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REVIEW

Immune regulation of bone metastasis

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Metastases to bone occur in about 70% of patients with metastatic prostate and breast cancers. Unfortunately, bone metastases result in significant morbidity and mortality and treatment options are limited. Thus, significant effort has focused on understanding the mechanisms that drive tumor dissemination to bone. Bone metastases are typically characterized by a self-perpetuating 'vicious' cycle wherein tumor cells and bone-resorbing cells (osteoclasts) are locked in a cycle that leads to osteoclast-driven bone destruction and the release of bone-stored factors that in turn stimulate tumor cell proliferation and survival. To break this 'vicious' cycle, potent antiresorptive agents such as zoledronic acid (ZOL) have been used. However, in the clinical setting, ZOL failed to improve the overall survival of cancer patients even though it inhibited osteoclast resorptive activity. Thus, other cells in addition to osteoclasts are likely involved in modulating tumor growth in the bone. The immune system has the ability to eliminate tumor cells. Nevertheless, tumor cells can acquire the ability to escape immune control. Our recent observations indicated that a decline in the ability of the immune cells to recognize and kill the tumor drives tumor dissemination to bone even when osteoclasts are inhibited by potent antiresorptive agents. This review focuses on the antitumor and protumor effects of various immune cell populations involved in the bone metastatic process. We also discuss strategies to enhance antitumor immune responses and bypass cancer immune resistance.

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Introduction

Cancer immunosurveillance occurs when the immune system identifies danger signals such as tumor-specific antigens or stress ligands on transformed cells that have escaped cell-intrinsic tumor suppressor mechanisms and eliminates them before they can establish malignancy.[1] Unfortunately, antitumor immune responses are not always efficient in eliminating incipient tumors, thus allowing the transformed cells to escape immune control. Many mechanisms are involved in the escape phase including intrinsic cancer cell alterations and tumor-induced immunosuppression.[1] The result of the escape phase is the tumor outgrowth and dissemination to distant sites.

The skeleton is the predominant metastatic site for many cancers, including breast, prostate and lung cancers.[2–5] Tumor invasion into bone is associated with marked skeletal-related events (SREs) such as fractures, bone pain, hypercalcemia and spinal cord compression.[6] The current model for the pathophysiology of bone metastasis centers on the interaction between tumor cells and osteoclasts (OCs) and is known as the 'bone tumor vicious cycle'. Tumor cells secrete a plethora of factors and cytokines that can directly activate the OC or increase their maturation by stimulating osteoblast-mediated production of receptor activator of nuclear factor-κB ligand (RANKL). Once mature OCs start to resorb the bone, they release bone-stored factors such as tumor growth factor-β (TGF-β) that further stimulate tumor cell recruitment and proliferation.[7] Thus, potent antiresorptive agents such as zoledronic acid (ZOL) and the anti-RANKL monoclonal antibody (Ab) such as Denosumab have become common treatments to minimize the risk of fractures in patients with bone metastasis.[4,8,9] Despite the central role that the OC has in creating a hospitable niche for tumor colonization and growth in the bone microenvironment, the antitumor effects of ZOL are controversial. The AZURE trial did not support the use of ZOL as adjuvant therapy in breast cancer.[10] Further analysis indicated that ZOL adjuvant therapy improved the disease-free survival in postmenopausal breast cancer patients but not in premenopausal women. These findings were in contrast to another study, the ABCSG-12 trial, showing improved disease-free survival in premenopausal early-stage breast cancer patients receiving ZOL adjuvant therapy.[11] Thus, there are likely other cells/factors modulating the ZOL antitumor effects in addition to targeting the OCs. Emerging evidence suggests that immune populations have a critical role in controlling local tumor growth within the bone microenvironment.[12–14] This review aims to discuss the recent findings showing the antitumor and protumor

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effects of immune cells in skeletal metastases and their interplay with ZOL.

CD4$^+$ and CD8$^+$ T Cells

During the antitumor immune response, the capture of tumor antigens by dendritic cells (DCs) induces their maturation and migration to tumor-draining lymph nodes where DCs cross-present these antigens to T cells leading to their activation. Tumor-specific cytotoxic CD8$^+$ T cells (CTLs) participate in the killing of antigen-positive tumor cells,[15,16] and activated CD4$^+$ T cells are shown to further facilitate the development of CTLs. Thus, the presence of activated T cells at tumor sites or in circulation generally has a good prognostic value. Although the percentage of T cells in the bone marrow is usually very low, activated CD4$^+$ and CD8$^+$ T cells have been observed in the bone marrow of untreated patients with breast cancer,[17] thus suggesting that they may have a protective role in the bone metastatic dissemination. However, the authors also observed increased memory T cells in breast cancer patients with disseminated tumor cells (DTCs) in the bone marrow compared with those without detectable DTCs. As the presence of DTCs is associated with an increased risk of bone metastasis,[18,19] clinical data correlating patient immune profiles with progression of tumors in the bone need to be further evaluated.

In recent years, a few laboratories have started investigating the role of T cells in tumor growth in the bone using various animal models of bone metastases. Our laboratory demonstrated that both CD4$^+$ and CD8$^+$ T-cell populations exert antitumor effects in the context of bone metastases.[13] Depletion of either cell type, alone or in combination, significantly increases the growth and metastatic dissemination of melanoma tumors in bone. Upon activation, T cells produce interferon-γ (IFNγ), a cytokine that has a critical role in antitumor immune responses. Human T-cell leukemia virus type 1 (HTLV-1) Tax transgenic mice develop spontaneous bone tumors. In this tumor model, genetic deletion of IFNγ increases the number of bone tumors.[20] This result indicates that IFNγ, mainly produced by HTLV-1-specific T cells (and possibly natural killer cells), has protective effects on Tax($+$) tumor cell growth in the bone.

Activation of T-cell responses via injection of ipilimumab, an Ab that blocks the inhibitory signal CTLA4 expressed on T cells, suppresses tumor growth in the bone following intracardiac injection of B16 melanoma cells.[13] Ipilimumab injection was approved by the Food and Drug Administration (FDA) in 2011 for the treatment of unresectable or metastatic melanoma. However, its protective effects in patients with bone metastases have only been indirectly evaluated in a recent phase III clinical trial. This trial included 799 metastatic castration-resistant prostate cancer (mCRPC) patients with progressive disease and at least one bone metastasis at the time of inclusion. Unfortunately, no significant differences between the ipilimumab group and the placebo group were found in terms of overall survival.[21] Nevertheless, an improved 3-month progression-free survival and a marked reduction in the prostate-specific antigen were observed in the ipilimumab group.[21] Even though α-CTLA4 Ab has been shown to stimulate T-cell responses in preclinical or early clinical studies in metastatic prostate or breast cancers,[22–25] the immune activation in this trial needs to be evaluated to understand why it was not fully successful. Nevertheless, this first finding suggests that patients with bone metastases might benefit from T-cell-activating therapies.

Another small study examined the tumor-protective effects of a T-cell adoptive transfer in breast carcinoma patients, some of which presented skeletal metastases at the time of inclusion.[26] In this pilot study, memory T cells isolated from the patient bone marrow were reactivated *ex vivo* with autologous DC pulsed with lysate from the MCF-7 breast cancer cells as a source of tumor antigens and adoptively transferred back into the patients. Regrettably, patients with bone metastases at the time of inclusion were not responsive to the treatment. As a high proportion of immunosuppressive regulatory T cells (Tregs) was found in the T-cell cultures of the non-responding patients, depletion of Tregs before the adoptive transfer should be considered.

Bisphosphonates, such as ZOL, are commonly used in patients with skeletal metastases to protect from SREs because of their OC inhibitory actions. Interestingly, bisphosphonates have also shown the ability to activate $\gamma\delta$ T cells. $\gamma\delta$ T cells, similar to CD4$^+$ and CD8$^+$ T cells, can exert antitumor effects. This T-cell population is found at tumor sites *in vivo* and can induce tumor cell cytolysis *in vitro*.[27] Thus, a phase I clinical trial in mCRPC patients with bone metastases has recently tested the immunomodulatory effects of ZOL via stimulation of $\gamma\delta$ T cells. Patients were divided into two groups, one receiving ZOL plus interleukin-2 (IL-2) (to further activate T-cell proliferation) and another receiving ZOL alone.[28] A favorable clinical response, or at least disease stabilization, was observed in six out of nine patients treated with the combined therapy, and the results correlated with increased peripheral $\gamma\delta$ T cells. As patients treated with ZOL alone did not show $\gamma\delta$ T-cell expansion, this study supports the need for IL-2 to maintain $\gamma\delta$ T-cell activation *in vivo*.

The efficacy of ZOL in reducing the incidence of bone metastases in early-stage breast cancer patients has been controversial. Meta-analysis studies by Valachis *et al.*[29] did not show significant differences for the disease-free survival outcome or incidence of bone metastases. He *et al.*,[30] found improved bone metastasis-free survival in the ZOL adjuvant therapy group compared with placebo or delayed ZOL administration. The reasons for these differential effects are unknown, and the immune status of these patients has not been evaluated. We have recently shown that, in animals with bone metastases, T-cell deficiency reduces the antitumor effects of ZOL compared with immunocompetent mice.[13] Interestingly, the antiresorptive therapy is still able to suppress tumor-induced bone loss, but ZOL loses its efficacy in constraining tumor growth in the bone. This observation has important clinical implications, as it suggests that reduced T-cell numbers or impaired T-cell activation might be the cause for the failure of ZOL to reduce tumor burden and increase survival in breast cancer patients. More studies to evaluate the role of T cells in bone metastatic dissemination using clinically relevant models of bone metastases, such as prostate and breast cancers, need to be performed.

Regulatory T Cells and T Helper Type 17

Among CD4$^+$ T cells, Tregs are known to be potent immune suppressors that protect tissues from autoimmune reactions by suppressing self-reactive cells. Unfortunately, activated Tregs

have been observed in cancer patients, including with invasive breast carcinoma, and their presence predicts worse relapse-free survival and decreased overall survival.[31,32] The mechanisms underlying immunosuppression include production of TGF-β, IL-35 and IL-10, and also direct T-cell cytolytic effects through the release of perforin and granzyme (reviewed in Linehan and Goedegebuure[33]). Tregs are also responsible for impaired γδ T-cell proliferation.[34] Several studies in animal models of myeloma, leukemia, sarcoma and lung metastases have shown the antitumor effects of Treg depletion using anti-CD25 Ab.[35,36] However, opposite results in which Treg depletion showed no beneficial effects were also reported.[37–39] As suggested by the authors, different animal models and/or cell lines may explain the controversial effects of targeting Tregs in cancer.

In addition to their immunosuppressive capabilities, infiltrating Tregs have also been recently shown to be a major source of RANKL,[40] the critical cytokine required for OC differentiation. As RANKL has been reported to regulate cancer cell mobility and bone metastasis,[41] it is possible that RANKL$^+$ Tregs could favor skeletal tumor dissemination.

Clinical studies have correlated the beneficial effects of antitumor approaches with a reduction in the Treg population. Low doses of cyclophosphamide, a DNA alkylating agent, have been shown to selectively deplete Tregs and restore T- and natural killer cell effector functions in end-stage cancer patients.[42] In another study conducted in metastatic breast cancer, including in patients with skeletal metastases, the low-dose regimen of the alkylating agent induced a 40% reduction in circulating Tregs.[43] Despite having only transitory effects on Treg depletion, patients receiving cyclophosphamide showed increased tumor-reactive T cells in blood, and increased overall survival by ~3 months.[43] Despite these exciting results, cyclophosphamide administration has been associated with increased circulating myeloid-derived suppressor cells (MDSCs) in animal models and cancer patients[44] that might counterbalance the positive effects of Treg depletion. Thus, further studies are required to fully characterize the antitumor effects of this agent and in particular of Treg depletion.

T helper type 17 (Th17) cells are another subset of CD4$^+$ T cells that might be more harmful than beneficial in the context of bone metastases. These IL-17 producing T lymphocytes were shown to induce osteoclastogenesis and bone damage through RANKL production in the context of inflammatory arthritis.[45] To study the link between arthritis-mediated inflammation and secondary metastases from breast cancer, Roy *et al.*[46] injected arthritis-inducing type II collagen in MMTV-PyVMT mice that develop spontaneous metastases to the lung and bone. Interestingly, the treatment with anti-IL-17 Ab in those arthritic mice reduced the percentage of secondary metastases compared with controls.[46] Monteiro *et al.*[47] further investigated the role of tumor-specific Th17 cells on OC activation in the context of bone metastases. Tumor-specific Th17 cells promote OC activation and induce osteolytic bone disease via the production of RANKL, and the effects in the bone microenvironment are observed before arrival of tumor cells. Furthermore, tumor-specific RANKL$^+$ Th17 cell adoptive transfer into mice orthotopically injected with 4T1 breast cancer cells increases tumor colonization to bone. Surprisingly, the growth of the tumor at the primary and metastatic sites was not affected by Th17 adoptive transfer.[47] Interestingly, although high IL-17 levels are observed *in vivo*, its blockade does not abolish the pro-osteoclastogenic activity of tumor-specific RANKL$^+$ Th17 cells. Nevertheless, another study indicated that human bone marrow-derived stem cells produce IL-17 and favor skeletal metastatic dissemination of breast cancer cells overexpressing IL-17 receptor (IL-17R).[48] Altogether, these data suggest that Th17 cells favor tumor growth in the bone, a process that is likely to be mediated by IL-17 and RANKL production.

Dendritic Cells

DCs are important regulators of T-cell activation by virtue of their antigen-presenting capacities (APCs). Thus, DC-based vaccines have been used in various cancers to induce tumor-specific T-cell responses. Sipuleucel-T was the first therapeutic vaccine approved by the FDA to treat mCRPC. Sipuleucel-T consists of the injection of *ex vivo* processed APCs, including DC, that express a key tumor antigen to stimulate patient's T-cell responses. In a phase III clinical trial, men receiving Sipuleucel-T experienced an overall reduced risk of death and improved overall survival of ~20 months compared with placebo.[49] However, the beneficial antitumor effects of Sipuleucel-T in mCRPC patients older than 65 years remain controversial.[50] Results from the ongoing clinical trial combining ipilimumab and Sipuleucel-T will help define new strategies to improve anti-tumor immunotherapy efficacy.

Unfortunately, like CTLs, DCs can be the target of immunosuppressive effects used by the tumor. Data suggest that tumor-infiltrating DCs remain in an immature state, which suppresses their ability to properly activate T cells.[51] Tumor-infiltrating DCs have also been reported to suppress CD8$^+$ T cells through the production of TGF-β, nitric oxide, IL-10, VEGF (vascular endothelial growth factor) and arginase I.[52–54] Furthermore, the accumulation of DCs with immunosuppressive properties at a tumor site promotes the recruitment of other immunosuppressive populations such as Tregs and MDSCs, thus supporting tumor progression and metastasis.[55]

In a recent study, Sawant *et al.*[56] reported elevated numbers of plasmacytoid DC (pDCs) within the bone of mice inoculated with 4T1 mammary cancer cells. Depletion of pDCs by PDCA-1 showed reduced lung and bone metastases. Interestingly, accumulation of Tregs and the monocytic fraction of MDSCs within the bone was also reduced by PDCA-1 Ab. As Tregs and MDSCs have been shown to have a critical role in late stages of tumor development, it would be interesting to test the therapeutic effects of the PDCA-1 Ab on established tumors.

Myeloid-derived Suppressor Cells

MDSCs are a heterogeneous cell population of immature myeloid cells derived from the bone marrow, which potently suppress T-cell-mediated antitumor responses. MDSCs are identified by the coexpression of α_m integrin (CD11b) and Gr-1 in mice and CD11b and CD33 in humans.[57–63] Stimulated by tumor, MDSCs leave the bone marrow and are found in high numbers in circulation, spleen and tumor sites.[64] Up to a 10-fold increase in MDSC numbers is also detected in the blood of cancer patients.[65] Recent evidence indicates a correlation between MDSC numbers, stage of malignancy and poor prognosis, with the highest numbers observed in advanced cancers with worst prognostic outcomes. Mechanistically,

MDSCs exert their proneoplastic effects through the release of small soluble oxidizers, impairment of T-cell/antigen recognition and depletion of essential amino acids from the local extracellular environment, all ultimately leading to T-cell suppression.[61,62,66,67] In addition, it has been suggested that MDSCs can induce the expansion of Tregs.[68] Furthermore, MDSCs can favor tumor proliferation through the overproduction of cytokines and angiogenic factors.[69] In a recent phase I study with advanced stage of small-cell lung cancer patients, the addition of all-*trans* retinoic acid (ATRA) induced a greater immune response to DC-expressing p53 vaccine. As ATRA has been shown to induce MDSC apoptosis, this result suggests that MDSC depletion may benefit patients with metastatic disease. Nevertheless, further studies are required to validate these combined therapies prospectively in patients with bone metastases.

The role of MDSCs in bone metastases has only become apparent in recent years. We found that tumor-bearing PLCγ2$^{-/-}$ mice have an increased percentage of MDSCs in the spleen and bone marrow.[12] Interestingly, despite the blockade of OC differentiation and resorption, tumor growth in the bone of PLCγ2$^{-/-}$ mice was significantly higher than that in the WT mice because of the inhibition of antitumor T-cell responses.[13] The effects of PLCγ2 in the T-cell population were not intrinsic, as PLCγ2 is not required for T-cell activity. Thus, modulation of T-cell responses in this context is likely due to aberrant expansion/activation of MDSCs. This study indicates that the immune phenotype of PLCγ2$^{-/-}$ animals overrides the requirement for active OC in promoting tumor growth in the bone.[13] These data support the assumption that immunosuppressive myeloid populations modulate tumor growth in the bone, independent of the OC status, by affecting T-cell responses.

However, a role for MDSCs in promoting bone metastases through OCs has also been proposed. Sawant *et al.*,[70] using an immunocompetent model of breast cancer bone metastases, showed that MDSCs isolated from the tumor–bone microenvironment differentiate into resorbing OCs *in vitro*. Remarkably, OC differentiation does not occur if MDSCs are isolated from tumor-free mice or tumor-bearing animals without bone metastases.[70] Similarly, in the context of multiple myeloma (MM), Zhuang *et al.*[71] discovered that MDSCs from tumor-bearing mice have increased osteoclastogenic potential. Importantly, coinjection of tumor-challenged MDSCs together with MM cells leads to increased tumor burden and osteolytic lesions, an effect that is inhibited by administration of ZOL. Despite the evidence that MDSCs can become OCs *in vitro* or can induce OC activation *in vivo* when adoptively transferred into tumor-bearing mice, the study with PLCγ2$^{-/-}$ mice, which have intrinsic OC defects, suggests that MDSCs can enhance tumor growth in the bone independent of their ability to differentiate into OCs.[12]

Macrophages

Macrophages are mature tissue-resident myeloid cells[72] that originate from circulating bone marrow-derived monocytic precursors, suggested to be a subset of MDSCs.[57,66,73] In recent years, macrophages have been divided into two major subsets (proinflammatory M1 and anti-inflammatory M2) with many other subsets in between. M1 macrophages detect endogenous danger signals present in the debris of necrotic cells through Toll-like receptors 2, 5 and 6, intracellular pattern recognition receptors and the IL-1R. Once activated, M1 macrophages produce high levels of proinflammatory cytokines such as IL-1, IL-6, IL-12, IL-23 and IFN-γ,[72,74] and participate in the elimination of tumor cells.[75] Distinct from M1, M2 macrophages, also called tumor-associated macrophages (TAMs), have a negative prognostic value for the overall survival in patients with breast, gastric, ovarian and thyroid cancers.[76] Tumor-derived factors such as IL-4, IL-10, IL-13, TGF-β and prostaglandin 2 promulgate an anti-inflammatory macrophage polarization associated with tumor progression. M2 macrophages are characterized by high expression of IL-1Rα (IL-1 decoy receptor), mannose receptors, scavenger receptors and elevated CCL17 and CCL22 secretion. Activated M2 macrophages also produce high levels of IL-10 and TGF-β, which alter the activation of CD4$^+$ and CD8$^+$ T cells.[77] Furthermore, their ability to express VEGF, matrix metalloproteinase 9 (MMP9) and other proangiogenic factors has been associated with their positive role in metastatic dissemination.[78,79] Thus, depleting agents targeting the infiltrating macrophage population are under intensive investigation for their antitumor effects.

Recent studies using clodronate-coated liposomes, which target phagocytic cells including macrophages, showed reduced incidence of bone metastases, as well as a number of metastatic lesions in the hindlimbs of nude mice injected with human lung cancer cells.[80] In this study, macrophages and OCs were reduced at the tumor sites. The anti-mouse CD115 mAb (CSF1R antagonist) reduces the recruitment of TAMs to the primary sites in the MMTV-PyMT mammary tumor model[81] and decreases the osteolytic bone lesions in nude mice injected intracardiacally with breast tumor MDA-MB-231 cells.[82] Altogether, these studies suggest a role for macrophages in supporting tumor growth in bone. Nevertheless, so far there is no direct evidence suggesting that the antitumor effects of targeting TAMs in the context of bone metastases are not simply because of the inhibition of OC differentiation.

Neutrophils

Polymorphonuclear neutrophils (PMNs) are the predominant leukocyte subset in human peripheral blood. They are released from the bone marrow to the blood as mature cells. PMNs have been extensively studied for their proinflammatory role in host defense against microorganisms. Based on similarities in inflammatory reactions between cancer and infection, PMNs have recently emerged as new infiltrating myeloid cells in the tumor microenvironment. Indeed, PMNs represent a significant proportion of the inflammatory cell infiltrate in human cancers, leading to the term tumor-associated neutrophils or TANs.[83–85] Some *in vitro* studies reported that PMNs may display antitumor effects by inducing tumor cell lysis via Ab-dependent cell-mediated cytotoxicity.[86] However, more recent studies in humans suggest the involvement of TANs in tumor progression rather than in antitumor responses.[83–85] Several groups proposed the neutrophil-to-lymphocyte ratio as a useful prognostic biomarker for predicting overall survival in metastatic colorectal cancer[87,88] and advanced gastric cancer.[89] In addition, neutrophils are able to release VEGF and MMP9, two factors involved in the metastatic process.[90] Considering the abundance of neutrophils in bone marrow, their involvement in favoring tumor growth in bone requires further investigations.

Conclusion

Unfortunately, there is no curative treatment for bone metastasis. Tumor cells that reach the bone environment are usually resistant to current antitumor therapeutic approaches. The only options for these patients are palliative treatments to reduce pain and prevent additional bone destruction.

The presence of $CD8^+$ tumor-infiltrating lymphocytes in primary breast tumors correlates with reduced metastatic invasion and increased overall survival.[91] In this review, we describe several strategies to stimulate therapeutic antitumor cytotoxic T-cell responses. We also discuss that immunosuppressive cells are major obstacles in the development of active immunotherapy for cancer patients. Unfortunately, because of the limited number of orthotopic bone metastatic tumor models available in immunocompetent mice, the role of immunosurveillance in bone metastasis is understudied. There are still major holes in the relevance of specific immune cell types regulating metastasis to bone, especially in breast or prostate cancer. Additional studies are needed to further elucidate the key immunosuppressive mechanisms at play and to identify possible biomarkers that would predict responsiveness to therapy. Completion of this task could lead to the design of appropriate clinical trials based on the identification of immunosuppressive/promoting phenotypes in a particular cancer type.

Conflict of Interest

The authors declare no conflict of interest.

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