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**REVIEW**

# Bone specific immunity and its impact on metastasis

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**Bone is one of the most common sites of metastasis in solid malignancy. Contributing to this osteotropism are the dynamic interactions between tumor cells and the numerous cell types resident in the normal bone, particularly osteoclasts and osteoblasts, which create a tumor supporting microenvironment. However, disseminated cells are detected in the bone marrow long before evidence of metastatic outgrowth, and it is likely that prolonged survival is also reliant on immunoescape. Compared with other peripheral organs such as the lung and spleen, the bone marrow constitutes a unique immune cell compartment that likely provides an immune privileged niche for disseminated tumor cells. This includes the large proportions of immunosuppressive cells, including myeloid derived suppressor cells and regulatory T cells, that blunt the activity of cytotoxic lymphocytes involved in tumor immunosurveillance. This review highlights key aspects of the osteoimmune landscape and emerging mechanisms by which tumor cells create or co-opt an immunosuppressed niche to support their outgrowth in bone. Future studies in this field are likely to shed light on the differences in immunoregulation between the bone and other sites including the primary tumor, and the potential for immunotherapeutics in treating disseminated disease in the bone. However, more immunocompetent models, that recapitulate tumor heterogeneity and bone metastasis need to be developed to accelerate this field.**

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## Introduction

Metastasis, or the spread of a cancer from the site of initiation, is a complex multistep process that inevitably depends on an invasive tumor cell reaching a growth-supporting microenvironment.[1] In line with this, many cancers preferentially metastasize to organs such as the bone, which appears to provide the most congenial 'soil'.[2] In some cancers, such as breast and prostate, the dissemination of tumor cells (DTCs) in bone can occur even before primary tumor diagnosis.[3] In fact, DTCs can be detected in up to 30% of breast and 20% of prostate cancer patients at the time of diagnosis.[4,5] This suggests that the rate-limiting steps in the metastatic cascade occur after extravasation, including the stimulation of angiogenesis and the creation of a favorable, growth-promoting niche. It is also conceivable that evading antitumor immunosurveillance in the bloodstream and/or bone microenvironment is a critical event in bone metastatic outgrowth. For solid malignancies, the ability of disseminated tumor cells to resist anoikis is a major factor in determining their viability once at the metastatic site. However, an emerging paradigm that links transformed and stressed cells to enhanced immunogenicity would suggest that even those that remain viable in the circulation are at risk of detection by the immune system. Although we are only now beginning to understand the importance of the immune system in restraining malignant disease, recent successes with immunotherapies indicate great potential for the field.

Immunosurveillance, a term coined by Burnett and Thomas in 1957, is defined as the ability of immune cells to detect, control and/or eliminate tumor cells. The importance of immunosurveillance in regulating cancer initiation and progression has been demonstrated in a variety of models.[6,7] Although studies that directly demonstrate a role for immunosurveillance in the later steps of metastasis are less common, this field has been hampered by the frequent use of immunocompromised metastasis models. Models that have been extensively used to date to dissect the mechanisms of bone metastasis are experimental and require injection of tumor cells directly into the circulation of immunocompromised hosts. However, recent work by our group and others using immunocompetent and spontaneous metastasis models provide evidence that the formation of bone metastases is indeed influenced by components of the innate and the adaptive immune system.[8,9] In fact, the bone is a unique immune environment that is likely to promote the lodgement and survival of disseminated cancer cells. Many questions remain as to how tumor cells interact with immune cells specifically in the bone microenvironment, and if current therapeutics aimed at targeting bone metastasis

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influence the immune mileu (the latter point as discussed in Capietto and Faccio[10]). This knowledge is critical not only for understanding the mechanisms of metastasis but also for identifying novel prognostic and diagnostic markers and developing new immune-based therapeutic strategies to prevent or treat metastatic bone disease.

## The Bone Microenvironment: A Unique Site for Metastasis

The bone is a common and preferred site for metastasis in a number of cancers, especially those that are associated with long latency periods between primary tumor diagnosis and detection of distant metastases. In fact, the incidence of bone metastasis in advanced breast and prostate cancer patients can be as high as 80% and 90%, respectively (Australian Institute of Health and Welfare, Canberra, ACT, Australia). Reasons for this preference to bone include efficient delivery into the red marrow, chemokine gradients, lodgement in hematopoietic stem cell (HSC) niches[11] and the growth-promoting soil supplied by areas of bone remodeling.[12]

The tightly regulated activity of bone-resorbing osteoclasts and bone-forming osteoblasts is a critical component of bone homeostasis. It is now well known that this process can be corrupted by tumor cells and/or associated immune cell infiltrates to provide a favorable growth environment for bone metastases. The 'vicious cycle' of bone degradation and tumor growth during breast cancer bone metastasis has been extensively studied using intracardiac experimental metastasis models, and recent studies have suggested that the recruitment and activation of osteoclasts is actually a key event in the outgrowth from dormancy.[13] Interestingly, tumor cell-induced immune cell subsets in the bone, such as immunosuppressive myeloid derived suppressor cells (MDSCs), have been demonstrated to directly contribute to osteoclastogenesis via secretion of immunosuppressive cytokines such as transforming growth factor-β (TGF-β).[14]

The specific immune cells and immune-derived signals that may influence tumour cell dormancy and outgrowth independent of the vicious cycle remain largely unknown, owing to the fact that immunocompetent dormancy models are scarce, if not non-existent. Importantly, it is highly likely that immune control in the bone does not always mirror that observed in the primary tumor or other sites of metastasis such as the lung. This was demonstrated by our recent study where enhanced type I IFN signaling in the aggressive 4T1.2 breast cancer model significantly reduced spontaneous bone metastases while having no impact on the occurrence of lung metastases.[15]

In fact, the bone represents a site of particularly dampened immunity that is thought to protect the critical hematopoietic stem cell compartment.[16] In addition to site-specific immune control, it should also be noted that the tumor cells arriving and growing in distant organs may be distinct from the majority in the primary tumor. It is known that the immune system sculpts a neoplasm to become more aggressive and immunoevasive,[17] and the study of clonal evolution and plasticity has highlighted the genetic heterogeneity of tumors and metastases.[18] However, less is known about the immunoreactivity towards these divergent metastatic populations. Taken together, it appears that the bone is a particularly amenable site of metastasis where reduced immunoreactivity may support a less rigorous requirement for tumor cells to become immunoevasive.

## Bone: A Unique Immune Milieu

Many organs display unique populations of resident and recruited immune cells during homeostasis or an immune response. In humans and mice, peripheral blood mononuclear cells consist of 45–75% T lymphocytes, including 25–60% of $CD4^+$ T cells and 5–30% of $CD8^+$ T cells. In the bone marrow, T-cell proportions drop to $<5\%$ of mononuclear cells, with $CD8^+$ T cells more abundant than $CD4^+$ T cells.[19,20] Not only are $CD8^+$ T cells important during antigen-dependent pathogen control, they have been effectively used as an adoptive immunotherapy strategy in patients with cancers such as metastatic melanoma, chronic lymphoid lymphoma and metastatic synovial carcinoma.[21–23] Importantly, the bone marrow is a preferred site for the retention of $CD44^+$ memory T cells that can elicit a potent response upon antigen restimulation and one that is known to induce antitumor activity in patients.[24] However, it is currently not clear if this composition is maintained during, or impacts on, the development of bone metastasis.

Similar to the scarcity of T cells, natural killer (NK) cells represent only 1–2% of lymphocytes in the bone marrow despite it being the major site of their development.[25] The majority of bone marrow-resident NK cells display markers of immaturity,[26] with only a small proportion of terminally differentiated NK cells present in the bone parenchyma and sinusoids.[27] Unlike antigen-specific T cells, NK-mediated elimination of infected, stressed or transformed cells relies upon the sum of activating and inhibitory receptors engaged by ligands on a potential target.[28] Interestingly, NK cells appear to fulfill an important role in 'missing self' recognition of cells that manage to evade $CD8^+$ T-cell immunity through the loss of major histocompatibility complex class I (MHC-I) expression. Conversely, adequate MHC-I expression is known to induce NK cell tolerance, except in situations of strong positive stimuli. Such circumstances include NKG2D recognition of cognate ligands (MICA/B and ULBP1-4 in humans or RAE-1α-ε, MULT-1 and H60 in mice) displayed by transformed cells that lead to perforin/granzyme-mediated cytotoxicity or agonism of the target cell's extrinsic apoptosis (death receptor) pathway.

Numerous non-cytotoxic immune cell populations also reside in the bone, including a group of $CD4^+$ T cells, of which 40% are functional regulatory T cells ($T_{regs}$).[29] $CD4^+$ $T_{regs}$ constitutively express CD25 and the transcription factor FoxP3 and function to keep the immune system in a balanced state by avoiding excessive immunoactivation and immune-mediated pathology, such as autoimmunity. MDSCs constitute another abundant immunosuppressive population in the bone marrow of healthy individuals, representing 10–20% of immune cells. However, during cancer progression this population expands further leading to potent inhibition of $CD4^+$, $CD8^+$ T cells and NK cells[30–32] through multiple mechanisms that have been reviewed elsewhere.[33]

Taken together, the bone appears to have a small pool of effective cytotoxic cells and a relatively large accumulation of immature or suppressor immune cell types. This imbalance is further skewed toward a suppressive state during the development of a malignancy as discussed below and likely

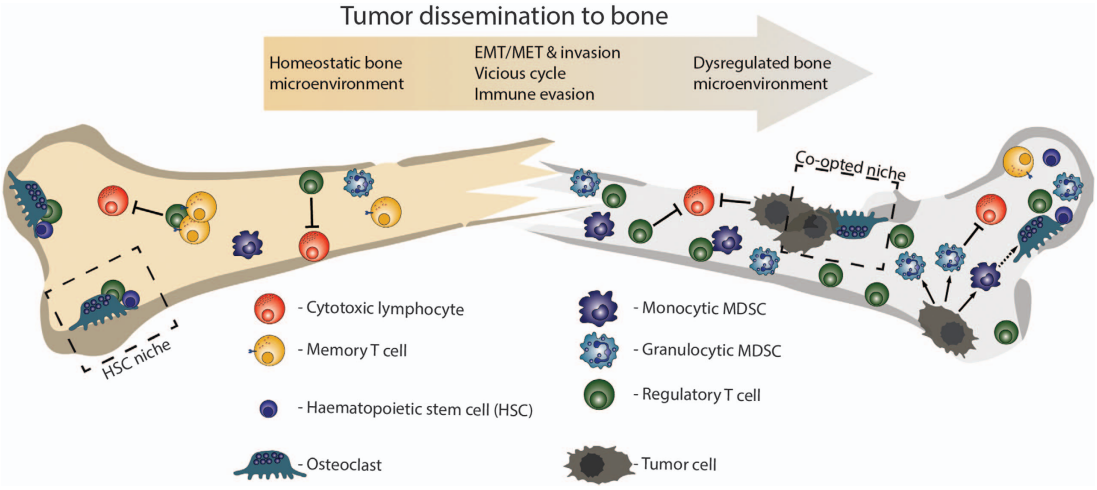


**Figure 1** Broken bones: Tumor cells exploit sites of dampened immunity. The bone microenvironment is a site of tempered immunoreactivity in comparison with many other organs and has been shown to be a particularly congenial 'soil' for disseminated tumor cells. The homeostatic bone marrow contains numerous cell types (myeloid suppressor cells (MDSCs) and regulatory T cells (Tregs)) that act to dampen immunity and protect the hematopoietic stem cell (HSC) population. Co-option of HSC niches and bone remodeling (the vicious cycle) have been suggested to support disseminated tumor cell survival, with metastatic outgrowth aided by the expansion of suppressive cell types to further blunt antitumor immunity.

provides an ideal site to harbor disseminated tumor cells (**Figure 1**).

## Antimetastatic Immune Response

Evidence continues to mount in support of the critical importance of the host immune system in dictating metastatic spread. For example, it is well established that the cytotoxic lymphocytes are crucial in restraining metastasis to multiple organs.[34,35] Studies into the immunoregulation of bone metastasis are less common, although NK cell activity has been shown to underpin the efficacy of a targeted interleukin-2 (IL-2) therapy in a preclinical model of bone metastatic neuroblastoma.[36] Likewise, in our hands, NK cells (along with $CD8^+$ T cells) were necessary for Irf7 (interferon regulatory factor 7)-driven bone metastasis suppression in the immunocompetent 4T1.2 breast cancer metastasis model.[8]

Apart from direct cytotoxicity functions, NK cells also produce a panel of proinflammatory cytokines such as Mip-1α (macrophage inflammatory protein 1α), TNF (tumor necrosis factor), IL-2 and IFN-γ (interferon-). IFN-γ production links the innate and adaptive immune response and leads to $CD8^+$ T-cell, dendritic cell (DC) and macrophage activation and an overall shift to a T helper type 1 immune response. Furthermore, IFN-γ can directly inhibit tumor cell proliferation and synergize with antitumor immunity in experimental models of lung metastasis.[37] Another study further highlighted the pleiotropic roles of IFN-γ in Tax viral oncogene driven malignancy, showing that IFN-γ directly inhibits osteoclast formation and as a result $Tax^+$ IFN-$\gamma^{-/-}$ mice develop significantly higher numbers of osteolytic skeletal tumors.[38] Finally, although some protumorigenic roles of IFN-γ have been reported, its role is likely context dependent and probably beneficial in supporting antimetastatic immunity.

Infiltration of $CD8^+$ T cells into established primary tumors is commonly observed, suggesting that tumor cells are recognized, but not necessarily eradicated. Their role in preventing spontaneous tumor initiation and progression has been documented in mouse models of cancer.[39,40] Evidence, albeit limited, also suggests that $CD8^+$ T cells can reduce tumor burden in the bone in experimental models of melanoma metastasis[41] and impact lung and bone metastasis-free survival in models of spontaneous breast cancer dissemination.[8]

Cytokines well known to stimulate both NK and $CD8^+$ T activity are the type I IFNs. A function of this pathway in restraining metastatic seeding and growth has been supported by the acceleration of B16 lung metastasis[42] and 4T1.2 bone metastasis[8] in mice lacking the type I IFN receptor. However, similar to IFN-γ, the type I IFNs have diverse effects on non-immune cells including osteoclasts that need to be further investigated to fully understand their mechanisms of metastasis suppression.

## Tumor Cell Escape From Immune Surveillance

As stated earlier, it is likely disseminating tumor cells escape immune surveillance to survive in the circulation. The mechanisms by which this occurs are now being uncovered.

One mechanism is that of a physical nature, whereby tumor cells avoid contact with immune cells by forming aggregates with platelets in the bloodstream. Covered by platelets, tumor cells are protected against TNF-α-mediated cell death[43] and perforin /granzyme-mediated NK cell cytotoxicity.[44] Owing to the stressful nature of metastatic dissemination, it is also likely that tumor cells that have acquired resistance to apoptosis are selected for during the metastatic cascade and that these cells are hence resistant to NK cell- or cytotoxic T cell-mediated killing. Recently, Akfirat *et al.*[45] demonstrated an increase in the expression of the prosurvival proteins BCL-2, MCL-1 and survivin-C in bone metastases compared with soft tissue metastases in prostate cancer patients.[45] Interestingly, there was enhanced expression of another prosurvival protein, survivin-N, in soft tissue metastasis, indicating the importance of site-specific survival strategies.

A common direct antigen-dependent immune escape strategy is the partial or complete loss of MHC class I

expression on tumor cells, preventing antigen presentation. In metastatic disease, a total deficiency in MHC class I molecules has been reported in ~44–90% of cases.[46–48] Expression of T-cell-suppressive ligands has also been implicated in immune escape. Certain tumors express programmed death ligand 1 (PD-L1) upon immune attack, which, upon binding to its receptor PD-1 on T cells, inhibits T-cell activation. However, to date the expression of PD-L1 has not been established as a biomarker of bone metastatic relapse. In fact, in a study with 63 melanoma patients, PD-L1 expression could not be correlated with progressive metastatic disease,[49] highlighting that immune escape strategies are likely to be cancer-type-, tissue site- and patient-dependent.

Our work has implicated suppression of type I IFN signaling as an immune escape mechanism. In both bone metastatic mouse models and in breast cancer patient cohorts, the loss of a tumor cell inherent type I IFN signature was associated with an increased risk of bone metastasis.[8] We demonstrated that restored type I IFN secretion from tumor cells suppressed bone metastasis via an NK and $CD8^+$ T-cell-dependent mechanism,[8] suggesting that loss of this pathway was a possible mechanism of immune escape during dissemination.

Taken together, these studies suggest that escaping immune control is a critical component of dissemination and metastatic growth. Studies in this area will likely uncover numerous other immune escape mechanisms that will have important implications in response to immunotherapies.

### Indirect Effects on the Immune Milieu

Apart from influencing the primary tumor microenvironment, it is now known that tumor cells can induce an immunosuppressed microenvironment in metastatic sites before their arrival via the secretion of immunosuppressive cytokines. For example, tumor cell and bone cell secretion of TGF-β has been associated with immunosuppression in the tumor microenvironment. Apart from its well-known role in promoting bone resorption during the 'viscous cycle',[50] TGF-β suppresses the immune response by repressing the production of effector molecules such as perforin, granzymes, Fas-ligand and IFN-γ by cytotoxic immune cells[51] and by downregulating the expression of the NKG2D receptor on NK cells in numerous human cancers.[52] Additionally, TGF-β targets the maturation of DCs and the antitumor functionality of macrophages and neutrophils.[53,54] In fact, 75% of human breast cancer bone metastases have been demonstrated to have functional and active TGF-β signaling.[55] In line with the important roles of TGF-β in bone remodeling and immune suppression, blocking TGF-β signaling in models of preclinical models of breast cancer and melanoma markedly decreased the incidence of bone metastasis in immunocompetent[56] as well as in immunocompromised models.[57,58] As recently reviewed by Buijs *et al.*,[59] several agents that target TGF-β signaling show promise in preclinical models and may be promising agents that warrant future clinical trials in patients with skeletal metastasis.

A hallmark of advanced bone metastasis is the prevalence of immunosuppressive cell populations such as MDSCs and $T_{regs}$. During tumorigenesis, the secretion of several factors such as IL-4, IL-13, VEGF (vascular endothelial growth factor), GM-CSF (granulocyte–macrophage colony-stimulating factor), GCSF (granulocyte-colony-stimulating factor) and TGF-β leads to the expansion, activation and recruitment of MDSCs. In patients with metastatic bone disease, the fraction of peripheral MDSCs can rise from <2% up to 25%. This is also observed in aggressive bone metastasis models such as the 4T1.2 model, where MDSC proportions can rise to over 65% of the leukocyte population.[60] Not only do MDSCs have multiple roles in immune suppression, they also promote the expansion of $T_{regs}$ and support tumor progression by promoting extravasation of metastatic cells via vascular remodeling, polarization of macrophages into a tumor-promoting M2 phenotype and simulating osteoclastogenesis.[33] In fact, MDSCs have been demonstrated to differentiate into functional osteoclasts,[61,62] cells well known to contribute to tumor cell growth in bone.

Elevated $T_{regs}$ are also associated with metastatic progression and tumor immunosuppression.[63,64] In prostate cancer patients bearing bone metastases, $T_{regs}$ accumulate at higher levels than in healthy individuals,[65] suggesting an important role in metastasis. Likewise, patients with stage IV metastatic breast cancer have more circulating $T_{regs}$ than stage I, II or III patients that do not have distant metastases.[64] Their accumulation in bone is likely to interfere with immunosurveillance by secreting the immunosuppressive cytokines IL-10, TGF-β and RANKL (receptor activator of nuclear factor κB ligand). $T_{regs}$ have also been implicated in bone remodeling, with reports of stimulating osteoclastogenesis via secretion of RANKL,[63] or conversely suppressing osteoclast differentiation and function, and promoting osteoblastic lesions.[65,66]

This work suggests that although the bone already represents an immune privileged site, tumor cells can further skew the balance of immune effector and suppressor cells towards an immunosuppressed niche to promote their outgrowth in bone (**Figure 1**).

### Conclusion

The bone is a unique microenvironment that appears to foster the dormancy, survival and outgrowth of disseminated cells. Mechanisms other than the well-established 'vicious cycle' that regulate bone metastasis are scarce; however, an emerging body of evidence suggests that tumor cells exploit the bone marrow's immunoprivileged status to evade antitumor immunity. As a consequence, it is tempting to speculate that the reduced selective pressure for immune evasive clones in the marrow effectively increases the pool of suitable 'seeds' in the circulation, contributing to the relatively high clinical incidence of bone metastases. It is also apparent that tumor cells exaggerate the immunosuppressed environment via direct and indirect mechanisms to allow their progression from a dormant or micrometastatic state to an overt clinically detectable metastasis. Understanding those mechanisms used by disseminated tumor cells to escape dormancy will be critical for not only predicting metastatic progression in patients but also in the development of immune-based therapeutics aimed at preventing the formation of overt metastases in bone. The future of this field relies on the development of spontaneous metastasis models that incorporate models of dormancy and retain tumor–immune cell interactions.

### Conflict of Interest

The authors declare no conflict of interest.

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