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Bone Metastasis Diminishes Extraosseous Responsiveness to Checkpoint Blockade Immunotherapy through Osteopontin-producing Osteoclast

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SUMMARY

Bone metastatic lesions typically associate with suboptimal responses to immune checkpoint blockade (ICB) therapies. In this study, we observed that across multiple clinical cohorts and a variety of mouse models, the presence of osseous metastases induces ICB resistance in extraosseous tumor. Mechanistically, this long-distance communication is mediated by osseous tumor-conditioned osteoclasts producing osteopontin (OPN). Through circulation, OPN reprograms the extraosseous tumor microenvironment and impairs T cell recruitment and differentiation of $CD8^{+}TCF1^{+}$ precursor cells, an essential population for ICB efficacy. In mice, ICB responsiveness is restored by αRANKL blockade of osteoclastogenesis, neutralization of OPN in circulation, or tissue-specific depletion of OPN in osteoclasts. Both the mode-of-action and therapeutic benefit were validated in clinical cohorts with the αRANKL-ICB combinatory regimen. These findings establish bone as a specific immunoregulatory organ exploited by tumor metastasis, and suggest osteoclastogenesis as a promising target to improve ICB prognosis in patients with bone metastasis.

INTRODUCTION

Therapeutic advances in immune checkpoint blockade (ICB) treatments have rapidly emerged within the past few years. Despite the successful application of ICB immunotherapy across multiple solid tumor types, the durable survival benefit remain limited to a minority of patients.[1,2] It has been the focus of the field to identify mechanistic-driven biomarkers to predict patients outcome; and, several prognosis biomarkers were generated to reflect tumor-intrinsic resistance,[3,4] tumor type-specific[5,6] or mutation-specific[7,8] tumor immune microenvironment (TIME), or systemic impact from comorbidity.[9] However, even in biomarker-stratified patient populations, mostly based on primary tumor analyses, responsiveness to ICB treatment remains generally unsatisfactory. This suggests that sophisticated interplays between tumor and host immunity need to be interpreted in the context treating the whole body as an integrated system.

Recently, one more layer of complexity was added to the picture. Especially for patients with metastases, the specific organ in which the metastasis resides was discovered as a crucial factor for ICB prognosis. Tumor cells actively adapt to the microenvironment of different resident organs, resulting in heterogeneity in the malignant phenotype and treatment responsiveness.[10] Tumors metastasizing to the liver are generally more life-threatening and likely to resist therapy, whereas those metastasizing to lymph nodes are more likely to exhibit a strong response to ICB immunotherapy.[11-14] Studies focusing on impacts associated with metastasis to organs have provided insights into the immune-related mechanisms responsible for disparities in ICB treatment response.[15-17] Therefore, the concept of organ-specific regulation is increasingly appreciated in the field of cancer immunology, especially as it relates to ICB immunotherapy.[18]

Bone is a particularly prevalent organ for metastases, especially in breast, lung, or prostate carcinoma.[19] Clinical trials have suggested bone metastasis as a prognostic factor for unfavorable clinical outcomes in patients treated with ICB.[20,21] Several studies have characterized a distinct tumor microenvironment (TME) in bone metastatic lesions.[22,23] Specifically, TGF-β released during bone resorption inhibits differentiation of Th1 lineage cells while promoting Th17 polarization and resistance to ICB treatment.[22] Bone metastatic lesions are also characterized by enrichment with CCL20-producing inflammatory monocytes and dysfunctional $CD8^+$ T cells. Disruption of the CCL20/CCR6 axis can reduce T cell exhaustion and extend survival time after ICB treatment.[23] Notably, recent work has revealed that both extraosseous tumors[24,25] and bone metastasis[26] may alter hematopoiesis in the bone to favor immune tolerance, extending the scope of osteo-immune interplay to extraosseous sites.

As the pivotal site of hematopoiesis, osteoclasts, osteoblasts, and osteocytes shape an osteo-immune environment. In both physiological homeostasis and pathological inflammation, these cells respond to stimuli and regulate the hematopoiesis outcome.[25]

In this study, we explored the interplay between bone metastases and osteoclast, and the remote impact on the extraosseous tumor response to immunotherapy. We found that bone metastasis diminished ICB treatment response in extraosseous solid tumors. Mechanistically, we identify osteopontin (OPN)-producing osteoclasts as essential mediators of extraosseous ICB resistance. Furthermore, using preclinical models and data from multiple clinical cohorts spanning several tumor types and treatment regimens, we show that an osteoclastogenesis-blocking strategy can restore ICB treatment responsiveness in mice and in human patients.

RESULTS

Bone metastasis predicts unfavorable therapeutic outcomes and reduced extraosseous tumor responses to ICB

To investigate how metastases in different organs affect the responsiveness to ICB immunotherapy, we first examined the metastatic organ-specific survival in two independent pan-cancer cohorts of patients with metastatic solid tumor who received ICB treatment: AMU1 (N = 927) and AMU2 (N = 343) (**Supplementary Table 1**). We observed that progression-free survival (PFS) times varied according to the metastatic organ **(Supplementary Figure 1A, 1B)**. Since the PFS is not a measurement limited to the existing metastases rather mostly related to the primary tumor changes, this strongly suggests that the site of metastasis has differential and systemic impact on ICB efficacy. While the remote immunosuppression of liver metastasis was reported previously,[26] we also observed a strong prognosis differentiation made by bone metastasis in comparison to the cohort average (AMU1, $^{**}p = 0.003$; AMU2, $^{**}p = 0.009$, **Supplementary Figure 1A, 1B**), or by grouping patients based on the presence or absence of bone metastasis (**Figure 1A, 1B**).

To rule out potential confounding factors of primary tumor bias, we extended our analysis to single tumor type cohorts: TUSM1, a non-small cell lung cancer (NSCLC) real-world study cohort with 280 patients; TUSM2, an ICB trial against liver-metastatic NSCLC cohort with 48 patients; and TUSM3, an NSCLC ICB monotherapy trial with 70 patients (**Supplementary Table 1**). All analyses confirmed that the presence of bone metastasis was consistently associated with faster progression in ICB-containing treatments **(Figure 1C-1E)**. Stratified analysis showed that bone metastasis is generally correlated with decreased PFS regardless of age, gender, and treatment regimens, as well as combination of metastases in other organs **(Figure 1F-1H)**. Notably, such correlation was not observed in bone metastasis patient cohorts receiving chemotherapy alone: no matter in pan-cancer cohort (AMU4, N = 81), NSCLC cohort (AMU3, N = 237); or a small cell lung cancer cohort (AMU5, N = 71) **(Supplementary Figure 2)**. This suggested that bone metastasis stratifies patient outcome is in the context of immunotherapies.

In all metastatic sites associated with shorter than average PFS, bone is the only

residency organ that metastases are unlikely life-threatening. We further evaluated the bone metastasis impact on ICB in overall survival (OS) data-available cohorts: TUSM2, TUSM3, and NM2019 (a published melanoma cohort with 144 patients),[27] and observed a clear separation of patients' outcomes **(Figure 1I-1K)**. Generic inverse variance meta-analysis also confirmed that baseline bone metastasis was consistently associated with shorter survival times across multiple cohorts (**Figure 1L**).

We next analyzed objective response rate (ORR), a standard measurement of tumor shrinkage according to RECIST 1.1 criteria.[28] Since, following such guidelines, objective response reflects the change in the sum of the diameters of measurable lesions, but bone lesions are almost unmeasurable, the ORR in patients with bone metastasis largely reflect responses in extraosseous lesions. Across all five ORR data-available cohorts, we observed that baseline bone metastasis is correlated with ORR to ICB therapies: it was consistently associated with higher frequency of progressive disease (PD), and fewer patients achieving partial or complete response **(**PR/CR, **Figure 1M)**. A pooled analysis also confirmed that the ORR to ICB therapies was significantly lower in patients with bone metastasis **(Figure 1N)**.

The extent of tumor shrinkage could serve as a proxy for long-term treatment outcomes against solid tumors.[29] We focused on the burden of extraosseous lesions in the AMU6 (pan-cancer, N = 40) and AMU7 (pan-cancer, N = 59) cohorts to directly assess the long distance impact of bone metastasis (**Figure 2A; Supplementary Table 1**). In AMU6 cohort, we found that the extraosseous tumor burden was significantly reduced after ICB treatment in patients without bone metastasis (**Figure 2B, left panel**), whereas no significant tumor shrinkage was observed in ICB-treated bone metastatic patients (**Figure 2B, right panel**). Accordingly, patients with bone metastasis who received ICB treatment showed substantially less changes in extraosseous tumor burden than those without bone metastasis (**Figure 2C**). This reduced shrinkage of extraosseous tumors was also observed in the larger sample size of AMU7 cohort (**Figure 2D, 2E**).

To evaluate whether the impact of bone metastasis on extraosseous tumor response to ICB therapies is organ-specific, we reviewed radiological follow-up data for patients with lung as the primary tumor site in cohort TUSM2. In this 48 NSCLC patient cohort , we found significantly less tumor shrinkage in lesions located in the lungs as primary lesions (N = 48, $p = 0.050$), intrapulmonary metastasis (N = 20, $*p = 0.023$), livers (N = 47, $*p = 0.042$), lymph nodes (N = 13, $*p = 0.030$), and pleural nodules (N = 9, $*p = 0.029$) **(Figure 2F)**. Collectively, these findings suggest that bone metastasis may lead to unfavorable therapeutic outcomes in patients receiving ICB immunotherapy through a systemic impact on extraosseous tumors.

The presence of osseous tumors diminishes extraosseous αPD-L1 response in mice

To examine the causality between the bone metastasis and potential extraosseous

resistance to ICB therapies, we set up a mouse model of bone metastasis (**Supplementary Figure 3A)**. Following protocol developed previously,[22] tumor cell suspension was injected into the proximal tibia cavity through a plateau (intratibial injection; designated as IT mice). *In vivo* bioluminescence imaging (BLI) confirmed the formation of osseous tumor colony at 10 days post transplantation in 100% tested animals **(Supplementary Figure 4)**. Micro-computed tomography (μCT) was employed to collect osseous tumor image and followed by 3D reconstruction **(Supplementary Figure 3B-3E)**. The imaging data revealed typical radiological manifestations of bone metastasis in our model: In limbs with intratibially implanted tumor cells but not with saline controls, defects developed in cortical bones and followed by erosive bone remodeling **(Supplementary Figure 3C-3E)**. H&E staining confirmed the establishment of an osseous lesion **(Supplementary Figure 3F, 3G)**.

Simultaneously, tumor cells (B16F10-luciferase melanoma shown as an example in **Figure 3A**) were implanted in IT mice subcutaneously as an extraosseous tumor (designated as SC+IT mice). Subcutaneous tumor-only mice (designated as SC mice) serve as controls. BLI showed that, compared to the isotype control group, αPD-L1 treatment resulted in a significant reduction of tumor burden in SC control mice **(Figure 3B)**. The therapeutic effect was not observed for osseous tumors in SC+IT mice, consistent with previous magnetic resonance imaging-based evaluation of osseous tumor burden.[22] Notably, subcutaneous tumors in SC+IT mice also showed no significant response to αPD-L1 treatment, recapitulating extraosseous resistance to ICB therapies in patients **(Figure 3C)**. The osseous tumor induced ICB resistance in subcutaneous tumor was also observed in B16F10-OVA, MC38 and CT26 colorectal carcinoma, and LLC-OVA NSCLC models **(Figure 3D, 3E, 3G** and **Supplementary Figure 5A-5C)**. Consistently, shortened survival was observed in all aforementioned models **(Figure 3F, 3G** and **Supplementary Figure 5D-5F)**. We also used B16F10-OVA model to study extraosseous ICB resistance in lung metastasis. In the pulmonary metastasis model, B16F10-OVA cells were injected intravenously **(**designated as IV+IT mice, **Figure 3H)**. While nodule formation was significantly reduced upon αPD-L1 treatment in osseous tumor-free IV control littermates, pulmonary nodule formation showed no obvious response in αPD-L1-treated IV+IT mice **(Figure 3I)**.

To rule out the possibility that the diminished αPD-L1 response in extraosseous tumors is purely due to increased tumor burden from forming osseous lesions, we compared mice with unilateral subcutaneous MC38 tumors to those implanted bilaterally (**Figure 3J**). In the bilateral implantation group, we adjusted the cell number so that the pre-treatment subcutaneous tumors reached a similar burden as the intratibially injected osseous tumors (**Figure 3K, 3L**). Upon treatment with the αPD-L1 antibody, we observed that both tumors implanted bilaterally responded efficiently, similar to unilaterally implanted ones. Additionally, doubling the baseline number of tumor cells did not impair the treatment efficacy (**Figure 3M**). Taken together, we reasoned that the suppression of extraosseous responses to ICB by osseous tumors is not due to the increased tumor burden.

Next, to investigate whether osseous tumor-associated ICB resistance is tumor antigen-specific, in mice bearing osseous B16F10 tumors, we subcutaneously implanted either MC38 or E.G7-OVA tumors, both of which are highly sensitive to ICB **(Figure 3N)**. The subcutaneous tumors, whether MC38 **(Figure 3O)** or E.G7-OVA **(Figure 3P)**, resisted to αPD-L1 treatment. This observation suggests that the immunosuppression generated by osseous tumors is unlikely limited to osseous tumor-specific antigen, but rather affects a much broader range of anti-tumor immunity.

Osseous lesions impair CD8$^+$ T cell immunity in extraosseous tumor

To investigate the effects of bone metastasis on extraosseous tumors, we harvested subcutaneous tumors from SC or SC+IT mice 10 days post tumor implantation for RNA-seq to comprehensively explore the TIME. Our Immune Map analysis[30] showed that, prior to any treatment, the bone lesions generated a "cold" TIME at baseline, indicating an unfavorable TIME to ICB therapies (**Figure 4A**). Specifically, focusing on a well-established 18-gene signature predicting clinical ICB responsiveness,[31] we observed a broad suppression of expression (**Figure 4B**). Gene set enrichment analysis (GSEA) against three clinically validated ICB-responsive signature panels — CheckMate-275,[32] POPLAR,[33] and KEYNOTE-028[34] — confirmed that the IT-implantation-generated bone lesion produced an extraosseous TIME less sensitive for ICB responses (**Figure 4C**). GSEA further revealed that the most significant suppression of the baseline transcriptome involved genes related to pro-inflammatory responses, such as IL2-STAT5 signaling and interferon-α/γ responses, which are critical for the responsiveness to ICB therapies in extraosseous tumors **(Figure 4D)**.

To depict osseous lesion-induced suppression of the extraosseous TIME at the cellular level, we employed mass cytometry (CyTOF) and multi-dimensional scaling (MDS) analysis, which separated the subcutaneous tumors between groups with or without IT implantation (**Figure 4E**). A total of 423,722 intratumoral CD45$^+$ cells were clustered in an unsupervised manner into 10 populations (**Figure 4F, 4G**). Overall, the TIME of SC mice was enriched with cell populations associated with anti-tumor immunity, such as CD8$^+$ T cells, CD4$^+$ T cells, and NK cells, whereas pro-tumor immunoregulatory cells, such as macrophages and M-MDSCs, were augmented in the TIME of SC+IT groups (**Figures 4H, 4I**). Principal component analysis (PCA) further validated this bifurcational polarization of lymphoid versus myeloid enrichments in the SC versus SC+IT TIME, respectively (**Figure 4J**).

With the CyTOF data, we further analyzed the CD8$^+$ T cell phenotypes. Within the CD8$^+$ population, we detected that IFN-γ-producing cells and progenitor exhausted T cells (T_{pex}, CD8$^+$CD44$^+$PD-1$^+$TCF-1$^+$TIM-3$^-$) were reduced in the SC+IT TIME **(Figure 4K)**. It has been well-documented that T_{pex} cells are the dominant population responding to ICB treatment for proliferation and prolonged anti-tumor efficacy.[35,36] Since the overall infiltration of CD8$^+$ T cells was suppressed in the SC+IT TIME, other

subgroups, including early reactivated ($CD28^+CD69^+$) and proliferating ($Ki\text{-}67^+$) $CD8^+$ T cells, were also reduced in absolute numbers **(Figure 4L)**. All these observations were subsequent confirmed by independent experiments with flow cytometry analyses **(Figure 4L** and **Supplementary Figure 6)**. These data suggest that osseous lesions remotely reprogram the extraosseous TIME to diminish $CD8^+$ T cell infiltration and effector differentiation necessary for ICB responses.

Osteoclast mediates extraosseous ICB resistance

We hypothesized that the reprogramming of the extraosseous TIME occurs through remote communication with the osseous lesion. To test this hypothesis, we collected serum from tumor-free, SC, or IT mice donors and subsequently transferred it to recipient mice with subcutaneous tumors only. αPD-L1 antibody or the isotype control was concurrently administered to the serum recipients **(Figure 5A)**. Sera collected from mice carrying bone tumors, but not from tumor-free or subcutaneous tumor-bearing donors, recaptured the bone lesion effect in diminishing subcutaneous tumors' response towards αPD-L1 treatment, in both B16F10-OVA **(Figure 5B)** and MC38 **(Figure 5C)** tumor model. Furthermore, we transferred sera from donors carrying B16F10-OVA bone tumors to MC38-bearing recipients **(Figure 5D)**. Sera from mice carrying unmatched bone tumors also significantly attenuated the MC38 tumor's response to αPD-L1 **(Figure 5E)**, which was consistent with observed ICB resistance in s.c. tumors derived from unmatched osseous lesions **(Figure 3N-3P)**.

To identify signals sent to extraosseous tumors that attenuate ICB responsiveness, we performed quantitative proteomic profiling on serum from IT, SC, and tumor-free mice. PCA analysis unbiasedly grouped the sera from these sources into three distinct clusters **(Figure 5F)**. Compared to tumor-free sera, we detected elevated levels of 259 proteins and decreased levels of 94 proteins in sera from IT mice. In comparison to sera from SC mice, despite both donors carrying the same type of tumor, the osseous location of the tumors reshaped the sera protein spectrum by upregulating 161 proteins and downregulating 163 proteins **(Figure 5G, 5H** and **Supplementary Figure 7)**.

In this dataset, we observed an enrichment in IT mice sera of proteins promoting bone remodeling (**Supplementary Figure 7F)**. Bone remodeling is a hallmark of established bone metastasis,[37,38] and coordinated by the activity between osteoblast and osteoclast.[39] In mice bearing osseous or subcutaneous tumors, the difference in serum composition tips the balance between osteoblast and osteoclast: an over-representation of osteoclastogenesis-promoting molecules such as Fbn1 and Rhoa in the sera from IT mice, and an over-representation of osteoblast-favoring molecules such as Clic1, Ltf, and Igf1 in the SC sera **(Figure 5I)**. This serum profile predicts bone loss.

We evaluated osseous tumor-related bone disruption *in vivo* with a fluorescent bisphosphonate derivative, OsteoSense 750EX, to label active bone mineralization. Fluorescence molecular tomography analysis of the tibial plateau showed substantially

reduced hydroxyapatite deposits in limbs implanted with B16F10-OVA **(Figure 5J)** or MC38 **(Supplementary Figure 8A)** tumors. The heightened osteoclast activity was also reflected by the substantial loss of bone mineral density in the tumor-bearing limbs **(Supplementary Figure 8B, 8C)**.

To establish the contribution of osteoclast to the ICB resistance of extraosseous tumors, as previously described,[40,41] we differentiated and matured bone marrow mononuclear cells into osteoclasts in culture. The osteoclast-conditioned medium was collected and intraperitoneally administered to subcutaneous tumor-bearing recipients **(Figure 5K)**. TRAP staining confirmed the functional maturation of cultured osteoclasts **(Figure 5L)**. Analysis of subcutaneous tumor progression showed that osteoclast-conditioned medium largely blunted responsiveness to αPD-L1 blockade in both B16F10-OVA **(Figure 5M)** or MC38 **(Figure 5N)** tumor-bearing recipients. These data collectively suggested that the signal remotely reprogram the ICB responses of extraosseous tumors may be generated by osteoclasts.

Overproduction of Osteopontin by tumor-conditioned osteoclasts induces ICB resistance

As a subclass of macrophage, the phenotypic plasticity of osteoclasts is well-documented,[42] prompting us to investigate whether osteoclasts have unique traits in the presence of osseous tumors. To signify the effects of tumors and avoid the confounding influence of other environmental factors *in vivo*, we employed a reductionist approach to characterize *in vitro* differentiated osteoclasts in the absence or presence of tumor-conditioned culture medium **(Figure 6A)**. RNA-seq analysis revealed that tumor-conditioning substantially reprogramed the osteoclast transcriptome, with significant upregulation of 3,467 genes **(Figure 6B)**. Intersecting this gene set with the serum proteomics dataset identified 19 overlapping candidates whose mRNAs were upregulated in the tumor-conditioned osteoclast culture and whose proteins were over-represented in the serum of bone tumor-carrying mice **(Figure 6C).** Among them, *Spp1*, which encodes osteopontin (OPN) in both *Homo sapiens* and *Mus musculus*, is the only secreted protein and has been previous reported to reprogram the TME.[43,44]

We confirmed that adding tumor cell-conditioned medium significantly upregulated *Spp1* mRNA and effectively increased OPN protein secretion in both bone marrow-derived osteoclasts **(Figure 6D)** and isolated primary osteoclasts **(Figure 6E)**. Additionally, we confirmed that establishing intratibial tumor elevates OPN concentration in sera **(Figure 6F)**. It has been previously reported that deletions of the *Spp1* gene in both tumor and recipient mice leads to intratumoral immune activation.[43] GSEA analyses with RNA-seq data collected from subcutaneous tumors accompanied by or without bone lesions (**Figure 4A**) against a published dataset of OPN-sufficient versus OPN-deficient tumors[43] showed concordant suppression of genes involved in T cell proliferation, IFN-g production and responses, as well as anti-tumor cytotoxicity **(Figure 6G)**.

To validate the contribution of OPN to extraosseous ICB resistance, we neutralized OPN in serum from IT donor mice using an antibody, then transferred the treated serum to SC recipient mice **(Figure 6H)**. During αPD-L1 treatment, the resistance generated by OPN-neutralized IT mouse sera was significantly reduced **(Figure 6I)**. Additionally, the direct combination of αOPN neutralizing antibody with αPD-L1 **(Figure 6J)** significantly sensitized the subcutaneous tumor of mice bearing bone lesions to benefit from ICB treatment **(Figure 6K)**.

Since OPN can be expressed in a wild range of cells,[45] we investigated whether osteoclast-produced OPN alone was sufficient to diminish extraosseous ICB responses. Osteoclast-specific OPN depletion was achieved in mice through cathepsin K (Ctsk)-driven Cre expression[46] in mice with *Spp1* alleles conditioned **(Figure 6L)**. Osteoclast-specific OPN depletion significantly decreased serum OPN concentration at 10 days after SC+IT implantation (prior to αPD-L1 administration), suggesting osteoclast as a major producer of OPN in bone tumor-bearing model **(Figure 6M)**. In comparison to control mice ($Spp1^{f/f}$), extraosseous tumor responses to αPD-L1 in the presence of bone tumor were significantly improved in *Spp1*-deleted mice ($Ctsk^{Spp1}$) **(Figure 6N)**, and survival time length was extended **(Figure 6O)**. FACS analyses of extraosseous tumor-infiltrating cells showed improved baseline $CD8^+$ T cell infiltration, including those surrogate tumor antigen-specific (Ova-tetramer$^+$) T cells in $Ctsk^{Spp1}$ mice. Additionally, we observed heightened $CD8^+$ T cell proliferation, increased IFN-g and TNF-a-producing effector T cell, as well as enhanced differentiation of T_{pex} cells **(Figure 6P, 6Q)**.

To further characterize the features of intratumoral $CD8^+$ T cells, we performed scRNA- and scTCR paired-sequencing with $CD8^+$ T cells collected from treatment-naïve tumors hosted by $Ctsk^{Spp1}$ or $Spp1^{f/f}$ mice. After filtering, a total of 16,878 $CD8^+$ T cells with transcriptomic data passed quality control and were categorized into four general phenotypes: non-exhausted, progenitor of exhaustion, exhausted, and proliferating cells **(Figure 6R** and **supplementary Fig. S9)**. In agreement with FACS assays, we observed an increase in T_{pex} cells in s.c. tumors from SC+IT $Ctsk^{Spp1}$ mice and a reciprocal decrease in exhausted T cells (T_{ex}) in percentage **(Figure 6T, 6U)**.

Within these 16,878 cells, TCRs were cloned for 12,715 cells and 2,600 clonotypes were identified, allowing us to track T cell differentiation at the individual clone level. For all 2,600 clonotypes of TCRs, we compared their relative T_{pex} to T_{ex} ratio. Overall, this ratio increased more than twofold upon deletion of the *Spp1* gene in hosts' osteoclasts **(Figure 6V)**. Zooming in on those T_{pex} cells, we found that, upon OPN depletion, the frequency distribution of the T_{pex} repertoire was more dominated by highly expanded clonotypes (frequency $\geq 0.5\%$[47]), showing more efficient clonal expansion of this critical subset determining the ICB efficacy **(Figure 6W)**. Overall, these findings indicated that OPN produced by osteoclasts is the major signal sent by bone lesions to reinforce extraosseous tumor resistance to αPD-L1 treatment.

αRANKL-mediated blocking of osteoclastogenesis improves extraosseous tumor response to ICB in patients with bone metastasis

Since tumor-conditioned osteoclasts are the major source of OPN, we reasoned that targeting osteoclastogenesis may relieve ICB resistance in the extraosseous tumor. RANK-RANKL signaling is essential for osteoclastogenesis,[48] and αRANKL-neutralizing antibody efficiently blocks osteoclast differentiation and maturation *in vivo*.[49] Therefore, we evaluated a therapy strategy combining RANKL blocking and αPD-L1 treatment for mice with subcutaneous tumors and bone lesions **(Figure 7A)**. The application of αRANKL is able to sensitize ICB responses in subcutaneous tumors in the presence of bone lesion **(Figure 7B)**. When B16F10-OVA cells were intravenously transferred **(Figure 7C)**, the same strategy also effectively reduced lung nodule formation **(Figure 7D)**. At the molecular level, in mice treated with αRANKL, either as monotherapy or in combination with aPD-L1, we observed that OPN concentrations are significantly reduced in sera, in accordance with our proposed mode of action **(Figure 7E)**.

While either RANKL or OPN neutralization can sensitize extraosseous ICB treatment, denosumab, a RANKL-neutralizing antibody, is clinically approved for patients with osseous lesions to mitigate bone resorption and destruction.[50] Therefore, a significant portion of ICB-treated cancer patients are also treated with Denosumab. In our retrospective cohort AMU8 **(Supplementary Table 1)**, a pan-cancer cohort of bone-metastatic patients under ICB therapies, two types of drugs were prescribed for patients' bone protection: denosumab (advanced treatment) or bisphosphonate (standard of care). Patients with concurrent treatment with denosumab and ICB showed a significant reduction in extraosseous tumor burden, while bisphosphonate failed to deliver such efficacy **(Figure 7F, 7G)**. The efficacy of denosumab/ICB regimen was confirmed in a similar bone-metastatic pan-cancer cohort, AMU9, with a larger sample size **(Figure 7H, 7I)**. This enhanced anti-tumor responses indeed delivered significant clinical benefit: the PFS in patients treated with the denosumab/ICB regimen was doubled **(Figure 7J)**.

In a specific case from patient with metastatic nasopharyngeal carcinoma, initially supported by bisphosphonate, the patient resisted three cycles of αPD-1 treatment with rapid progression of lung and liver lesions. After switched the regimen to denosumab plus αPD-1, extraosseous lesions stabilized after four rounds of treatment, and a 50.4% tumor reduction was achieved after eight cycles **(Figure 7K)**. These data provide clinical validation supporting a positive association between RANKL inhibition and increased treatment response in extraosseous tumors, improving ICB efficacy in patients with bone metastasis.

We also investigated the correlation between bone metastasis and levels of OPN in the sera, as well as its clinical relevance. While established tumors generally elevated

OPN levels in sera, patients with bone metastasis had significantly higher elevations **(**cohort AMU10, **Figure 7L)**. Moreover, stratified analysis indicated that increased OPN concentration during ICB treatments was associated with reduced extraosseous tumor shrinkage **(Figure 7M)** and poorer overall prognosis, measured by PFS **(Figure 7N)**. Furthermore, a high baseline level of OPN in patient sera itself predicted unfavorable PFS outcomes in ICB immunotherapy **(Figure 7O)**. These results support the serum concentration of OPN as a mechanism-driven biomarker for the prognosis of ICB treatment in patients with bone metastasis.

DISCUSSION

Although correlations between sites of metastasis and prognostic cancer outcomes have been extensively reported,[10,14,51,52] the impact of recipient organs for metastases has been barely considered in current therapeutic paradigms. An adequate immune response demands efficient communications and coordination among a network of cells and organs. Since breaking or hijacking such communication is an effective route for immune escape,[24,53,54] in the era of immunotherapy, we are obligated to contemplate anti-tumor immunity in the whole body: Tumor invasion could alter the immunoregulatory function of recipient organ and therefore reshape body's immunity systemically.

This underlying principle triggered our studies to investigate more than 3,000 cancer patients with various types of primary tumor and metastatic sites across multiple ICB-treated cohorts. In organs with high metastasis prevalence and as part of immune system, patients' clinical outcomes were significantly affected by metastasis in liver and bone. It has been elegantly demonstrated that metastases in the liver extensively reprogram anti-tumor responses by enlisting macrophage for tumor antigen-specific T cell elimination,[26] and/or through differentiation of tumor antigen-specific regulatory T cells.[55] Although similarly impactful to ICB resistance of primary tumors, metastasis in the bone utilize a distinct mechanism to promote remote immunosuppression. This suppression is not restricted to the existing anti-tumor T cell immunity; rather, it reprograms the TIME of primary tumor remotely by tumor-conditioned osteoclasts. The activation of osteoclasts for the remolding the bone tissue is necessary to accommodate metastatic tumor in the bone.[56] Metastatic tumors further exploited osteoclast activation to generate a primitive mechanism to suppress attacks in their primary origin. This suppression is less sophisticated than those discovered for live metastasis, however, it has a wider range and is less dependent on tumor antigen presentation at the metastatic site.

As the host for hematopoiesis and the birthplace of major part of immunity, recent studies have implicated that bone structural cells are integral parts of immunoregulation. It has been shown that ablation of osteocytes leads to severe lymphopenia;[57] osteoblasts supply the differentiation of Siglec-F^{high} neutrophil to facilitate lung tumor growth;[53]

and, apoptotic bodies generated by the physiological turnover of osteoclasts impair local naïve T cell priming, a mechanism favoring bone metastasis of breast cancer.[58] All these bone niche-dependent regulations have systemic implications for anti-tumor immunity. What we discovered here through unbiased proteomics and single-cell transcriptomics approaches is a more classical way of communicating—local tumor-conditioned osteoclasts send protein messengers, OPN, through circulation.

OPN is a multifunctional immunoregulator secreted by a variety of cellular sources, including T cells, NK cells, macrophages, tumor cells and osteoblasts.[45] Its intracellular and secreted isoforms have distinct functions in hematopoietic cell development[59] and T cell differentiation.[60] The secreted form of OPN, cytokine-like, reduces the progression of acute GVHD[61] and promotes tolerogenic macrophage differentiation and tumor growth.[62] In our animal models bearing bone lesions, OPN cytokine, ~70% of which is produced by tumor-conditioned osteoclasts, reprograms the extraosseous TIME to suppress T cell infiltration, proliferation, and effector differentiation. Specifically, the depletion of $CD8^+$ T_{pex} and accelerated exhaustion were observed in the majority of TCR clonotypes, likely impairing T cell differentiation across a large antigen affinity range. While both high- and moderate-affinity TCRs contribute to acute ICB responses, lower-affinity TCRs become dominant for memory formation and sustained ICB efficacy.[63] This implies that OPN may contribute to both primary and acquired ICB resistance in patients with bone metastasis.

To overcome ICB resistance in patients with bone metastasis, our findings strongly suggest that OPN-producing osteoclasts are actionable targets. Both αRANKL blockade and αOPN neutralization sensitized extraosseous ICB responses in preclinical models. In clinical applications, we favor αRANKL because it targets upstream osteoclast maturation. Besides blocking the major source of OPN, it also provides benefits for bone protection. Indeed, beside our cohorts, accumulating evidences have shown that αRANKL treatments improve the efficacy of ICB.[64-66] Importantly, although both αRANKL (e.g., denosumab) and bisphosphonates (e.g., zoledronic acid) can treat bone damage, the αRANKL/ICB regimen demonstrated superior efficacy in our cohort compared to the bisphosphonate/ICB combination. This suggests that the immunoregulatory role of αRANKL, mostly through reducing serum OPN, is essential for therapeutic efficacy. Conversely, in the phase III SPLENDOUR trial, denosumab failed to deliver additional clinical benefits over zoledronic acid in NSCLC patients receiving standard platinum-based doublet chemotherapy,[67] strongly supporting an immunoregulatory mechanism underlying the synergistic effects of denosumab/ICB treatment. Our studies urge for more prospective trials to define an optimal denosumab/ICB combinatorial regimen, which will improve the clinical benefits of ICB treatment in patients with bone metastasis.

Limitations of this study should be carefully considered. Although our findings demonstrate that OPN-producing osteoclasts are previously unrecognized factors for ICB resistance, the mechanism by which bone metastasis regulates OPN secretion by

osteoclasts remains unclear. Additionally, all clinical observations reported here were retrospective and largely based on baseline clinical documentation without long-term follow-up. Again, prospective clinical trials are needed to examine the contribution of bone metastasis in mediating extraosseous ICB resistance.

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AUTHOR CONTRIBUTION

J-N.C., Q.J., and B.Z. proposed the original conceptualization and designed experiments. J-N.C., Z.J., Q.J., B.Z. and Q-J.L. contributed to writing the manuscript and producing the figures. C.S., T.J., X.Z., and T.Y. collected and analyzed clinical cohort information. J-N.C. conducted most experiments and analysis. Z.J. and H.C. performed bioinformatic analysis. X.Z., J.G, X.L., J.J., Q.Z., X.D., Y.Z., and X.Y. coordinated and performed mouse model, morphology, and detection. X.C., H.Z., D.C., Y.Y.W., X.L., L.Y., H.T., and A.L.J.S. contributed critical intellectual guidance to this study. Q-J.L., Q.J., and B.Z. supervised the project. All authors reviewed the manuscript.

DECLARATION OF INTEREST

The authors declare no competing interests.

METHODS&MATERIALS

Cohort study

Patients in cohorts AMU1, AMU2, AMU3, AMU4, AMU5, AMU6, AMU7, AMU8, AMU9, AMU10, and AMU11 were recruited through the Institute of Cancer, Xinqiao Hospital, Third Military Medical University. Patients in cohorts TUSM1, TUSM2, and TUSM3 were recruited through the Department of Medical Oncology, Shanghai Pulmonary Hospital, Tongji University School of Medicine. Cohort identifier, number of cases, tumor type, intervention, related figures, and available clinical information were summarized in **Supplementary Table 1**. Clinical characteristics and radiological following-ups were retrospectively obtained with the approval of Institutional Review Broad. For publicly available cohort, Liu *et al*., NM 2019,[27] all relevant clinical documents were downloaded from supplementary material online.

Cohort AMU1 represents patients with metastatic solid tumors who received ICB-containing regimen at 1st ward in Institute of Cancer, Xinqiao Hospital from Aug 2017 to Jul 2023. Cohort AMU2 represents patients with metastatic solid tumors who received ICB-containing regimen at 2nd ward in Institute of Cancer, Xinqiao Hospital from Aug 2017 to Jul 2023. Cohort AMU3 represents patients with metastatic NSCLC who received chemotherapy (either chemotherapy alone or combining with radiotherapy or anti-angiogenic agents) at 1st ward in Institute of Cancer, Xinqiao Hospital from Jan 2015 to Dec 2018. Patients receiving EGFR/ALK/ROS1/RET target therapy were excluded from cohort AMU3. Cohort AMU4 represent patients with metastatic solid tumors who received chemotherapy (either chemotherapy alone or combining with radiotherapy, anti-angiogenic agents, anti-Her2 treatment, or endocrine therapy) at 2nd and 3rd wards in Institute of Cancer, Xinqiao Hospital from Jan 2015 to Dec 2018. Patients receiving EGFR/ALK/ROS1/RET target therapy were excluded from cohort AMU4. Cohort AMU5 represents patients with extended stage small cell lung cancer who received chemotherapy (either chemotherapy alone or combining with radiotherapy) at 1st ward in Institute of Cancer, Xinqiao Hospital from Jan 2015 to Dec 2018. Cohort AMU6 and AMU7 represent patients with metastatic solid tumors who received ICB-containing regimen at Institute of Cancer, Xinqiao Hospital from Jul 2018 to Aug 2022, and from Jul Sep 2022 to Aug 2023, respectively. Sum of diameters of extraosseous measurable lesions in pre- (baseline) and post-ICB-treated (radiological scans after 2~4 cycles of ICB-containing treatment) radiological imaging were recorded for patients in cohort AMU6 and AMU7. Cohort AMU8 and AMU9 represents patients with bone-metastatic solid tumors who received ICB-containing regimen, either combining with denosumab or bisphosphonate/zoledronic acid therapy, at Institute of Cancer, Xinqiao Hospital from Jul 2018 to Aug 2022, and from Sep 2022 to Aug 2023, respectively. Sum of diameters of extraosseous measurable lesions in pre- (baseline) and post-ICB-treated (radiological scans after 2~4 cycles of ICB-containing treatment) radiological imaging were recorded for patients in cohort AMU8 and AMU9. Cohort AMU10 represent healthy volunteers, treatment-naive cancer patients with bone metastasis or not. Peripheral blood samples were collected from patients in cohort AMU10 detect OPN concentration. Cohort AMU11 were solid cancer patients with bone-metastasis who received ICB-containing treatment. Sum of diameters of extraosseous measurable lesions in pre- (baseline) and post-ICB-treated (radiological

scans after 2~4 cycles of ICB-containing treatment) radiological imaging and PFS were recorded.

Cohort TUSM1 represents patients with metastatic NSCLC who received ICB-containing regimen at Department of Medical Oncology, Shanghai Pulmonary Hospital, Tongji University School of Medicine from Jan 2018 to Dec 2020. Cohort TUSM2 represents patients with metastatic NSCLC who received ICB-containing regimen at Department of Medical Oncology, Shanghai Pulmonary Hospital, Tongji University School of Medicine from Jan 2018 to Dec 2020. All patients were liver-metastatic and brain-metastasis-free in cohort TUSM2. Cohort TUSM3 represents patients with metastatic NSCLC who received ICB monotherapy at Department of Medical Oncology, Shanghai Pulmonary Hospital, Tongji University School of Medicine from Jan 2018 to Dec 2020.

In each participating site, two associate chief physicians or senior attending physicians reviewed the radiological archives to define the presence of measurable lesions and evaluate the diameter of each measurable lesion. Objective response rate was determined according to RECIST 1.1 criteria. PFS and OS were calculated from the initial administration of ICB agents.

Animal studies

Six-to-eight-week-old male C57BL/6 and BALB/c mice were ordered from the Center of Experimental Animals of Third Military Medical University (TMMU). Mice were housed under standard pathogen free conditions. Ctsk-2A-Cre mice (Cat. NO. NM-KI-190019) and *Spp1*-Flox mice (Cat. NO. NM-CKO-210205) were purchased from Shanghai Model Organisms Center, Inc.. The *Ctsk*-2A-Cre mice and *Spp1*-Flox mice were crossed to generate *Spp1* conditional knockout ($Ctsk^{Cre}$; $Spp1^{f/f}$) mice. All mice experiments performed at Xinqiao Hospital were following the institutional animal care and Laboratory Animal Welfare and Ethics Committee of TMMU (NO: AMUWEC20210386; animal production license number: SCXK20170002; animal use license number: SYXK20170002).

Cell lines

The mouse colon adenocarcinoma cell line MC38, mouse melanoma cell line B16F10 and colon carcinoma cell line CT26 were obtained from the CAMS (Cell Resource Center, Institute of Basic Medicine, Chinese Academy of Medical Sciences). Luciferase-expressing B16F10 tumor cells (B16F10-luciferase) and ovalbumin-expressing B16F10 or MC38 tumor cells (B16F10-OVA or MC38-OVA) were established by transfection with lentivirus and selected with puromycin. Cells were cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS), penicillin (100 IU/mL), streptomycin (100 μg/mL), 2 mM L-glutamine, and 1 mM sodium pyruvate in a humidified incubator at 37°C and 5% CO_2.

Tumor models

For subcutaneous tumor models, B16F10-luciferase (1×10^6), B16F10-OVA (1×10^6), B16-F10 tumor cells (1×10^6), E.G7-OVA (5×10^5), MC38 tumor cells (5×10^5),

and CT26 tumor cells (5×10^5) were suspended in 100 μL DMEM medium and subcutaneously injected on the right hindlimb. For osseous tumor-bearing mouse models, mouse tumor cell line B16F10-luciferase (5×10^5), B16F10-OVA (5×10^5), B16-F10 (5×10^5), E.G7-OVA (2.5×10^5), MC38 (2.5×10^5) or CT26 (2.5×10^5) tumor cells were digested and resuspended of 5×10^7/mL cells in DMEM medium. Mice were anesthetized with pentobarbital (80 mg/kg) and a superficial incision about 0.5 cm was made to expose the patellar ligament. Firstly, a 25-gauge needle was used to create a route from the patellar ligament to tibial cavity. Then the needle was replaced with a 10 μL microinjection syringe containing suspension of tumor cells. After slowly injecting tumor cells, the incision was covered with gelatin sponge to prevent liquid leakage. Tumor size was measured every other day and calculated as (length × width^2)/2. Mouse survival was monitored every other day. For lung metastasis model, single-cell suspensions of B16F10-OVA tumor cells (1×10^6) were intravenously injected into the tail vein of mouse. Treatment was started on days 10, and lungs were harvested for counting surface tumor nodules, weighing and H&E staining on days 17.

In vivo imaging

For *in vivo* bioluminescence imaging (BLI), B16F10-luciferase tumor cells were used to establish mouse tumor model. Mice were anesthetized with isoflurane (2% in 1 L/min oxygen) and given an intraperitoneal injection of 200 μL D-luciferin (Selleck, 15 mg/mL). BLI images were acquired with the IVIS Spectrum system (PerkinElmer). For Micro-CT (μCT), mice were anesthetized with isoflurane and assayed by PerkinElmer Quantum FX system. For OsteoSense signal detection, mouse hair from hind legs and lower abdomen were removed, 4 nmol/100 μL OsteoSense 750 EX (PerkinElmer, NEV10053EX) was injected intravenously into the mouse. The optimal imaging time point is 3-24 hours post injection according to the instructions using an imaging system BLT (AniView100).

Osteoclast induction

Bone marrow from the femur and tibia of six to eight-week-old mouse was flushed out with cold DMEM medium and processed into a single-cell suspension without red blood cells. After cultured in incubator at 37°C for 30 minutes, nonadherent cells were collected through centrifuge at 2200 rpm for 5 minutes. Cells were resuspended in osteoclast induction medium of DMEM complete medium with 50 ng/mL M-CSF (R&D) and 50 ng/mL RANKL (R&D) at a concentration of 1×10^5 cells/mL. The medium was replaced by fresh culture medium every two days, and 7-10 days later osteoclasts can be harvested for detection with K-ASSAY TRAP staining kit (Kamiya Biomedical Company) according to the manufacturer's instructions.

Mouse treatment protocol

For immune checkpoint blockade therapy, 200 μg anti-mouse PD-L1 (BioXCell, BP0101) or isotype IgG2b (BioXCell, BP0090) was given intraperitoneally on days 10, 13, 16. For anti-mouse RANKL (BioXCell, BE0191) treatment, mice were given 100 μg antibody or isotype IgG2a (BioXCell, BE0089) by intraperitoneally injection every

other day, and four times in total. For zoledronic acid monohydrate (Selleck, S5244) treatment, 100 μg/kg agent was given subcutaneously daily for totally seven times. For anti-mouse osteopontin (BioXCell, BE0373) intervention, 200 μg antibody or isotype IgG2c (BioXCell, BE0366) was given intraperitoneally every three days, and three times in total. For mouse serum transfer experiments, mouse orbital blood from tumor-free mouse, osseous tumor-bearing mouse, or subcutaneous tumor-bearing mouse was collected to separate serum through centrifugation. The number of serum donor mice was the same as that of the recipient mice. The collected serum was mixed thoroughly and then transfused to the subcutaneous tumor-bearing mice by intraperitoneally injection every other day, and three times in total. For osteoclast-condition medium treatment, successfully induced osteoclasts were washed with PBS to exclude cytokines and then cultured with serum-free medium for two days before the conditioned medium was collected for transfer experiment. When tumor-induced osteoclast supernatant transfer, the tumor conditioned medium is incubated with osteoclasts for an additional two days before *in vivo* transfer.

Histological analysis

For histologically analysis of mouse osseous tumor, leg specimens were harvested and fixed with 10% formalin for two days, then the muscle tissues around bone were removed carefully for EDTA decalcification. Check the specimen daily for adequate decalcification solution coverage and change the solution every three days. 14 days later the specimens were embedded in paraffin and cut into 5-micrometer-thick sections for H&E staining. The specimens were scanned by MoticDSM System.

Flow cytometry

Tumor tissues were cut into little specimens and digested with collagenase IV (1 mg/mL) and deoxyribonuclease (DNase I) (0.2 mg/mL) (both from Sigma-Aldrich). For intracellular staining, lymphocytes were incubated with RPMI 1640 complete medium supplemented with Cell Activation Cocktail (with Brefeldin A, 423304, BioLegend) at 37°C for 4 hours. For extracellular staining, the cells were pre-cultured with CD16/CD32 (clone 2.4G2, BD Bioscience) at 4°C for 30 minutes to block non-antigen specific binding of immunoglobulins. The cells were incubated with extracellular antibodies at 4°C for 30 minutes, then washed and fixed by Fix/Perm solution (BD Bioscience) for 1 hour, and finally intracellular staining for 40 minutes. The following antibodies were used: CD45 (clone 30-F11), CD3 (clone 17A2), CD8α (clone 53-6.7), CD4 (clone GK1.5), CD11b (clone M1/70), CD25 (clone PC61), NK1.1 (clone PK136), CD44 (clone IM7), Ki67 (clone 16A8), F4/80 (clone BM8), Gr-1 (clone R86-8C5), GZMB (clone GB11), TNF-α (clone MP6-XT22), CD45.1 (clone A20), CD45.2 (clone 104), TCR Vα2 (clone B20.1), Tim-3 (clone B8.2C12), LAG-3 (clone C9B7W), 2B4 (clone m2B4 (B6)458.1), Slamf6 (clone 330-AJ), TIGIT (clone 1G9), CD279 (clone 29F.1A12), APC Annexin V Apoptosis Detection Kit with 7-AAD (640930), above antibodies were all purchased from BioLegend, and others were detailed mentioned: IFN-γ (clone XmG1.2, BD Biosciences), TCF-1 (clone S33-966, BD Biosciences), Fixable Viability Dye eFluor 780 (65-0865, Thermo Fisher

Scientific), CellTrace Violet (CTV) (Thermo Fisher Scientific, C34557), FOXP3 (clone FJK-16S, Thermo Fisher Scientific), TOX (clone TXRX10, Thermo Fisher Scientific), CountBright Plus Absolute Beads (C36995, Thermo Fisher Scientific), H-2K^b p15E Tetramer (KSPWFTTL, MBL), H-2K^b OVA Tetramer (SIINFEKL, MBL).

Quantitative proteomic sequencing and data processing

The orbital blood from tumor-free mice, subcutaneous tumor-bearing mice, and osseous tumor-bearing mice were collected respectively after mice were anesthetized with pentobarbital (80 mg/kg), and serum samples were separated by centrifugation at 1000g for 15 minutes at 4℃ and stored in aliquots at -80℃. Subsequent project procedures including protein extraction, process, peptide in vitro label of Tandem Mass Tag (TMT) technique and Liquid Chromatography-Mass Spectrometry LC-MS/MS analysis were conducted by EVbio (Beijing). Briefly, mouse serum was collected for protein identification using HPLC-MS/MS technique. Sample was dissolved in lysis buffer for protein extraction and digestion, the total protein concentration was measured by the Bradford protein assay. After TMT labeling, the acquired peptide fractions were loaded into the nano UPLC-MS/MS system (Thermo Fisher Scientific). The resulting MS/MS data were quantification and processed using Proteome Discover 2.4.

The original data was filtered with null values and performed median normalization to eliminate the experiment error. The proteins with fold change more than 1.5 or less than 0.66 and p-value less than 0.05 were recognized as differentially expression proteins. For GSEA analysis, fgsea package with Gene Oncology (GO) gene sets were applied.[68]

RNA sequencing and data processing

Mouse tumor tissues and osteoclasts were stored in TRIzol reagent (Thermo Fisher Scientific) and prepared by Novogene Bioinformation Technology Co, Ltd. for transcriptome sequencing (Beijing, China). RNA was extracted from the samples according to standard methods, and total amounts and integrity of RNA were assessed using the RNA Nano 6000 Assay Kit of the Bioanalyzer 2100 system (Agilent Technologies, CA, USA). Total RNA was used as input material for the mRNA purified and subsequent library construction. Raw reads of fastq format were firstly processed to remove reads containing adapter, reads containing poly-N and low-quality reads. All downstream deep analyses were based on the clean data with high quality.

The clean data were further aligned to GRCm38 reference genome using Hisat2.[69] Uniquely mapped reads were counted with featureCounts using Ensembl GRCm38 GTF transcript annotations,[70] and normalized based on the length of the gene and reads count mapped to this gene. To perform the "Immune maps", a random forest machine learning algorithm using 244 input variables.[30] RandomForest R package was used to run this analysis. MDS was used to assign a spatial location to each sample according to the proximity matrix from machine learning and 244 dimensions of defined immune-related features.

To identify the differentially expression genes between conditions, log2 fold change (log2FC) and adjusted *p*-value were calculated through DESeq2.[71] The GSEA

function from clusterProfiler package with GO, HALLMARK, REACTOME, and BIOCARTA pathway gene sets of MSigDB were used to perform GSEA analysis based on ranked genes.[72-74]

Quantitative RT-PCR

Total RNA was extracted from tumor tissues using the TRIzol reagent (Thermo Fisher Scientific), followed by complementary DNA (cDNA) synthesis using a PrimeScript RT reagent kit according to the manufacturer's protocol (Takara). Gene expression was assessed using a CFX384 Real-Time PCR detection system with Premix Ex Taq II (Takara) and gene-specific primers as shown in Key resources table. Cycling conditions included a single denaturing step at 95℃ for 30 seconds, followed by 40 cycles at 95℃ for 5 seconds and 60℃ for 34 seconds.

Mass Cytometry sample preparation

The sample preparation was performed according to the manufacture's instruction (PLT-Tech Inc, Hangzhou, China). Briefly, tumor tissues were mechanically dissociated with the GentleMACS Dissociator (Miltenyi Biotec) and digested into single cells with 0.2 mg/mL Deoxyribonuclease (DNase I), Collagenase IV and hyaluronidase (Sigma-Aldrich) for 30 minutes at 37℃. Red cells were removed using ACK Lysing Buffer (PLT-Tech). 3×10^6 single cells were pulsed with 0.25 μM Cell-IDTM Cisplatin-194Pt (Fluidigm, Cat# 201194) and then blocked with anti-CD16/CD32 (clone 2.4G2, BD Bioscience). Qualified cells were stained with forty-one metal-conjugated antibody cocktail (Key resources table), followed by fixation with 250 nM Cell-IDTM Intercalator-Ir (Fluidigm, Cat# 201192B) in Fix and Perm Buffer (Thermo Scientific, Cat# 00-5123-43, Cat# 00-5223-56). After incubated with intracellular antibody cocktail, the cells were washed with Maxpar Fix and Perm Buffer (Fluidigm, Cat# 201067) and mixed with EqBeads before analysis on a CyTOF system (Helios, Fluidigm).

Analysis of mass cytometry data

After acquisition, FCS files were generated and imported into FlowJo to manually gate living $CD45^+$ singlets. The data of gating cells were integrated and transformed using arcsinh with cofactor 5 to make the expression value comparable.[75] To perform the similarity of samples, pseudobulk-level Multi-Dimensional Scaling (MDS) plot was calculated based on the median expression of all 41 proteins with pbMDS function from CATALYST package.[76,77] The Rphenograph clustering algorithm,[78] available through the cytofkit package,[79] was applied to generate clusters. Clusters were annotated into 10 major cell types, such as $CD8^+$ T cell, $CD4^+$ T cell, and Macrophage, according to canonical immune cell markers. The annotation data was visualized using *t*-Distributed Stochastic Neighbor Embedding (*t*-SNE) via cytof_dimReduction function through cytofkit package, and density plots via the ggpointdensity package.[80] To investigate domain major features which cause the difference between two conditions, principal component analysis (PCA) was performed on the fraction matrix of major cell type, and the contribution of each major cell type was calculated through factoextra

package.[81]

ELISA

Protein levels in the serum or condition medium were quantified using ELISA kit according to the manufacturer's instructions: Mouse RANKL ELISA kit (MTR00, R&D), Human RANKL ELISA kit (E-EL-H5813c, Elabscience), Mouse OPN ELISA kit (MOST00, R&D), Human OPN ELISA kit (DOST00, R&D). The concentration of protein was investigated in duplicate and calculated by extrapolating values according to standard curve following manufactures guidelines.

Single-cell RNA-seq library and single-cell TCR-seq library preparation and sequencing

Single-cell libraries were prepared using the Chromium Single Cell 5' Library and Gel Bead Kit, according to the manufacturer's instructions. Briefly, cells were washed with 0.04% BSA DPBS three times and resuspended to a concentration of 700-1200 cells/μL (viability ≥ 85%) as determined by Countess® II. Around 10,000 cells (targeted cell recovery rate ~90%) were encapsulated in droplets with barcoded beads and underwent reverse transcription to generate barcoded cDNA. After the RT step, emulsions were broken and barcoded cDNA was purified with Dynabeads, followed by PCR amplification. Amplified cDNA was used for both gene expression (GEX) and TCR/BCR library construction. For GEX libraries, fragmented cDNA (50 ng) underwent end-repair, size selection with SPRIselect beads (optimal size range not specified), and adapter ligation for Illumina sequencing. The final library was sequenced on a NovaSeq platform, generating 150 bp paired-end reads. For TCR/BCR libraries, TCR transcripts were specifically amplified with 10 PCR cycles. The VDJ region-enriched libraries were size-selected with SPRI beads (optimal size range not specified) and sequenced on a NovaSeq 6000 instrument, generating 150 bp paired-end reads.

Single-cell RNA-seq and single-cell TCR-seq data processing

The single-cell RNA and TCR sequencing data from the same sample were processed using the Cell Ranger multi pipeline (version 7.1.0) from 10x Genomics. The scRNA-seq data was aligned to mouse reference genome (GRCm38), assigned cell barcodes, and used to generate the unique molecular identifier (UMI) matrix. A R-based toolkit, Seurat (version 4.3.0),[82] was used for analyzing the scRNA-seq data. Cells with less than 500 UMIs, less than 200 genes detected, and more than 10% mitochondrial gene counts were filtered out. The TCR sequences were aligned to the same mouse reference genome and filtered to retain high-quality, full-length, productive, and assigned with a valid cell barcode and an unambiguous chain type. ScRepertoire (version 2.0.0) was applied to remove chains without values and keep the chain with the highest UMI count if a cell had two or more qualified chains of the same type.[83] The combined TCR information were integrated into the same Seurat object using AddMetaData() function.

Identification of exhaustion progenitor CD8$^+$ T cells

To accurately identify the exhaustion progenitor CD8$^+$ T cells, we employed a two-round analysis strategy. In the first-round, each sample was analyzed independently, for each sample, the top 2,000 highly variable genes (HVGs) were identified using the "vst" method. The normalized data was obtained by the "LogNormalize" method and scaled with the mean and standard deviation of the HVGs. Principal component analysis (PCA) was performed on the variable gene matrix to reduce noise, and the top 30 out of 50 principal components were used for downstream analyses. A K-nearest neighbor (KNN) graph was constructed based on the Euclidean distance in PCA space. The edge weights between any two cells were refined based on the co-expression in their local neighborhoods. The Louvain algorithm was applied to identify cell clusters with a resolution of 0.5. We then used the same principal components for non-linear dimension reduction to generate the Uniform Manifold Approximation and Projection (UMAP) for visualization. Cell clusters with low expression of CD45, CD3E, and CD8A, and/or high expression of ITGAM, were removed. We first annotated the terminally exhaustion CD8$^+$ T cell clusters in each sample based on the high expression of Havcr2, Lag3, Pdcd1, and Entpd1. Exhaustion progenitor CD8$^+$ T cells were then identified as cells sharing the same clonotype with terminally exhaustion cells. Here, the same clonotype refers to identical nucleotide sequences for both α- and β-chain. The remaining cells were annotated as proliferative CD8$^+$ T cells with high expression of Mki67, or non-exhaustion CD8$^+$ T cells.

After the first-round analysis, data from *Spp1*$^{f/f}$ and *Ctsk*^{Spp1} samples were integrated. The merged data were then re-normalized, dimensionally reduced, and visualized using the methods mentioned above. No obvious batch effect was not observed during the second-round integration analysis. The annotation was inherited from the first-round analysis for visualization.

Statistical analysis

Data were analyzed using GraphPad Prism Software (version 8.2.1), SPSS (IBM), and R Studio. Experiments were repeated two to three times, and comparisons between two groups were assessed by a two-tailed Student's *t*-test. Multiple comparisons of tumor sizes were assessed by two-way analysis of variance (ANOVA). Kaplan-Meier estimates and log-rank (Mantel-Cox) test were used to analyze survival curves. *P* values <0.05 were considered statistically significant.

Data availability

The raw FASTQ files of RNA-seq in this study can be obtained from Genome Sequence Archive (GSA) with an accession number of CRA012873. The raw data of Proteomics and CyTOF has been deposited on Zenodo with an identify of https://doi.org/10.5281/zenodo.8412402.

FIGURES AND FIGURE LEGENDS

Figure 1 | Bone metastasis on therapeutic response to ICB immunotherapy

(A-E) PFS following ICB treatment in patients with bone-metastatic (BnMets) or bone-metastasis-free (BnMets-free) disease in cohorts **(A)** AMU1, **(B)** AMU2, **(C)** TUSM1, **(D)** TUMS2, and **(E)** TUSM3. **(F-H)** Stratified analysis of bone metastasis on ICB treatment. Forest plots of PFS in indicated ICB-treated patient subsets are illustrated for cohort **(F)** AMU1, **(G)** AMU2, and **(H)** TUSM1. The hazard ratio with 95% confidential interval, *p*-value of log-rank test, and number of patients are shown. **(I-K)** OS following ICB treatment in patients with BnMets or BnMets-free disease in cohorts **(I)** TUSM2, **(J)** TUSM3, and **(K)** NM2019. The numbers of patients involved are shown in brackets. The hazard ratio with 95% confidential interval, *p*-value of log-rank test, and median survival time are shown. Orange, BnMets-free; green, BnMets. **(L)** Forest plot showing the hazard ratios in patients who received ICB treatment, stratified by the bone metastasis. **(M)** Stacked barplots showing the objective response to ICB treatment, stratified by the bone metastasis. *P*-values of Chi-square test are indicated. **(N)** Pie charts showing the responses of all patients in cohorts represented in **(M)**. The Chi-square test was used for statistical analysis. PD, progressive disease; CR, complete response; PR, partial response; SD, stable disease.

Figure 2 | Inferior extraosseous tumor shrinkage following ICB treatment in patients with bone metastasis

(A) Workflow for evaluating extraosseous tumor shrinkage after ICB administration. BM, bone metastasis. The extraosseous tumor burden was measured by calculating the sum of largest diameters of all extraosseously measurable lesions. **(B)** Comparisons of the extraosseous tumor burden before and after ICB administration, in patients with BnMets-free **(left panel)** or BnMets **(right panel)** disease in cohort AMU6. Two-tailed paired Student's *t*-test was used. **(C)** Comparison of change of extraosseous tumor burden between patients with BnMets-free and BnMets disease. Two-tailed Student's *t*-test was used. **(D, E)** Validation analysis with cohort AMU7. **(F)** Changes in the tumor burden of extraosseously measurable tumors in ICB-treated patients in cohort TUSM2. Dots indicated the changes in the tumor diameter following ICB treatment for each patient. Differences between the means are presented in grey shadow. Orange dots, BnMets-free patients; green dots, BnMets patients. PriLung, primary lung lesion; MetaLung, intrapulmonary metastatic lesion. Solid grey horizontal line, no change of diameters (0%); dashed grey horizontal lines, mean changes of tumor diameters in patients with or without bone metastasis. The metastatic organs and sample sizes are indicated. Two-tailed Student's *t*-test was used.

Figure 3 | Osseous tumors diminish the extraosseous response to αPD-L1 treatment in mouse model

(A) Schematic depicting strategy used to monitor intratibial and subcutaneous tumor growth by BLI. **(B)** Barplot **(left panel)** and representative images **(right panel)** showing the BLI from subcutaneous (extraosseous) tumors in SC mice treated with either αPD-L1 or IgG isotype. **(C)** Barplots **(left panel** for intratibial tumors, **middle**

panel for extraosseous tumors**)** and representative images **(right panel)** showing the BLI from SC+IT tumor-bearing mice treated with either αPD-L1 or IgG isotype. **(D)** Schematic depicting strategy used to monitor s.c. tumor growth by volume. **(E)** Growth curves of s.c. tumors in SC+IT mice treated with either αPD-L1 or IgG isotype. Two-way ANOVA with Tukey's post hoc test was used. **(F)** Survival curves of αPD-L1 or isotype-treated SC or SC+IT tumor-bearing mice. *p*-value of log-rank test are indicated. **(G)** Summary of s.c. tumor growth and survival outcome in MC38, CT26, and LLC-OVA-implanted mice. **(H)** Schematic depicting strategy used to evaluate pulmonary nodules via macroscopy. **(I)** Representative lungs **(left panel)** and summary of nodule formation **(right panel)** in intravenously (IV) implanted or intravenously/intratibially (IV+IT) implanted B16F10-OVA tumor-bearing mice, either treated with αPD-L1 or IgG isotype. Scale bars, 5 mm. Two-tailed Student's *t*-test was used. **(J)** Schematic depicting strategy used to evaluate the effect of the tumor burden on the efficacy of αPD-L1 treatment. **(K)** Representative IVIS images of mice with implanted tumors. Left panel, SC+IT mice; right panel, mice with bilaterally subcutaneously tumors. **(L)** Total BLI levels in bilaterally subcutaneously implanted mice and SC+IT mice. **(M)** Tumor growth curves in unilaterally **(left panel)** and bilaterally **(right panel)** subcutaneously implanted MC38 tumor-bearing mice, treated with either αPD-L1 or IgG isotype. **(N)** Schematic depicting strategy used to evaluate cell line-specific resistance to αPD-L1 treatment. **(O, P)** Growth curves in osseous tumor-bearing mice with subcutaneously implanted tumors, derived from **(O)** MC38 or **(P)** E.G7-OVA, that were treated with either αPD-L1 or IgG isotype.

Figure 4 | Bone metastasis altered the extraosseous immune microenvironment
(A) Scatterplot showing the Immune Map of subcutaneous tumor from SC and SC+IT B16F10 tumor-bearing mice. **(B)** Heatmap showing row-scaled normalized expression of signature genes in IFN-γ-related pathway. **(C)** GSEA showing lower expression of genes in the ICB-responsive transcription signature in s.c. tumors from SC+IT mice than those from SC mice. **(D)** Enrichment for different signature in HALLMARK collection in the MsigDB dataset. **(E)** MDS analysis of normalized protein expression data in replicate samples from SC (orange) and SC+IT (green) MC38 tumor-bearing mice. Concentration ellipses were added for each group. **(F)** Dot heatmap showing row-scaled expression of feature proteins in the detected $CD45^+$ cell subsets. The dot diameters represent the percent of cells positive for each marker, and the color indicates the average intensity of expression. **(G)** *t*-SNE projections of the detected cell clusters, colored by annotated immune cell population from **(left panel)** SC and **(right panel)** SC+IT tumors. **(H)** Unsupervised clustering of 423,722 $CD45^+$ cells by *t*-distributed stochastic neighbor embedding (*t*-SNE) projections, colored according to the density of detected immune cells. Contours are illustrated in the *t*-SNE map. Clustering for cells from s.c. tumors from 4 SC mice or 4 SC+IT mice. Tumors were harvested 10 days after MC38 tumor implantation. **(I)** Boxplot with jitter comparing annotated immune cell infiltration in SC and SC+IT mice. **(J)** Biplot showing contributing immune cell types identified via principal component analysis. **(K, L)** Percent composition of each cell type in s.c. tumors from SC or SC+IT mice. **(K)** Clustering of $CD8^+$ T cells in

CyTOF analysis. **(L)** $CD8^+$ T cells phenotype in MC38-OVA tumor-bearing mice by flow cytometry analysis. Two-tailed Student's *t*-test was used.

Figure 5 | Osteoclasts drive extraosseous ICB resistance

(A) Schematic depicting transfer strategy used to determine the impact of serum from osseous tumor-bearing mice on the efficacy of αPD-L1 treatment. **(B, C)** Growth curves in αPD-L1-treated s.c. tumor-bearing recipients who were transferred with serum from tumor-free mice, IT donors, or SC donors. An IgG antibody was administered as an isotype control. Tumor growth was measured either in **(B)** B16F10-OVA or **(C)** MC38 tumor-bearing mice. **(D)** Schematic depicting transfer strategy used to evaluate cell line-specific resistance to αPD-L1 treatment. **(E)** Growth curves in αPD-L1-treated s.c. MC38 tumor-bearing recipients who were transferred with serum from tumor-free mice or intratibially B16F10-OVA implanted donors. An IgG antibody was administered as an isotype control. Two-way ANOVA with Tukey's post hoc test was used. **(F)** PCA based on differentially expressed proteins showing the bio-similarity of serum samples from different sources. Dots represent replicates from each source. **(G)** Heatmap showing relative abundances of differentially expressed proteins in serum from tumor-free donor (green), SC donor (red), and IT donor (yellow). Color gradient indicating normalized fold changes in expression. **(H)** Barplot showing the number of differentially expressed proteins. IT, serum from intratibially implanted tumor-bearing mice; SC, serum from subcutaneously implanted tumor-bearing mice. **(I)** Jitter plot comparing expression of interested molecules related to osteoclast or osteoblast differentiation. **(J)** FMT-based detection of OsteoSence 750 EX signals were performed to show hydroxyapatite deposits in the regions of interest (ROI) for B16F10-OVA osseous tumor-bearing mice. **Left panel,** representative image with red dash line used to label the ROI. **Middle and right panel**, signals detected in the ROIs, quantified either as the total fluorescence **(middle panel)** or average fluorescence **(right panel)**. Contra, contralateral (tumor-free) limb; tumoral, intratibially implanted limb. **(K)** Schematic depicting transfer strategy used to evaluate the effect of osteoclast-conditioned medium in mediating extraosseous αPD-L1 resistance. **(L)** Tartrate-resistant acid phosphatase (TRAP) staining revealed the differentiated bone marrow mononuclear cell-induced osteoclasts. Scale bars, 500 μm. **(M, N)** Growth curves in αPD-L1-treated s.c. tumor-bearing recipients, who were transferred with osteoclast-conditioned medium. IgG isotype was also administered as control. Tumor growth was measured either in **(M)** B16F10-OVA or **(N)** MC38 tumor-bearing mice. Two-way ANOVA with Tukey's post hoc test was used.

Figure 6 | OPN in osteoclast-mediated extraosseous ICB resistance

(A) Schematic depicting strategy used to evaluate tumor-induced phenotypic change of osteoclasts *in vitro*. **(B)** Differentially regulated genes between osteoclasts cultured in the presence of tumor-conditioned medium or control medium. Red dots, upregulated genes; grey dots, non-significant genes; blue dots, downregulated genes. **(C)** Venn diagram outlining the strategy used to identify candidate mediators of extraosseous αPD-L1 resistance. Genes of interested were annotated. **(D, E)** Quantifications of

SPP1/OPN production from bone marrow-derived osteoclasts **(D)** and primary osteoclasts **(E)** after cultured in tumor-conditioned medium. Tumor CM, tumor-conditioned medium. Left panel, qPCR analysis; right panel, ELISA assay. **(F)** OPN concentration in serum samples from tumor-free mice (TF, green), SC mice (red), and IT mice (yellow). Sera were collected 10 days after tumor implantation. **(G)** Enrichment of OPN-regulated pathway in s.c. tumors. NES, normalized enrichment score. **(H)** Schematic depicting transfer strategy used to determine the impact of OPN on αPD-L1 treatment. **(I)** Growth curves in αPD-L1-treated s.c. tumor-bearing recipients who were transferred with serum from tumor-free mice, IT donor mice, or IT donor mice treated with αOPN neutralizing antibody. **(J)** Schematic depicting strategy used to evaluate OPN neutralization in extraosseous αPD-L1 resistance. **(K)** Growth curves of s.c. tumors in SC+IT mice treated with αPD-L1 and αOPN neutralizing antibody. **(L)** Schematic depicting strategy used to evaluate αPD-L1 resistance in osteoclast-specific *SPP1* depleted mice. **(M)** Barplot with jitter showing OPN concentration in serum from $Spp1^{f/f}$ or $Ctsk^{Spp1}$ SC+IT mice. **(N)** Growth curves of s.c. tumors in SC+IT mice treated with either αPD-L1 or IgG isotype. Tumor growth was tested in osteoclast-specific *SPP1* depletion mice (grey, $Ctsk^{Spp1}$) or control (black, $Spp1^{f/f}$) mice. Two-way ANOVA with Tukey's post hoc test was used. **(O)** Survival curves of αPD-L1-treated or control-treated $Spp1^{f/f}$ or $Ctsk^{Spp1}$ SC+IT mice. *p*-value of log-rank test are shown. **(P, Q)** $CD8^+$ T cells phenotype prior to ICB treatment (10 days after implantation) in subcutaneous tumor by flow cytometry analysis. **(P)** Percent in $CD45^+$ immune cell; **(Q)** Number per gram tumors. Two-tailed Student's *t*-test was used. **(R)** Annotation of exhaustion status of sequenced $CD8^+$ T cells. **(S)** Unsupervised clustering and distribution of $CD8^+$ T cells in s.c. tumor from $Spp1^{f/f}$ or $Ctsk^{Spp1}$ SC+IT mice. **(T, U)** Barplot showing percent of **(T)** T_{ex} and **(U)** T_{pex} cells in $CD8^+$ T cells. **(V)** Ratio of TCR clonal number of T_{pex} to T_{ex} cells, stratified according to *SPP1* depletion. Red line, median ratio. **(W)** Cumulative frequency expanded T_{pex} clones. Blue bars, highly expanded T cell clones. Mann-Whitney *U*-test was used.

Figure 7 | Denosumab/ICB combinational therapy in patients with bone metastasis **(A)** Schematic depicting strategy used to evaluate the effect of blocking osteoclastogenesis. **(B)** Growth curves of s.c. tumors in SC+IT mice treated with αPD-L1 and αRANKL neutralizing antibody. **(C)** Schematic depicting strategy used to evaluate pulmonary nodule formation by macroscopy. **(D)** Summary of nodule formation in implanted B16F10-OVA tumor-bearing mice, treated with either αPD-L1 or IgG isotype, plus αRANKL neutralizing antibody. Two-tailed Student's *t*-test was used. **(E)** Barplot with jitter showing OPN concentration in serum from SC+IT mice treated with αRANKL and/or αPD-L1 treatment. **(F, H)** Comparison of extraosseous tumor burden before and after ICB administration, in patients with bone metastasis treated with denosumab (**left panel**) or bisphosphonate (**right panel**) in cohort **(F)** AMU8 and **(H)** AMU9. Extraosseous tumor burden was quantified as the sum of diameters of extraosseous measurable lesions. Two-tailed paired Student's *t*-test was used. **(G, I)** Comparison of change of extraosseous tumor burden between patients with bone metastasis treated with denosumab (Deno) or bisphosphonate (BP) in cohort **(G)**

AMU8 and **(I)** AMU9. Two-tailed Student's *t*-test was used. **(J)** PFS outcomes in response to ICB immunotherapy in bone-metastatic patients treated with either denosumab or bisphosphonate. The hazard ratio with 95% confidential interval, *p*-value of log-rank test are shown. **(K)** Radiological scans showing dynamic changes in measurable lesions over time. The red arrows point to measurable lesions. Clinical course of representative case with bone-metastatic disease who received combinational therapy with denosumab and αPD-1 antibody. **(L)** Violin plot with jitter showing OPN concentration in peripheral blood samples from age-matched healthy volunteers, patients with BnMets disease, and patients with BnMets-free disease in cohort AMU10. Mann-Whitney *U*-test was used. **(M)** Tumor burden changes stratified based on the ratio of post-treatment/pre-treatment OPN concentration. Stratified based on > 100% (increased OPN), < 50% (significantly decreased OPN), or otherwise (modestly decreased OPN). Red line, mean tumor burden change of extraosseously measurable lesions. Two-tailed Student's *t*-test was used. **(N)** Kaplan-Meier survival curves of patients with metastatic solid tumors stratified based on change of serum OPN concentrations. **(O)** Kaplan-Meier survival curves of patients with metastatic solid tumors stratified based on baseline serum OPN concentrations.

SUPPLEMENTARY FIGURES AND LEGENDS

Supplementary Figure 1 | The impact of the metastatic sites on the survival to ICB treatment

(A, B) PFS outcomes in response to ICB immunotherapy in patients with metastatic lesions in the brain, lung, liver, bone, lymph node, and other organs, in comparison to the overall population (black curve). "Others" including metastatic lesions in chest wall, adrenal gland, soft tissue, omentum, mucosa, pancreas, and malignant effusions. The hazard ratio with 95% confidential interval, *p*-value of log-rank test are shown. **(A)** Cohort AMU1; **(B)** cohort AMU2.

Supplementary Figure 2 | Bone metastasis on survival to chemotherapy

(A-C) Survival outcomes to chemotherapy in patients with bone-metastatic or bone-metastasis-free disease from cohort **(A)** AMU3, **(B)** AMU4, and **(C)** AMU5. The hazard ratio with 95% confidential interval, *p*-value of log-rank test, and median survival time are shown. **(D)** Forest plot showing the hazard ratios of patients who received chemotherapy in cohort AMU3, AMU4, and AMU5. All cohorts were stratified by bone metastasis.

Supplementary Figure 3 | Establishment of an osseous tumor-bearing mouse model

(A) Schematic illustration of the strategy used to establish experimental mouse model of bone metastasis via intratibial injection in the proximal tibia. **(B)** Spatial orientation of the sectional view in Micro-computed tomography (μCT) scan. **(C)** μCT scans showing the cortical bone defect in each sectional view at 10 days after B16F10-OVA intratibial implantation. Red arrows point to the cortical bone defect. **(D)** Three-dimensional reconstruction of implanted limbs based on μCT scans. The yellow dash

square show zoomed in views of the implanted or contralateral control tibia. Red arrows point to the cortical bone defect. A, axial axis; C, coronal axis; S, sagittal axis. **(E)** Three-dimensional reconstruction of tibias in saline injected control mice. **(F, G)** Hematoxylin and eosin staining showed the bone involvement in **(F)** B16F10-OVA tumor implanted or **(G)** saline injected limbs. Scale bars, 100 μm.

Supplementary Figure 4 | Dynamics of osseous tumor formation
IVIS spectrum assays showing the bioluminescent images of mice implanted intratibially with B16F10-luciferase cells at 2, 4, 6, 8, and 10 days after implantation.

Supplementary Figure 5 | Extraosseous tumor growth in osseous tumor-bearing mice
(A-C) Growth curves of s.c. tumors in SC+IT mice treated with either αPD-L1 or IgG isotype. Two-way ANOVA with Tukey's post hoc test was used. **(A)** MC38, **(B)** CT26, and **(C)** LLC-OVA tumor-bearing mice were used. **(D-F)** Survival curves of αPD-L1 or control-treated SC or SC+IT tumor-bearing mice. Survival was tested in **(D)** MC38, **(E)** CT26, and **(F)** LLC-OVA tumor-bearing mice. *p*-value of log-rank test are indicated.

Supplementary Figure 6 | Flow cytometry analysis
Gating strategy and summary of $CD8^+$ T cell infiltration in s.c. tumor from SC or SC+IT MC38-OVA tumor-bearing mice. Two-tailed Student's *t*-test was used.

Supplementary Figure 7 | Quantitative mass spectrometry analysis identify proteins with different abundance
(A-C) Heatmap showing relative levels of differentially presented proteins in the serum samples from tumor-free mice (grey), subcutaneously implanted tumor-bearing mice (green), and intratibially implanted tumor-bearing mice (orange). Color gradient, normalized fold change. Dendrograms for clustering are shown. **(A)** The serum samples from tumor-free and subcutaneously implanted mice. **(B)** The serum samples from tumor-free and intratibially implanted mice. **(C)** The serum samples from subcutaneously implanted and intratibially implanted mice. **(D-F)** Volcano plots showing significantly differentially presented proteins between the serum samples from **(D)** tumor-free versus subcutaneously implanted mice, **(E)** tumor-free versus intratibially implanted mice, and **(F)** subcutaneously implanted versus intratibially implanted mice.

Supplementary Figure 8 | Effect of bone metastasis on bone density and remodeling
(A) FMT-based detection of OsteoSence 750 EX signals was performed to show hydroxyapatite deposits in the regions of interest (ROI) for MC38 osseous tumor-bearing mice. **Left panel,** representative image with red dash line used to label the ROI. **Middle and right panel**, signals detected in the ROIs, quantified either as the total fluorescence **(middle panel)** or average fluorescence **(right panel)**. Contra, contralateral (tumor-free) limb; tumoral, intratibially implanted limb. **(B)** Bone mineral

density in tumor-free or tumor-bearing limbs. Two-tailed Student's *t*-test was used. **(C)** TRAP staining to detect osteoclasts from tumor-free or tumor-bearing limbs.

Supplementary Figure 9 | Clustering strategies of scRNA- and scTCR-sequencing cells

Dot heatmap showing row-scaled expression of feature genes in the detected $CD8^+$ T cells. The dot diameters represent the percent of cells positive for each marker, and the color indicates the average intensity of expression.

KEY RESOURCE TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Flow: anti-mouse CD16/CD32, clone 2.4G2	BD Bioscience	Cat# 553142; RRID: AB_394656
Flow: anti-mouse CD45, clone 30-F11	BioLegend	Cat# 103137; RRID: AB_2561392
Flow: anti-mouse CD3, clone 17A2	BioLegend	Cat# 100235; RRID: AB_2561455
Flow: anti-mouse CD8, clone 53-6.7	BioLegend	Cat# 100707; RRID: AB_312746
Flow: anti-mouse CD4, clone GK1.5	BioLegend	Cat# 100431; RRID: AB_893329
Flow: anti-mouse CD11b, clone M1/70	BioLegend	Cat# 101229; RRID: AB_2129375
Flow: anti-mouse CD25, clone PC61	BioLegend	Cat# 102012; RRID: AB_312861
Flow: anti-mouse NK1.1, clone PK136	BioLegend	Cat# 108707; RRID: AB_313394
Flow: anti-mouse CD44, clone IM7	BioLegend	Cat# 103005; RRID: AB_312956
Flow: anti-mouse Ki67, clone 16A8	BioLegend	Cat# 652403; RRID: AB_2561524
Flow: anti-mouse F4/80, clone BM8	BioLegend	Cat# 123107; RRID: AB_893500
Flow: anti-mouse Gr-1, clone R86-8C5	BioLegend	Cat# 108411; RRID: AB_313376

Flow: anti-mouse GZMB, clone GB11	BioLegend	Cat# 515408; RRID: AB_2562196
Flow: anti-mouse TNF-α, clone MP6-XT22	BioLegend	Cat# 506321; RRID: AB_961435
Flow: anti-mouse CD45.1, clone A20	BioLegend	Cat# 110729; RRID: AB_1134170
Flow: anti-mouse CD45.2, clone 104	BioLegend	Cat# 109814; RRID: AB_389211
Flow: anti-mouse Tim-3, clone B8.2C12	BioLegend	Cat# 134019; RRID: AB_2814028
Flow: anti-mouse LAG-3, clone C9B7W	BioLegend	Cat# 125225; RRID: AB_2715763
Flow: anti-mouse 2B4, clone m2B4 (B6)458.1	BioLegend	Cat# 133503; RRID: AB_1595624
Flow: anti-mouse Slamf6, clone 330-AJ	BioLegend	Cat# 134609; RRID: AB_2728154
Flow: anti-mouse TIGIT, clone 1G9	BioLegend	Cat# 142109; RRID: AB_2566572
Flow: anti-mouse CD279, clone 29F.1A12	BioLegend	Cat# 135207; RRID: AB_10550092
Flow: anti-mouse IFN-γ, clone XmG1.2	BD Bioscience	Cat# 505825; RRID: AB_1595591
Flow: anti-mouse TCF-1, clone S33-966	BD Bioscience	Cat# 566692; RRID: AB_2869822
Flow: anti-mouse FOXP3, clone FJK-16S	Thermo Fisher Scientific	Cat# 25-5773-82; RRID: AB_891552
Flow: anti-mouse TOX, clone TXRX10	Thermo Fisher Scientific	Cat# 50-6502-82; RRID: AB_2574265
Flow: H-2K^{b} p15E Tetramer	MBL	Code No. TS-M507-1
Flow: H-2K^{b} OVA Tetramer	MBL	Code No. TS-5001-1C
Flow: anti-mouse CD366 (Tim-3), clone RMT3-23	BioLegend	Cat# 119715; RRID: AB_2571932

Flow: anti-mouse CD3, clone 17A2	BioLegend	Cat# 100245; RRID: AB_2565882
Flow: anti-mouse Ki-67, clone 11F6	BioLegend	Cat# 151215; RRID: AB_2876504
Flow: anti-mouse CD69, clone H1.2F3	BioLegend	Cat# 104505; RRID: AB_313108
Flow: anti-mouse CD44, clone IM7	BioLegend	Cat# 103037; RRID: AB_10900641
Flow: anti-mouse CD45, clone 30-F11	BioLegend	Cat# 103174; RRID: AB_2860600
Flow: anti-mouse TCR β chain, clone H57-597	BioLegend	Cat# 109218; RRID: AB_493346
Flow: anti-mouse CD279 (PD-1), clone 29F.1A12	BioLegend	Cat# 135220; RRID: AB_2562616
CyTOF: anti-mouse CD45 (clone 30-F11), 89-Y	BioLegend	Cat# 103102; RRID: AB_312967
CyTOF: anti-mouse CD3ε (clone 145-2C11), 115-ln	BioLegend	Cat# 100302; RRID: AB_312667
CyTOF: anti-mouse CD28 (clone 37.51), 141-Pr	BioLegend	Cat# 102102; RRID: AB_312867
CyTOF: anti-mouse CD95 (Fas) (clone SA367H8), 142-Nd	BioLegend	Cat# 152602; RRID: AB_2629777
CyTOF: anti-mouse MHC II (I-A/I-E) (clone M5/114.15.2), 143-Nd	BioLegend	Cat# 107602; RRID: AB_313317
CyTOF: anti-mouse CD44 (clone IM7), 144-Nd	BioLegend	Cat# 103002; RRID: AB_312953
CyTOF: anti-mouse CD69 (clone H1.2F3), 145-Nd	BioLegend	Cat# 104502; RRID: AB_313105
CyTOF: anti-mouse CD206 (MMR) (clone C068C2), 146-Nd	BioLegend	Cat# 141702; RRID: AB_10900233
CyTOF: anti-mouse Ly-6G (clone 1A8), 147-Sm	BioLegend	Cat# 127602; RRID: AB_1089180

CyTOF: anti-mouse Ly-6C (clone HK1.4), 148-Nd	BioLegend	Cat# 128002; RRID: AB_1134214
CyTOF: anti-mouse CD19 (clone 6D5), 149-Sm	BioLegend	Cat# 115502; RRID: AB_313637
CyTOF: anti-mouse CD186 (CXCR6) (clone SA051D1), 150-Nd	BioLegend	Cat# 151102; RRID: AB_2566544
CyTOF: anti-mouse CD161(NK-1.1) (clone PK136), 151-Eu	BioLegend	Cat# 108702; RRID: AB_313389
CyTOF: anti-mouse IFN-γ (clone XMG1.2), 152-Sm	BioXCell	Cat# BE0055; RRID: AB_1107694
CyTOF: anti-mouse iNOS (clone CXNFT), 153-Eu	eBioscience	Cat# 14-5920-82; RRID: AB_2572890
CyTOF: anti-mouse CD194 (CCR4) (clone 2G12), 154-Sm	BioLegend	Cat# 131202; RRID: AB_1227524
CyTOF: anti-mouse CD103 (clone 2E7), 155-Gd	BioLegend	Cat# 121402; RRID: AB_535945
CyTOF: anti-mouse CD62L (clone MEL-14), 156-Gd	BioLegend	Cat# 104402; RRID: AB_313089
CyTOF: anti-mouse CD25 (clone 3C7), 157-Gd	BioLegend	Cat# 101902; RRID: AB_312845
CyTOF: anti-mouse TIGIT (VSTM3) (clone 2190A), 158-Gd	R&D	Cat# MAB72671
CyTOF: anti-mouse F4/80 (clone Cl:A3-1), 159-Tb	Bio-rad	Cat# MCA497G; RRID: AB_872005
CyTOF: anti-mouse TCR β chain (clone H57-597), 160-Gd	BioLegend	Cat# 109202; RRID: AB_313425
CyTOF: anti-mouse CD64 (FcγRI) (clone X54-5/7.1), 161-Dy	BioLegend	Cat# 139302; RRID: AB_10613107
CyTOF: anti-mouse FOXP3 (clone FJK-16s), 162-Dy	eBioscience	Cat# 14-5773-82; RRID: AB_467576
CyTOF: anti-mouse CD172a (SIRPα) (clone P84), 163-Dy	BioLegend	Cat# 144002; RRID: AB_11203711
CyTOF: anti-mouse CD11c (clone N418), 164-Dy	BioLegend	Cat# 117302; RRID: AB_313771

CyTOF: anti-mouse TCF7(TCF1) (clone 812145), 165-Ho	R&D	Cat# MAB8224
CyTOF: anti-mouse CD27 (clone LG.3A10), 166-Er	BioLegend	Cat# 124202; RRID: AB_1236456
CyTOF: anti-mouse CD184 (CXCR4) (clone L276F12), 167-Er	BioLegend	Cat# 146502; RRID: AB_2562589
CyTOF: anti-mouse CD49b (clone DX5), 168-Er	BioLegend	Cat# 108902; RRID: AB_313409
CyTOF: anti-mouse Ki-67 (clone SolA15), 169-Tm	eBioscience	Cat# 14-5698-82; RRID: AB_10854564
CyTOF: anti-mouse T-bet (clone 4B10), 170-Er	BioLegend	Cat# 644802; RRID: AB_1595503
CyTOF: anti-mouse CD279 (PD-1) (clone 29F.1A12), 171-Yb	BioLegend	Cat# 135202; RRID: AB_1877121
CyTOF: anti-mouse CD127 (IL-7Rα) (clone A7R34), 172-Yb	BioLegend	Cat# 135002; RRID: AB_1937287
CyTOF: anti-mouse CD223 (LAG-3) (clone C9B7W), 173-Yb	BioLegend	Cat# 125202; RRID: AB_961187
CyTOF: anti-mouse CD196 (CCR6) (clone 29-2L17), 174-Yb	BioLegend	Cat# 129802; RRID: AB_1227495
CyTOF: anti-mouse Siglec-F (clone E50-2440), 175-Lu	BD Bioscience	Cat# 552125; RRID: AB_394340
CyTOF: anti-mouse CD366 (Tim-3) (clone RMT3-23), 176-Yb	BioLegend	Cat# 119702; RRID: AB_345376
CyTOF: anti-mouse CD4 (clone RM4-5), 197-Au	BioLegend	Cat# 100576; RRID: AB_2832266
CyTOF: anti-mouse CD8a (clone 53-6.7), 198-Pt	BioLegend	Cat# 100746; RRID: AB_11147171
CyTOF: anti-mouse CD11b (clone M1/70), 209-Bi	BioLegend	Cat# 101202; RRID: AB_312785
In vivo: anti-mouse PD-L1, clone 10F.9G2	BioXCell	Cat# BP0101; RRID: AB_10949073
In vivo: rat IgG2b isotype, clone LTF-2	BioXCell	Cat# BP0090; RRID: AB_1107780

In vivo: anti-mouse RANKL, clone IK22/5	BioXCell	Cat# BE0191; RRID: AB_10949003
In vivo: rat IgG2a isotype, clone 2A3	BioXCell	Cat# BE0089; RRID: AB_1107769
In vivo: anti-mouse osteopontin, clone 103D6	BioXCell	Cat# BE0373; RRID: AB_2927510
In vivo: mouse IgG2c isotype, clone DV5-1	BioXCell	Cat# BE0366; RRID: AB_2894738
Bacterial and virus strains		
Biological samples		
Chemicals, peptides, and recombinant proteins		
D-luciferin	Selleck	Cat# S6580
OsteoSense 750 EX	PerkinElmer	Cat# NEV10053EX
Recombinant Mouse M-CSF Protein	R&D	Cat# 416-ML-010/CF
Recombinant Mouse TRANCE/RANK L/TNFSF11 Protein	R&D	Cat# 462-TR-010/CF
Zoledronic acid monohydrate	Selleck	Cat# S5244
Collagenase IV	Sigma-Aldrich	Cat# 9001-12-1
DNase I	Sigma-Aldrich	Cat# 10104159001
Cell Activation Cocktail	BioLegend	Cat# 423304
Fix/Perm solution	BD Bioscience	Cat# 554722
Fixable Viability Dye eFluor 780	Thermo Fisher Scientific	Cat# 65-0865
CellTrace Violet	Thermo Fisher Scientific	Cat# C34557
CountBright Plus Absolute Beads	Thermo Fisher Scientific	Cat# C36995
Critical commercial assays		
K-ASSAY TRAP staining kit	Kamiya Biomedical Company	Cat# KT-008
APC Annexin V Apoptosis Detection Kit with 7-AAD	BioLegend	Cat# 640930
Mouse RANKL ELISA kit	R&D	Cat# MTR00
Human RANKL ELISA kit	Elabscience	Cat# E-EL-H5813c
Mouse OPN ELISA kit	R&D	Cat# MOST00
Human OPN ELISA kit	R&D	Cat# DOST00
EasySep™ Mouse $CD8^+$ T Cell Enrichment Kit	STEMCELL	Cat# 19753

Deposited data		
mouse tumor tissues RNA-Seq		
osteoclast RNA-Seq		
serum Proteomics		
CyTOF		
mouse scRNA-seq		
Experimental models: Cell lines		
B16F10	Chinese Academy of Medical Sciences	1101MOU-PUMC000473
MC38	Chinese Academy of Medical Sciences	1101MOU-PUMC000523
CT26	Chinese Academy of Medical Sciences	1101MOU-PUMC000275
E.G7-OVA	TMMU	N/A
Experimental models: Organisms/strains		
C57BL6/J	TMMU	N/A
BALB/c	TMMU	N/A
Ctsk-2A-Cre mice	Shanghai Model Organisms Center, Inc.	Cat# NO. NM-KI-19001
Spp1-Flox mice	Shanghai Model Organisms Center, Inc.	Cat# NO. NM-CKO-210205
Oligonucleotides		
Actin primer (mouse): Fw: ATGGAGCCGGACAGAAAAGC; Rv: CTTGCCACTCAGGGAAGGA	Beijing Tsingke Biotech Co., Ltd.	N/A
SPP1 primer (mouse): Fw: AGCAAGAAACTCTTCCAAGCAA; Rv: GTGAGATTCGTCAGATTCATCCG	Beijing Tsingke Biotech Co., Ltd.	N/A
Recombinant DNA		
CV146-OVAL	Genechem	N/A
CV146-Luciferase	Genechem	N/A
Software and algorithms		
GraphPad Prism	GraphPad Software	www.graphpad.com
FlowJo Software (verison 10.9.0)	TreeStar	www.flowjo.com
Living Image	PerkinElmer	www.perkinelmer.com
Proteome Discover-2.4	Thermo Fisher Scentific	https://www.thermofisher.com

R-4.2.1	84	https://www.r-project.org
fgsea-1.18	68	https://github.com/ctlab/fgsea/
Hisat2-2.2.0	69	http://daehwankimlab.github.io/hisat2/
featureCounts-2.0.1	70	https://subread.sourceforge.net/featureCounts.html
DESeq2-1.32.0	71	https://bioconductor.org/packages/release/bioc/html/DESeq2.html
clusterProfiler-4.8.2	72	https://github.com/YuLab-SMU/clusterProfiler
CATALYST-1.20.1	76,77	https://github.com/HelenaLC/CATALYST
cytofkit-1.10.0	79	https://github.com/JinmiaoChenLab/cytofkit
ggpointdensity-0.1.0	80	https://github.com/LKremer/ggpointdensity
ggplot2-3.4.3	85	https://github.com/tidyverse/ggplot2
factoextra-1.0.7	81	http://www.sthda.com/english/rpkgs/factoextra
Cell Ranger-7.1.0	10x Genomics	https://www.10xgenomics.com
Seurat-4.3.0	82	https://satijalab.org/seurat/
scRepertoire-2.0.0	83	https://github.com/ncborcherding/scRepertoire
randomForest-4.6-14	10.32614/CRAN.package.randomForest	https://cran.r-project.org/web/packages/randomForest/index.html
tidyverse-2.0.0	https://doi.org/10.32614/CRAN.package.tidyverse	https://cran.r-project.org/web/packages/tidyverse/index.html

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