSCs according to their CD14 or CD15 expression. It should be noted that CD15-positive cells usually exhibit a granulocytic morphology, while CD14-positive cells exhibit monocytic features.

Recent reports have confirmed that early bone marrow mesenchymal stem cells (e-BMSCs) can serve as precursors of PMN-MDSCs and M-MDSCs. MDSCs interact most closely with T lymphocytes of all immune cells, and these interactions can lead to the production of reactive oxygen species (ROS) and ablate L-arginine in the tumor microenvironment to suppress the proliferation and boost the apoptosis of T lymphocytes and weaken T lymphocyte-mediated immunity. Different MDSC subtypes can also suppress the activity of T lymphocytes in different ways. For example, PMN-MDSCs can upregulate nicotinamide adenine dinucleotide phosphate (NADP) oxidase and activate STAT-3 to generate ROS, while M-MDSCs can modulate inducible nitric oxide synthase and activate STAT1 to release NO to suppress the function of T lymphocytes. Notably, MDSCs can inhibit not only the acquired antitumor immune response but also the innate antitumor immune response. For instance, MDSCs can impair the antigen presentation of DCs and phagocytosis of NK cells to promote tumor immune escape. In addition to suppressing immunity in the tumor microenvironment, MDSCs can also actively participate in the management of the immune microenvironment and tumor metastasis. Interestingly, under the stimulation of the hypoxic tumor microenvironment, MDSCs usually produce high levels of

basic FGF, VEGF, VEGF analog Bv8, and MMP-9 to promote angiogenesis and the creation of a premetastatic niche, indicating a close association with pulmonary metastasis of osteosarcoma. Extracellular vesicles (EVs) in the immune microenvironment of osteosarcoma

Cancer cell-derived EVs are a group of heterogeneous nanovesicles secreted into the tumor microenvironment or circulation, encapsulating intact organelles, proteins, nucleic acids, and lipids (such as eicosanes, cholesterol, and fatty acids). Exosomes, a subclass of EVs, are produced by direct outward budding of the cell membrane. They induce the proliferation, metastasis, and chemotherapy resistance of osteosarcoma cells. These functions may be derived in part from interactions between tumor-derived exosomes and bone cells that form a microenvironment conducive to cancer cell homing. Notably, tumor cell- and immune cell-derived exosomes have been shown to carry TAAs and activate antitumor immune responses, resulting in the elimination of established tumors by CD8+ and CD4+ T lymphocytes, as well as directly inhibiting tumor cell proliferation and malignant tumor progression. In addition, emerging evidence supports that tumor-derived EVs can typically form an immunosuppressive microenvironment. As an example, the release of soluble MHC-I chain-related proteins and NKG2D soluble receptors from osteosarcoma-derived exosomes can suppress NK cell or CTL activity, thereby creating a conducive environment for osteosarcoma immune escape. Another study suggested that the level of exosomal PD-L1 in osteosarcoma patients with pulmonary metastasis was much higher than that in patients without pulmonary metastasis. In the study, a mouse model was used to evaluate the roles of exosomes expressing PD-L1, and the study showed that pulmonary metastasis significantly increased after exposure to such exosomes. Based on these studies, it appears that osteosarcoma cells can secrete exosomal PD-L1 to promote lung metastasis; therefore, detecting the level of exosomal PD-L1 in serum might be an important means to identify pulmonary metastasis for clinical treatment. A potential mechanism behind the utility of this strategy is related to PD-L1-induced immunosuppression, which subsequently enables expression of epithelial-mesenchymal transition (EMT)-related proteins, including N-cadherin, vimentin, fibronectin, and laminin 5. Another study reported that in 36% of cancer patients, exosomes containing IDO, which can modulate antitumor immune responses by regulating tryptophan (Trp) consumption and is involved in the formation of an inflammatory microenvironment to facilitate tumor angiogenesis, were identified in the majority of bodily fluids. These molecules in osteosarcoma-derived exosomes plays an indirect role in immune responses and may influence DC-mediated neoantigen presentation. Therefore, reversing the immunosuppressive microenvironment by regulating exosome release may be a new strategy to enhance osteosarcoma immunotherapy.

Mesenchymal stem cells (MSCs) in the immune microenvironment of osteosarcoma

MSCs, considered competitive clones that promote tumorigenesis, are another reason for the substantial heterogeneity found in osteosarcoma, which causes chemotherapy resistance, recurrence, and metastasis. Based on the expression of tumor stem cell-related genes, osteosarcoma patients can be divided into two clusters (Cluster 1 and Cluster 2). The immune microenvironment of Cluster 1 patients has fewer follicular helper T lymphocytes and macrophages and more cytotoxic T lymphocytes, resulting in an immune infiltrating phenotype and superior therapeutic effects compared to those of Cluster 2 patients. This suggests that MSCs affect the immune microenvironment of osteosarcoma, and different types of MSCs might

lead to diverse immune infiltration phenotypes and outcomes. Moreover, a variety of studies have indicated that osteosarcoma might be derived from bone marrow-derived MSCs. MSCs are mature pluripotent stem cells found in different tissues, particularly in the dental pulp, bone, and adipose tissue, and play a significant role in modulating immunity, cell fusion and differentiation in osteosarcoma tumorigenesis. The histogenesis of osteosarcoma shows that naive MSCs and MSCs from tumors might play adverse roles in osteosarcoma progression. Naive MSCs generally have suppressive or supportive effects in cancer, while tumor-derived MSCs have the ability to facilitate epithelial-mesenchymal transition (EMT), thus exhibiting a robust immunosuppressive effect and promoting tumor cell proliferation. This MSC-mediated promotion of the proliferation and metastasis of osteosarcoma cells can be attributed to the following two points. On the one hand, the interaction between MSCs and osteosarcoma cells involves aquaporin 1 and IL-8. On the other hand, abnormal gene expression, including abnormal expression of TP53, Rb, C-MYC, IHH, and KRAS, can facilitate the reprogramming of MSCs into osteosarcoma cells. Notably, MSCs can also transform into tumor-associated fibroblasts (CAFs) after exposure to osteosarcoma cells, which can obviously promote osteosarcoma cell proliferation and metastasis. This process usually involves multiple substances, including monocyte chemotactic protein 1, growth-related oncogene-α, TGF-β, and intercellular adhesion factor. Moreover, CAFs can release extracellular matrix components to maintain cell proliferation and intercellular adhesion and communication to maintain malignant phenotypes and increase tumor heterogeneity. To some extent, osteosarcoma cells and MSCs have similar functions. For example, some studies have reported that osteosarcoma cells can also trigger the migration and invasion of endothelial cells and facilitate angiogenesis after exposure to MSCs. In regard to the immune response, MSCs can effectively release anti-inflammatory factors and suppress proinflammatory factors to assist osteosarcoma cells in immune escape, which is induced by autocrine or paracrine EVs, especially exosomes. An interesting study reported that MSCs can secrete EVs containing miRNAs/RNAs and proteins to suppress the proliferation and immune response of T lymphocytes. Additionally, TGF-β and IFN-γ secreted by MSC-derived EVs can induce the switch of mononuclear cells into Tregs. In addition to inhibiting T lymphocyte-related antitumor immunity, MSCs can also suppress the immune function of B lymphocytes. For example, MSC-derived exosomes can significantly increase the expression of MVB1 RNA and C-X-C motif chemokine ligand (CXCL) 8, which decreases the quantity of immunoglobulin M (IgM) in B lymphocytes. It should be noted that MSCs can also secrete IL-6 to promote the M2-like polarization of TAMs. Additionally, some cytokines, including IL10, hepatocyte growth factor (HGF), leukemia inhibitory factor (LIF), CXCL2, CXCL20, and VEGF-C, also play a significant role in the increased accumulation of MSC-EVs at tumor sites and inflammation suppression.

Circulating tumor cells (CTCs) in the immune microenvironment of osteosarcoma

Cancer cells, including osteosarcoma cells, can exist either in tumor tissues or in blood circulation, and such cells are named circulating tumor cells (CTCs). They have the ability to escape local therapy to survive at low levels with systemic interventions, such as radiotherapy, phototherapy and surgical resection, eventually leading to the metastasis and recurrence of osteosarcoma. Emerging evidence has also shown the important role of CTCs in the osteosarcoma immune microenvironment. For instance, previous studies have shown that

suppression of IL-6 can decrease the number of CTCs to improve osteosarcoma treatment effects. This phenomenon was also found in another in vitro study that suggested that human IL-6 could activate the Janus-activated kinase/STAT3 and mitogen-activated protein kinase/extracellular signal modulated kinase 1/2 (MEK-1/ERK) pathways. These pathways have been shown to promote osteosarcoma cell proliferation, but only the Janus-activated kinase/STAT3 pathway can drive the migration of osteosarcoma cells. Therefore, one can speculate that the STAT3 pathway might facilitate the spread of CTCs for the formation of an immunosuppressive osteosarcoma microenvironment. In addition to IL-6, IL-8 also participates in CTC-mediated osteosarcoma progression by recruiting and activating T or B lymphocytes, neutrophils, basophils, and eosinophils. IL-8 released from self-seeded CTCs can effectively induce osteosarcoma cell proliferation and pulmonary metastasis in ex vivo assays. Consequently, suppressing the activity and secretion of IL-8 might be a potential antitumor strategy that can possibly be used in combination with various agonists or antagonists of other cytokines. Although the mechanism underlying the relationship between immune responses and CTCs is not clear, CTCs have been identified as potential predictive biomarkers and drug intervention targets for enhanced osteosarcoma immunotherapy. Furthermore, elimination of CTCs is being recognized as a more thorough and effective antitumor strategy.

Conventional immunotherapy for osteosarcoma

To better understand the immune response in osteosarcoma, the concept of the “tumor immunity cycle” has been used to illustrate approaches to enhance the effects of immunotherapy. The crucial process of this cycle occurs in the tumor and regional lymph nodes, with immune cells traveling between these distinct regions. This immunity cycle starts with the release of neoantigens or TAAs from dying cancer cells, and these TAAs are subsequently captured and processed by APCs. After that, APCs present the processed TAAs on MHC-I and MHC-II molecules to naive T lymphocytes in draining lymph nodes, leading to the activation and production of CTLs to eliminate cells with specific TAAs. Activated CTLs multiply through clonal expansion, enter the blood circulation, and migrate from local lymph nodes to the tumor microenvironment. Once activated T lymphocytes arrive at tumor tissues, they can release cytotoxic substances such as perforin and granzyme B to eliminate tumor cells. These dying tumor cells in turn release more TAAs and costimulatory signals (such as DAMPs) to induce the antitumor immunity cascade. However, osteosarcoma can disrupt the essential elements of the tumor immunity cycle via an extensive negative feedback immunoregulatory mechanism, which is becoming increasingly recognized as a promising target for osteosarcoma immunotherapy.

Macrophage modulation strategies for osteosarcoma immunotherapy

Macrophages are capable of responding to multiple stimuli in the tumor immune microenvironment via broad activation phenotypes due to their plasticity. As mentioned above, the transition from M1- to M2-like polarization of TAMs plays a significant role in the pulmonary metastasis of osteosarcoma. Thus, modulating macrophage polarization has been considered a promising approach for osteosarcoma immunotherapy. Various agents have been used to modulate the polarization of macrophages to enhance antitumor immune responses, including Toll-like receptor (TLR) agonists, cytokines, and monoclonal antibodies. Moreover, several cytokines (including IFN-γ and IL-12) have been found to reprogram macrophages toward type

1-like phenotypes by activating the STAT signaling pathway. It should be noted that TLRs are important pathogen recognition receptors expressed by APCs, such as macrophages; therefore related agonists can mediate the switch of M2- to M1-like phenotypes to elicit an antitumor immune response. For example, Vidyarthi et al. found that murine colonic tumors polarized M2-like TAMs toward M1-like TAMs and inhibited tumor cell proliferation in an IFN-αβ signaling pathway-dependent manner by administering the TLR-3 ligand [poly (I: C)]. In addition to cytokines and TLR agonists, antibodies, such as anti-CSF1 and anti-CD40 antibodies, are also used to facilitate the polarization of TAMs. Moreover, several drugs have also been shown to reprogram TAMs and have showing promising results in osteosarcoma treatment. Lipopolysaccharide (LPS)-activated M1-like TAMs in combination with IFN-γ presented significant inhibitory effects on osteosarcoma cell proliferation, and these effects could be regulated by soluble substances released by TAMs in an IL-1/TNF-α-independent manner. For example, all-trans retinoic acid (ATRA) can effectively suppress M2-like polarization and the secretion of MMP-12 to inhibit the invasion and pulmonary metastasis of osteosarcoma. Metformin (Met), which repolarizes TAMs to elicit antiangiogenic and antitumor effects, also plays a significant role in suppressing osteosarcoma cell proliferation by reprogramming the metabolic polarization of TAMs. Notably, gefitinib (Gef), an efficient EGFR inhibitor, can also repolarize the pulmonary macrophage phenotype by disturbing macrophage receptor-interacting protein kinase 2 (RIPK2) expression to suppress the invasion and metastasis of osteosarcoma. Additionally, Gef can also relieve postoperation-accelerated osteosarcoma metastasis and prolong overall survival time in a mouse model of osteosarcoma. Various compounds have also been derived from natural products, including epimedokoreanin B (derived from Epimedii Herba), onion A1 (derived from allium sulfides), and oleanolic acid (OA)/corosolic acid (CA). In an osteosarcoma mouse model, these compounds significant suppressed STAT3 activation to modulate type 2 TAM polarization, protecting against osteosarcoma progression and metastasis. Another study explored M2-like TAM antagonists or modulators, such as esculetin, wogonin (derived from the roots of Scutellaria baicalensis), resveratrol and synthetic hydroxystilbenes, xanthoangelol and 4-hydroxyderricin, for enhanced osteosarcoma immunotherapy. These compounds all effectively inhibited the activation and differentiation of type 2 macrophages to suppress osteosarcoma cell proliferation and metastasis.

Osteosarcoma-associated vaccines

Osteosarcoma-associated vaccines are considered a novel immunotherapy method that exerts antitumor effects by activating a patient's own endogenous immunity. Studies on tumor antigens utilizing tumor-relevant substances composed of tumor proteins or peptides, autologous DCs, gangliosides, and autologous or allogeneic cancer cells to activate systemic immunity are ongoing. Adjuvants, cytokines, and other immunomodulators have also been applied in vaccine preparation to improve antitumor immunity. For instance, autologous tumor lysates were first used as tumor-associated vaccines and significantly prolonged the overall survival time of patients with osteosarcoma. Immune responses to peptides derived from the TAA papillomavirus binding factor (PBF) were found in 9 of 11 patients with refractory osteosarcoma. Moreover, T lymphocyte responses were detected in 20 of 28 patients with osteosarcoma and were related to long DFS (more than 5 years) in 2 patients with an anti-idiotypic antibody who received vaccination. The use of tumor vaccines is gaining momentum. Tumor vaccines are

mainly divided into autologous cancer cell- and immune cell-based vaccines and noncell-based vaccines. Of these types, immune cell-based vaccines take full advantage of the activation of effector T lymphocytes by innate immunocytes, such as macrophages, DCs, and γδT lymphocytes. At the same time, however, the feasibility of modulating migration and activation is a major concern, as these processes are regulated by immunoinhibitory substances in the tumor microenvironment and the quantity and quality of compromised immune effector cells in patients. On the other hand, autologous cancer cell- and noncell-based vaccines have shown potential clinical value, as they can circumvent these barriers. It should be noted that the mechanisms of action of autologous cancer cell-based vaccines are independent of HLA-I, and the function of the patients' immune system is to specifically select the most immunogenic antigen. Noncell-based vaccines generally have better antitumor effects and biocompatibility since they do not induce off-target effects.

For example, melanoma-associated antigen 1 (MAGE-A1) was isolated and the first HLA-restricted anti-MAGE-A1 T lymphocytes and epitopes were identified using autologous CTL identification. Canine osteosarcomas are the only other spontaneous osteosarcomas in large animals, and their immune infiltrates resemble those of human osteosarcomas. It should be noted that mifamurtide (MTP-PE), the most effective systemic agent in canine osteosarcoma, has been approved in Europe and Japan for clinical use. Another example, DXS31-164, a Listeria monocytogenes vaccine (recombinant Listeria monocytogenes expressing a chimeric human HER2/neu construct), effectively triggers a HER2/neu-specific immune response to suppress pulmonary metastasis and prolong overall survival in a canine osteosarcoma model. The study also suggested the excellent effects of DXS31-164 in the treatment of HER2/neu-positive osteosarcoma patients. A recent study showed that the human anti-idiotypic vaccine 105AD7 was effective in most young osteosarcoma patients in clinical trials, without notable side effects. Another study also demonstrated that this vaccine could effectively elicit T-lymphocyte-mediated immunity in osteosarcoma patients and target a natural antigen (CD55) with amino acid and structural homology with 105AD7. Furthermore, various trials have also suggested that protein-based tumor vaccines, including those composed of tumor-rejection antigens and papillomavirus binding factors, can be used for specific immunotherapy in osteosarcoma and other malignant cancers due to shared overexpressed TAAs.

Osteosarcoma is a primary malignant bone tumor that mainly develops in adolescence and has a poor prognosis. The immune microenvironment in osteosarcoma is complex, has high plasticity, and is closely associated with immune escape, uncontrolled proliferation, and metastasis of osteosarcoma cells. Therefore, remodeling the immunosuppressive microenvironment of osteosarcoma should be a strategy to remove small lesions and CTCs for efficacious osteosarcoma treatment.

In this review, we systematically discussed the roles of various immune-associated cells in the immune microenvironment in osteosarcoma and the progress of relevant immunotherapy regimens and clinical applications. In particular, we highlighted the use of nanotechnology to modulate the immunosuppressive osteosarcoma microenvironment for enhanced osteosarcoma

immunotherapy, thus providing guidance for the study, diagnosis, and treatment of osteosarcoma in the future.

Current immunotherapies for osteosarcoma, including tumor antigen vaccines, ICB strategies, and CAR-T therapy, have shown satisfactory therapeutic efficacy. However, the promotion of metastasis by and immunosuppressive nature of the tumor microenvironment remain two major barriers to successful therapeutic outcomes. Overall, modulating the immune microenvironment of tumors to overcome immunosuppression and favor an antitumor immune response may be an important strategy for overcoming the current challenges of tumor therapy. A growing amount of evidence supports a comprehensive approach that can mediate antitumor immunity while overcoming the immunosuppressive tumor microenvironment, which should significantly boost clinical therapeutic outcomes.

Although such immunotherapy approaches have many advantages, there are also some disadvantages, including the extremely high cost, significant individual differences in immune responses, poor in vivo pharmacokinetic characteristics, and substantial adverse effects of systemic delivery, all of which need to be overcome.

The research described above also has limitations. First, the immune microenvironment of osteosarcoma is complicated and dynamic and varies by disease type and duration and in different individuals. The current knowledge of the role of immune microenvironment components in osteosarcoma has been formed based on similar studies on other cancers, though the results may be both ambiguous and disease-specific. Moreover, most of the research is still in the preclinical stage, with insufficient evidence for translational medicine and nanotechnology-based drug delivery systems.

Furthermore, antitumor immunity affects the whole body, and immune-associated treatments need to be both biocompatible and efficient. Such treatments may also result in new and serious side effects if not used properly; therefore, the side effects of these therapeutic agents must be seriously considered.

Owing to strong genomic heterogeneity, targeted immunotherapy has not effectively improved overall survival for decades. Novel prognostic markers assessable at diagnosis are vital to identifying subsets of osteosarcoma. The focus of clinical trials has now shifted to serial phase II studies to evaluate the activity of novel agents in recurrent and refractory disease. In-depth analyses have revealed profound genomic instability and heterogeneity across patients, with nearly universal TP53 aberration. The complexity of the genome may support the role for immunotherapy.

Characteristic gene expression classifiers can be applied to distinguish different categories of patients at initial diagnosis or during the postoperation phase and can be helpful in guiding the formulation and modification of immunotherapy treatment strategies.

There has been continuous progress in nanomaterials and production technology, and nanotechnology provides great opportunities for effective control of specific immune responses and enhancement of osteosarcoma immunotherapy. However, osteosarcoma immunotherapy strategies based on nanotechnology are still in development but clearly have great potential.

Therefore, multivariate diagnostic models and grading systems for osteosarcoma that consider immune components to promote accurate treatment and diagnosis of osteosarcoma should be explored. Novel methods that are more efficient than current treatment options and are also safe need to be developed. Immunotherapy should be combined with other treatment modalities, and less expensive and more efficient production processes need to be pursued to achieve effective osteosarcoma treatment.

Furthermore, more specific biomarkers are required for recognizing immune and nonimmune cells, identifying the interactions between the components of the immune microenvironment, exploring more suitable therapeutic targets, and integrating multidisciplinary knowledge and multitechnological support.

Additionally, rational modification of the physicochemical properties of nanoparticles may be able to overcome immune escape, inhibit tumor cell proliferation and tumor progression, enhance cell targeting and drug accumulation in the tumor, and control drug release. In particular, the particle size and specific surface features of nanoparticles are special elements to be considered in the specific delivery of immunoregulators for effective targeting of the tumor microenvironment.

Nanoplatforms with a size of 100 nm can be exuded from blood vessels and target tumors, spreading over a limited range in the extracellular space. However, a large number of xenograft models have confirmed that 10-100 nm nanoplatforms can readily reach and accumulate in tumor tissues after entering the blood circulatory system. Notably, nanoparticles with suitable particle size and slightly positive or negative charges have repulsive effects, which can decrease phagocytosis and clearance by the reticuloendothelial system. Consequently, the control of surface charge and steric stabilization can minimize the nonspecific interactions of nanoplatforms and prevent the loss of nanoplatforms at nontargeted sites.
Looking ahead, the integration of artificial intelligence and machine learning with immunotherapy and nanotechnology holds tremendous promise for advancing osteosarcoma treatment. These computational approaches could help analyze complex tumor-immune interactions, predict patient responses to various immunotherapies, and optimize nanoparticle design for targeted drug delivery. Additionally, the development of personalized immunotherapy regimens based on comprehensive profiling of a patient's tumor microenvironment and immune status may significantly improve treatment outcomes. Emerging technologies such as single-cell sequencing and spatial transcriptomics are providing unprecedented insights into the cellular and molecular heterogeneity of osteosarcoma, which could lead to the identification of novel therapeutic targets. Furthermore, the combination of immunotherapy with emerging treatment modalities like oncolytic viruses or epigenetic modulators may help overcome current limitations and provide synergistic anti-tumor effects. As our understanding of osteosarcoma biology and

the tumor-immune interface continues to grow, so too will our ability to develop more effective and precise immunotherapeutic strategies for this challenging disease. The future of osteosarcoma treatment likely lies in multimodal approaches that combine immunotherapy with nanotechnology, targeted therapy, and conventional treatments, all guided by comprehensive molecular profiling of individual tumors.