The road of NSCLC stem cells toward bone metastases

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A B S T R A C T

Despite advancement in therapeutic options, Non-Small Cell lung cancer (NSCLC) remains a lethal disease mostly due to late diagnosis at metastatic phase and drug resistance. Bone is one of the more frequent sites for NSCLC metastatization.

A defined subset of cancer stem cells (CSCs) that possess motile properties, mesenchymal features and tumor initiation potential are defined as metastasis initiating cells (MICs). A better understanding of the mechanisms supporting MIC dissemination and interaction with bone microenvironment is fundamental to design novel rational therapeutic option for long lasting efficient treatment of NSCLC.

In this review we will summarize findings about bone metastatic process initiated by NSCLC MICs. We will review how MICs can reach bone and interact with its microenvironment that supports their extravasation, seeding, dormancy/proliferation. The role of different cell types inside the bone metastatic niche, such as endothelial cells, bone cells, hematopoietic stem cells and immune cells will be discussed in regards of their impact in dictating the success of metastasis establishment by MICs.

Finally, novel therapeutic options to target NSCLC MIC-induced bone metastases, increasing the survival of patients, will be presented.

1. Introduction

Lung cancer is often diagnosed when it is locally advanced or metastatic (Miller et al. 2019), and bone is a common metastatic site with about 30–40 % of advanced NSCLC patients developing bone metastases (Kuchuk et al., 2013; Riihimaki et al., 2014; D'Oronzo et al., 2019; Roato et al., 2008), which decrease the quality of life, since they are associated to skeletal related events (SREs). Indeed SRE, such as fractures, pain, bone marrow compression may occur in 30–60 % of bone metastatic patients and reduce survival (Kuchuk et al., 2013). In the bone microenvironment different populations of stem and mature cells live, interacting to regulate haematopoiesis, bone remodeling and immune cell activity. Bone metastases disrupt the equilibrium of bone microenvironment, that is endowed of some properties favoring homing and growth of tumor cells (Croucher et al., 2016). Osteolytic lesions are associated to activation of osteoclast (OC) activity and suppression of osteoblast (OB) one, neoangiogenesis and activation of immunosuppressive subsets of immune cells (Coleman et al., 2020a).

It is even more evident that bone metastatization can start early in the history of tumor, since disseminated tumor cells (DTCs), enriched for a subset of cancer stem cells (CSCs) responsible for metastasis initiation have been detected in bone marrow of patients long before the diagnosis of metastases (Mohme et al., 2017). CSCs contribute mainly to the heterogeneity of cancer and are endowed with different characteristics that account for primary tumor maintenance, aggressiveness, drug resistance, metastasis and tumor-immune-microenvironment remodeling (Su et al., 2020; Visvader and Lindeman, 2012). In NSCLC, a subset of CSCs, constituted by metastasis initiating cells (MICs), have been demonstrated able to promote bone metastasis initiation (Bertolini et al., 2015).

In recent years, immune checkpoint inhibitor (ICI) therapy completely changed the strategy of treatment of advanced NSCLC, which express programmed death-ligand 1 (PD-1 L) (Doroshow et al., 2019; Pasello et al., 2020), thus some data on the effect of ICI treatment on bone metastasis formation are emerging.

In this review, we will analyze the recent advancements in the knowledge of the different steps involved in the bone metastatic process initiated by NSCLC MICs. Finally, the results deriving from experimental and clinical studies on the effectiveness of anti-resorbing drugs and ICI in the treatment of bone metastases will be discussed.

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2. NSCLC CSCs generation

CSCs are comprised of heterogeneous and specialized cell subsets deputed to primary tumor maintenance, drug resistance, tumor escape/metastasis formation (Leon et al., 2016; Bocci et al., 2019). Indeed, CSCs are functionally defined as self-renewing cells responsible for tumor initiation and generation of differentiated tumor cells, comprising the bulk of the tumors (Visvader and Lindeman, 2008; Walcher et al., 2020).

The first evidence for the existence of a subset of CSCs in primary NSCLC was provided by Eramo et al.: the authors demonstrated that primary lung cancer cells expressing the surface marker CD133 were able to form tumor spheres in culture, that could be transplanted in immunocompromised mice (Eramo et al., 2008). Bertolini et al. demonstrated that CD133+ cells, sorted from NSCLC primary tumors and patient derived xenograft (PDX) models, possess the highest ability to initiate tumor when injected in mice and can maintain their tumorigenic ability during *in vivo* serial transplantation assay. CD133+ cells-derived tumors resemble the phenotype of original tumor, indicating the ability of CD133+ CSCs to reform the heterogeneity of bulk tumor (Bertolini et al., 2009).

The process of Epithelial to Mesenchymal transition (EMT) can provide tumor cells with stemness feature and migrating abilities necessary for successful establish metastases (Polyak and Weinberg, 2009). First evidence for the existence of MICs was reported in pancreatic adenocarcinoma, where the subset of CSCs expressing CD133

and the chemokine receptor CXCR4 determines the metastatic phenotype of the individual tumor cells (Hermann et al., 2007). In NSCLC, subset of CD133+CXCR4+ cells were also demonstrated to represent MICs, since they are endowed with both tumor initiation properties, mesenchymal and migration properties (Bertolini et al., 2015). By exploiting an original model of bone metastasis assay, generated by implanting a vital human bone fragment in the flanks of immunocompromised mice, the authors demonstrated the high bone tropism and metastasis formation ability of CD133+CXCR4+ MICs, sorted from NSCLC PDX models. Finally, the presence of CD133+CXCR4+ MICs within primary NSCLC was correlated with tumor relapse and poor patients' outcome (Bertolini et al., 2015).

Notably the subset of CD133+CXCR4+ MICs are preferentially modulated by microenvironmental stimuli that can trigger their expansion or *de novo* generation through the induction of EMT (Bertolini et al., 2015; Andriani et al., 2016). In particular, cancer associated fibroblasts (CAF) were demonstrated to play a pivotal role in modulation of CSC phenotype, through the release of factors such as tumor growth factor β (TGFβ), insulin growth factor II (IGF-II) and stromal-derived factor-1 (SDF-1) that can expand the subset of CSCs, also through the induction of EMT, and prime their metastatic dissemination toward distant site (Andriani et al., 2016; Chen et al., 2014), Fig. 1.

NSCLC MICs can also be spared by conventional chemotherapy and can be responsible for tumor recurrence and metastatic progression after therapy (Bertolini et al., 2009; Eramo et al., 2008; Levina et al., 2008). A
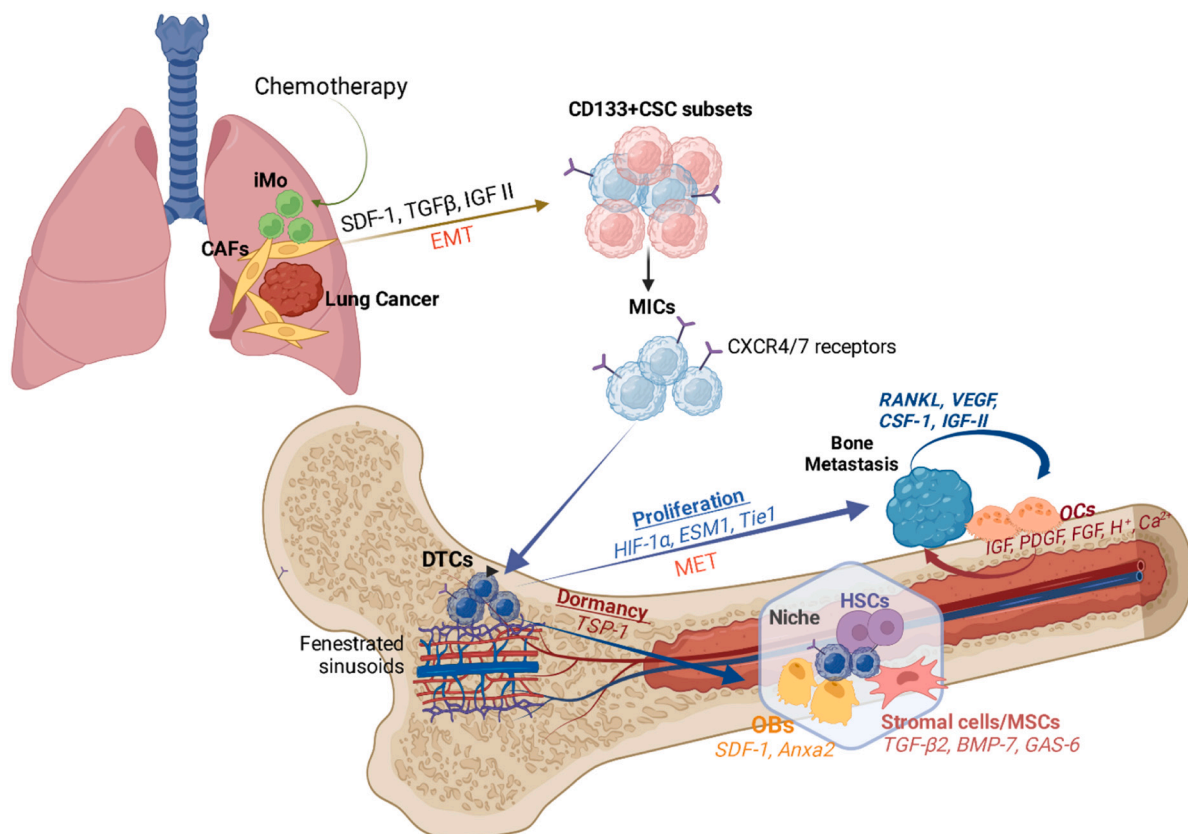
Fig. 1. The crosstalk between primary tumor and bone is mediated by CSCs, and particularly by their subset named MICs. Chemotherapy directly influences the tumor microenvironment promoting inflammatory monocytes (iMo) and cancer-associated fibroblasts (CAFs), all stimulating the formation of CD133+ CSCs and their subset of metastasis initiating cells (MICs), through the induction of epithelial-mesenchymal transition (EMT) and the release of factors such as SDF-1, TGF-β and IGF-II. Once reached the microvasculature of bone, MICs can find a favorable soil to live. Dormancy can be soon stimulated by the production of trombospondin-1 (TSP-1) by endothelial cells of the sinusoids. Moreover, MICs can compete with hematopoietic stem cells (HSCs) for the lodgment in the bone marrow niche, which is also constituted by osteoblasts (OBs), stromal cells and mesenchymal stem cells (MSCs), all contributing to the dormant state of MICs, through the production of many molecules, such as SDF-1, Anxa2, TGF-β2, BMP-7, GAS-6. If MICs receive stimuli promoting mesenchymal-epithelial transition (MET), they start to proliferate producing factors able to activate osteoclasts (OCs), such as RANKL, VEGF, CSF-1, IGF-II. OCs resorb bone causing the release of factors by the bone matrix, IGF, PDGF, FGF, H +, Ca^{2+}, which stimulates tumor cells, thus contributing to the establishment of the typical bone metastatic vicious cycle.

recent study demonstrated that cisplatin treatment of NSCLC pre-clinical models induces an increased release of both stromal and tumor SDF-1, resulting in the recruitment of both CXCR4+ inflammatory monocytes (IM), with pro-tumorigenic activities and MICs, Fig. 1. The crosstalk of IM and MICs at distant site determines massive increase of metastasis formation, that can be prevented by the combination with a CXCR4 inhibitor (Bertolini et al., 2021).

3. Factors regulating osteotropism

For years, cancer dissemination has been thought to be a late process in tumorigenesis, but now it is known that it can be an early and highly inefficient process by which tumor cells can enter vasculature, travel in circulation as circulating cancer cells (CTCs), extravasate and colonize distant sites. Indeed, also early-stage tumor can release several CTCs in circulation, that become DTCs once landed to specific organs in which they can find right condition for survival (Braun et al., 2005; Massague and Obenauf, 2016). To survive in a distant site, DTCs must activate mechanisms of immune escape and create a supportive niche, where they grow and then give origin to an overt metastasis (Chambers et al., 2002). Thus, osteotropism is regulated by different biological and molecular processes, which are activated by DTCs, according to their survival needs and allowing them to end with bone metastasis formation (Table 1). Below we will discuss some of the bone metastatic steps taken by CSCs in general, with a particular focus on NSCLC MICs.

3.1. Chemokine gradient promotes homing to bone

The chemokine receptor CXCR4 has been demonstrated to regulate several processes of tumor cells and in particular CSCs, by activating different pathways that can promote cell survival, proliferation, cancer cells dissemination and drug resistance (Wang et al., 2016).

The chemokine SDF-1 (also known as CXCL12) is the ligand of CXCR4 and it is expressed in the most common sites of metastasis such as lymph nodes, lungs, liver, and bone marrow. CXCR4/SDF-1 axis acts as driver of cancer cells from different tumors to their transfer in the bloodstream, and then to extravasate in secondary organs, such as bone, interacting with the bone marrow stromal components (Coniglio, 2018; Morein et al., 2020; Chatterjee et al., 2014). NSCLC stem cells, which highly express CXCR4, are attracted into the bone marrow, leading to bone metastatic process initiation (Bertolini et al., 2015; Phillips et al., 2003), Fig. 1. Moreover, the analysis of CXCR4 in primary tumors demonstrated that metastatic NSCLC show a higher expression of CXCR4 than non-metastatic NSCLC (Su et al., 2005; Zhou et al., 2015).

CXCR7 is a G-protein-coupled receptors, that can act both as signaling or non-signaling scavenger/decoy receptor for SDF-1 (Wang et al., 2018). CSCs of several tumor types exploit CXCR4/CXCR7 receptors for migration, dissemination and colonization of SDF-1 rich organs (Lopez-Gil et al., 2021). Several molecules targeting CXCR4/CXCR7/CXCL12 axis have been tested to impair cancer dissemination and to sensitize CSC to chemotherapy/immunotherapy (Duda et al., 2011; Daniel et al., 2020).

CXCR4/SDF-1 axis is known for hematopoietic stem cell (HSC) homing (Sharma et al., 2011) and it is also involved in bone healing after fractures (Yellowley, 2013), indeed SDF-1 has an important role in mesenchymal stem cell (MSC) migration at fracture site (Dar et al., 2006; Kitaori et al., 2009). Moreover, SDF-1 directly enhances osteoclastogenesis and regulates OC function (Okada et al., 2016; Hatano et al., 2018). Recently, it has also been showed that CXCR7 agonist inhibits the enhancement of SDF-1-induced osteoclastogenesis (Nugraha et al., 2022). Thus, the relevance in targeting SDF-1 pathway is due to its double effect, both of CSCs and OCs, resulting in the block of bone metastases.

3.2. The dense network of capillaries in the cancellous bone

In the bone marrow, endothelial cells are organized in fenestrated sinusoids, which allow for extravasation of CTCs, that find oxygen and nutrients in abundance, promoting bone marrow colonization. The cross-talk between bone and endothelial cells is commonly active during bone modeling, where angiogenesis and osteogenesis are coupled in an hypoxic microenvironment (Ramasamy et al., 2016; Grosso et al., 2017; Wang et al., 2007; Hulley et al., 2017). Endothelial cells stimulate osteogenic pathways for bone repair, and osteogenic precursors release angiogenic factors, such as vascular endothelial growth factor (VEGF) (Stucker et al., 2020). In tumors, hypoxia increases angiogenesis by stimulating the expression of VEGF and its receptors, angiopoietin, basal fibroblast growth factor (bFGF), platelet-derived growth factor (PDGF), stem cell factor (SCF), osteopontin (OPN), and matrix metalloproteinases (MMPs) (Krock et al., 2011). In bone marrow, VEGF can promote OB differentiation and activity, but also osteoclastogenesis, fueling the cancer-bone vicious cycle (Hu and Olsen, 2016; Kitagawa et al., 2005; Yang et al., 2008), Fig. 1. In particular, the perivascular niche that comprises endothelial cells, pericytes and BMSCs cells contribute to maintain metastatic CSCs in dormant state in breast cancer (Kusumbe, 2016; Ghajar et al., 2013). Moreover, it has been reported that endothelial cells can transdifferentiate into OB-like cells in prostate cancer, inducing bone metastasis (Lin et al., 2017). Such transition from endothelial cells to OBs is induced by tumor itself to promote its progression and it is part of the tumor-induced stromal reprogramming, which is one of the strategies engaged by tumor to modify the microenvironment (Yu et al., 2021).

3.3. The role of hypoxia on bone tumor progression

Hypoxia-inducible factor-1α (HIF-1α) is a mediator of the metastatic organotropism from the tumor cells, indeed it is highly expressed in metastases. Bone is a hypoxic tissue, and it is known that hypoxia regulates bone remodeling through the stabilization of HIF transcription factors. Furthermore, hypoxia contributes to the coupling between angiogenesis and osteogenesis (Wang et al., 2007), and control bone cell activity (Hulley et al., 2017; Bentovim et al., 2012). Hypoxia induces OBs and cancer cells to release RANKL, VEGF, colony stimulating factor (CSF-1), IGF-II, which stimulates OCs (Knowles, 2015). In the bone microenvironment, hypoxia regulates some immunosuppressive subset of immune cells, inducing the up-regulation of immune checkpoint in myeloid derived suppressor cells (MDSCs), that promote T cell exhaustion, thus contributing to the formation of the immunosuppressive environment (Doedens et al., 2010). This aspect will be treated in detail in a following chapter.

Hypoxia regulates OCT4, SOX2 and NANOG, the master genes of stemness (Mimeault and Batra, 2013; Abou Khouzam et al., 2020; Najafi

Table 1
Factors regulating osteotropism.

Survival strategies in bone	NSCLC DTC features	References
Chemokine gradient	Expression of CXCR4/CXCR7 receptors Migration to bone producing SDF-1	Bertolini et al., 2015; Coniglio et al., 2018; Morein et al., 2020
Plasticity	EMT confer stemness features and capability to migrate	Bertolini et al., 2009; Mani et al., 2008
Dormancy	Quiescent state confers chemotherapy-resistance and immune system escape	Esposito and Kang, 2014; Endo and Inoue, 2019; Phan and Croucher, 2020
Neoangiogenesis, hypoxia	VEGF production regulates OCs and OBs HIF-1α is highly expressed by NSCLC CSCs	Hu and Olsen, 2016; Pezzuto et al., 2019
Immune evasion	Creation of an immunosuppressive microenvironment	Abou Khouzam et al., 2020; Fortunato et al., 2020; Bertolini et al., 2022

et al., 2020), and HIF-1α promotes EMT of cancer cells, leading to the maintenance and the dormancy of CSCs (Tam et al., 2020). In breast cancer, HIF-1α increases circulating levels of SDF-1, thus activating the CXCR4 signaling, which is associated to the dissemination of cancer cells (Devignes et al., 2018). In NSCLC cells, HIF-1α has been shown to promote the stemness of cancer cells through the regulation of several pathways, including PI3K/Akt/mTOR (Gong et al., 2018). Moreover, the expression of HIF-1α is high in NSCLC, it correlates with bone metastases, and it is predictive of poor prognosis (Pezzuto et al., 2019). More recently, it has been shown that hypoxia can enhance NSCLC stem cells by up-regulating stemness, drug resistance, cell proliferation, migration and invasion through the endothelial cell-specific molecule-1 (ESM1), which is often overexpressed in different tumors (Gu et al., 2021). Furthermore, HIF-1α induces up-regulation of Tie1 (Tyrosine kinase with immunoglobulin and epidermal growth factor homology domains) expression, and it promotes cisplatin resistance through the stimulation of CSCs in NSCLC (Li et al., 2021).

3.4. Dormancy or overt metastatic growth in bone marrow

DTCs in bone marrow are less differentiated compared to the primary tumor cells (Coleman et al., 2020a) and are enriched for the subset of MICs, with mesenchymal traits and the capability to initiate metastasis (Yu-Lee et al., 2018). DTCs can establish a supportive niche, where they can lay in a dormant state or activate in overt metastasis because of different factors (Phan and Croucher, 2020), Fig. 1. The status of dormant cells allows them to be resistant at the conventional radio-chemotherapy since they are not proliferating, and contemporary to interact with the bone microenvironment and immune system, inducing a tumor supportive environment and an immunosuppressive (Endo and Inoue, 2019; Jahanban-Esfahlan et al., 2019).

Bone is an attractive site for DTCs, since they can compete with hosts HSCs for the lodgement in the niches (Calvi et al., 2003). Indeed, the same strategies adopted by HSCs to maintain their dormant phenotype and ability to switch in a proliferative status may also be adapted by DTCs (Esposito and Kang, 2014). Osteoblasts (OBs), which are a component of HSC niche, express SDF-1 and annexin-II (Anxa2), that attract both HSCs and cancer cells expressing their receptors, CXCR4 and Anxa2r, respectively (Shiozawa et al., 2008). HSC niches express adhesion molecules and secrete factors able to induce dormancy of DTCs, which can remain in a quiescent state in the bone marrow of cancer patients for years (Shiozawa et al., 2010). MSCs represent the other population of normal stem cell present in the bone marrow. They regulate HSCs, which in turn are mainly responsible for maintaining pluripotency of MSCs. MSCs interact also with DTCs inducing their doemant phenotype through (i) a direct intercellular communication mediated by miRNA and exosomes, able to block the G0/G1 transition (Bliss et al., 2016); (ii) the secretion of growth-suppressive factors, such as TGF-β2, bone morphogenetic protein 7 (BMP7), growth arrest specific 6 (GAS6) and leukemia inhibitory factor (LIF) (Eltoukhy et al., 2018). In bone marrow, the presence of a stable vasculature, for example characterized by the expression of thrombospondin-1 (TSP-1), is associated to dormancy (Ghajar et al., 2013). Conversely, a sprouting neo-vasculature, with endothelial cells producing tenascin C, fibronectin, periostin and collagen I accelerates the outgrowth of DTCs (Oskarsson et al., 2014).

Alterations of the acidic and hypoxic conditions, as well as the high extracellular calcium concentration are associated to an increased OC activation, with a consequent release of many growth factors, contained in the bone matrix such as IGF, platelet-derived growth factor (PDGF), fibroblast growth factors (FGFs) and calcium (Ca^{2+}), which promote cancer cell growth (Lou et al., 2011), Fig. 1.

Also, genetic and epigenetic mutations influence the capability of DTCs to form bone metastases, which is highly variable, reflecting the different rate of genomic alterations (Celia-Terrassa and Kang, 2018).

The immune system is involved in the control of dormancy state of DTCs, and the topic deserves a whole discussion, which is out of this revision, thus we suggest an interesting review outlining the mechanism of immune cells in regulating dormancy (Jiang et al., 2021).

4. Bone immune microenvironment

4.1. The immunosuppressive subsets of cells affect bone cells

Bone homeostasis is regulated by the activity of bone-resorbing OCs and bone-forming OBs. DTCs and immune cells interfere with the co-ordinated activity of OC and OBs, providing a favorable soil for bone metastases. DTCs, enriched in CSCs localize in the bone marrow, escape the immune system control, because this is an "immunocompromised area". Indeed, bone is endowed of a peculiar immune microenvironment, which is less immunoreactive than other organs, due to the presence of the HSC niches, which need protection (Abou Khouzam et al., 2020). Bone marrow is characterized by many immunosuppressive cells, such as MDSCs, regulatory T cells (Tregs), and tumor-associated macrophages (TAMs), which weaken the activity of cytotoxic lymphocytes involved in tumor immunosurveillance (Baschuk et al., 2015; Almand et al., 2001) and interact with bone cells.

MDSCs promote cancer progression in pleiotropic ways, such as by shaping tumor microenvironment and metastatic niches, by activating immunosuppressive mechanisms and inflammation (Wang et al., 2019). The infiltration of MDSCs within NSCLC tissues has been detected in a patient-derived xenograft model, and it was correlated with cancer progression and a poor patient's prognosis. These MDSCs directly promote metastasis through the EMT process, since they express CCL11, that activate Akt and Erk signaling pathway, promoting NSCLC metastasis (Lin et al., 2021). In pre-clinical model of NSCLC, a population of MDSCs, identified by CCR2/CXCR4 expression, was able to help CD133+CXCR4+ MIC extravasation at the metastatic site and to induce their expansion through the release of SDF-1 (Bertolini et al., 2021). In NSCLC, a higher frequency of a MDSC subset was associated with reduced recurrence-free survival (Zhang et al., 2015). Moreover, the increase of circulating MDSCs in NSCLC patients' peripheral blood is associated with a reduced overall survival (OS). Indeed, patients treated with anti-PD-1 immunotherapy showed reduced circulating MDSCs, a longer progression free survival (PFS) and OS (Koh et al., 2020).

Particularly relevant it is also the fact that MDSCs can differentiate into OCs, contributing to bone metastatic osteolysis in a mice model of bone metastatic breast cancer (Danilin et al., 2012). The accumulation and activity of MDSCs in bone microenvironment is also regulated by dickkopf-1 (DKK-1) Wnt signaling pathway inhibitor, indeed its neutralization reduced the number of MDSCs and the tumor growth (D'Amico et al., 2016). It has been shown that DKK-1 was expressed in tumor preferentially metastasizing to bone, where it down regulates OB activity, leading to increase bone resorption by OCs (Zhuang et al., 2017). Furthermore, DKK-1 promotes vasculogenic mimicry, by inducing EMT and CSCs in NSCLC (Yao et al., 2016). Thus, therapeutic strategy based on DKK-1 inhibition can exert a double effect hindering MDSC activity and controlling bone metastases.

In bone marrow, CD4+CD25+ Tregs traffic through CXCL12/CXCR4 signaling pathway (Zou et al., 2004). Treg cells inhibit osteoclastogenesis due to IL-4 and IL-10, which are dependent on CTLA4 (Kim et al., 2007), an immune checkpoint, currently associated with immunotherapy. CTLA4 can also bind to CD80/CD86 on OC precursors, promoting OC apoptosis (Zaiss et al., 2007), and preventing bone resorption (Zaiss et al., 2010). Conditioned medium from NSCLC MICs has been demonstrated able to promote Tregs, indeed targeting MICs with an anti-CXCR4 treatment prevented the stimulation of Tregs (Fortunato et al., 2020).

TAM promote the growth of bone metastases as shown in experimental model of OCs and macrophages depletion (Pollard, 2004). In in vitro experiments, Fortunato et al. showed that conditioned medium from NSCLC MICs induced TAM polarization of macrophages toward M2

phenotype, with the up-regulation of the immunosuppressive IL-10 and VEGF, and the decrease of the inflammatory cytokines IL-12 and IL-6. These data confirmed the immunosuppressive behavior of MICs, which also show a high expression of PD-L1 and CD38, CD73 enzymes able to catabolize the production of adenosine as well as high release of the immunosuppressive IL-10 (Fortunato et al., 2020). The level of extracellular adenosine in the bone microenvironment has been shown to be important for bone homeostasis (Sauer et al., 2009). Human OB precursors produce extracellular adenosine, which modulates the secretion of interleukin 6 (IL-6) and OPG, contributing to the regulation of bone resorption and formation (Evans et al., 2006). We recently demonstrated that NSCLC CSCs modulate the levels of adenosine in the presence of bone cells, such as OCs and OBs. Particularly, the increase of adenosine induced by NSCLC CSCs, lowers pH in the microenvironment, and activate OCs in an *in vitro* co-culture of OCs and CSCs (Bertolini et al., 2022). Therefore, the immunosuppressive ability of CSCs could generate at the bone metastatic site a proficient microenvironment for their seeding and colonization.

4.2. Bone cells affect immune cells

Besides being important components of the HSC niche, OBs promotes HSC proliferation through PTH receptor stimulation (Calvi et al., 2003) and support all differentiation stages of B cells (Zhu et al., 2007). OB conditional ablation in mice resulted in lack of B cell differentiation. Indeed, OBs release interleukin-7 (IL-7) and SDF-1, that are fundamental for survival and activity of B cells (Miller et al., 2002; Egawa et al., 2001).

OCs mobilize HSCs, by releasing cathepsin K, that degrades matrix in the areas of bone remodeling, inducing the release of SDF-1, stem cell factor (SCF), and osteopontin (OPN), which deprive the bone niche of HSC-binding sites (Kollet et al., 2006). In mice, where OC activity was suppressed, MSCs increased, but they were unable to differentiate into OBs, thus HSC homing to bone and HSC niches were impaired (Mansour et al., 2012). These mice also showed an impaired B cell maturation and T-cell activation (Blin-Wakkach et al., 2004). Mice knockout for RANK ligand (RANKL) had osteopetrosis due to the absence of OCs, but also showed the impairment of B and T lymphocytes development and lack of lymph node organogenesis (Yasuda et al., 1998; Dougall et al., 1999). OCs express immunosuppressive cytokines, such as IL-10 and metabolic enzymes as indoleamine 2,3-dioxygenase 1 (IDO1), which limits T cell activity (Li et al., 2010; Li et al., 2014; Kiesel et al., 2009).

Osteocytes release Receptor Activator of NFkB Ligand (RANKL), that supports OCs and lymphopoiesis. The absence of osteocytes leads to loss of lymphoid-supporting stroma in bone marrow and in thymus, resulting in a severe lymphopenia, which is reverted whether osteocytes are re-established (Sato et al., 2013). In a mice model of estrogen deficiency, RANKL released by osteocytes is responsible for the increase in B cells and bone loss (Fujiwara et al., 2016).

5. RANK/RANKL axis as target to block NSCLC CSCs

RANKL is crucial for OC formation, function, and survival (Boyle WJ, Simonet WS, Lacey DL. Osteoclast differentiation and activation. Nature 2003). RANK is expressed in each lung cancer histotype, particularly Rao et al. showed that 72 % of lung adenocarcinomas were positive for RANK (Rao et al., 2017), according to the previously published data, deriving from samples of primarily early stage treatment-naive resected NSCLCs (Botling et al., 2013). Moreover, the RANK/RANKL/OPG system is highly associated with tumor invasiveness and metastasis, indeed pre-clinical models of bone metastases demonstrated that RANKL inhibition was able to prevent tumor-associated bone metastases (Miller et al., 2014; Feeley et al., 2006; Tannehill-Gregg et al., 2006). Randomized clinical trials showed that the monoclonal antibody denosumab, which binds to RANKL, blocking the RANK/RANKL binding reduced the SREs and improved overall survival (OS) when compared

with zoledronic acid (ZA) in patients with lung cancer (Henry et al., 2011). Subsequently Scagliotti et al. showed that treatment with denosumab significantly prolonged survival, in patients with bone metastases by NSCLC adenocarcinomas and squamous tumors (Scagliotti et al., 2012). Notably, a case report showed the regression of an ALK-mutated primary lung adenocarcinoma upon treatment with denosumab, suggesting that inhibition of RANKL could reflect on ALK inhibition, leading to a regression of the tumor overexpressing ALK (Curioni-Fontecedro et al., 2013).

More recently, Sisay et al. showed that the inactivation of RANK decreased the NSCLC cancer initiation capabilities by reducing CSC formation, delaying the malignant lung tumor progression (Rao et al., 2017). The capability of denosumab to target CSCs is particular important in view to prevent bone metastases formation. A recent published review reported the experience of 10 years after approval of denosumab in adjuvant regimen to prevent bone metastasis induced SREs, showing that NSCLC patients with bone metastases and a life expectancy major than 3 months, must be treated with both denosumab or ZA (Coleman et al., 2020b). Moreover, combination of denosumab with ICIs, has been demonstrated effective in controlling bone metastases in lung cancer (Liede et al., 2018; Myoken et al., 2020).

6. Checkpoint inhibitors in bone metastases

Checkpoint pathways are involved in OC formation. For instance, the CD200/CD200R axis, which plays an inhibitory role in T cell response (Siva et al., 2008), also modulates differentiation of OCs and bone mass (Cui et al., 2007). Indeed, PD-1 deficiency leads to a reduction of osteoclastogenesis without altering the number of OBs (Nagahama et al., 2004). PD-1 was highly expressed in pre-OCs, while its expression was lost during OC formation *in vitro*. The addiction of PD-L1 in *in vitro* cell cultures could increase low-dose RANKL-induced OC differentiation. Moreover, in the presence of RANKL both pre-OCs and mature OCs increased Chemokine (C-C motif) ligand 2 (CCL2) secretion (Wang et al., 2020). In a mice model of Lewis Lung Carcinoma (LLC), Wang et al. reported that infiltrating tumor cells in bone marrow microenvironment express PD-L1 and CCL2 that stimulate OC differentiation, according to previously published works by other groups (Khan et al., 2016; Kim et al., 2006). CCL2 expression was upregulated by PD-L1 activation of JNK during OC differentiation, contributing to osteoclastogenesis. Blocking PD-1, using monoclonal antibody, resulted in an inhibition of CCL2 production and of OC formation (Wang et al., 2020). NSCLC patients who received ICIs as first-line therapy showed an increase in overall survival higher than patients treated in second line (Liang et al., 2020; Qiang et al., 2022). This result suggests that likely these patients had an immune system not completely suppressed, thus the checkpoint inhibitor helps the anti-tumor immune response. Despite the effect of checkpoint inhibitors on OC formation and activity, the role of immunotherapy on bone metastases is currently under investigation and results are not completely clear. Comparing advanced NSCLC patients with and without bone metastases, treated with anti-PD-1, no difference was observed in median progression-free survival (PFS) (Tamiya et al., 2018). Multicenter retrospective studies on patients treated with anti-PDL-1, reported contrasting results since a study did not show significant differences in PFS associated to bone metastases (Kawachi et al., 2020), while another one reported that the presence of bone metastases is associated to worse clinical outcomes (Cortellini et al., 2020). In line with this last result, Li et al.'s reported that bone metastases negatively affect the efficacy of ICI monotherapy, and neither palliative radiotherapy nor bisphosphonates could improve the overall survival (Li et al., 2020). In general, a greater number of bone metastases correlates with a more advanced stage of NSCLC and consequently with a poor response to immunotherapy (Nakata et al., 2020). Nonetheless, these same Authors suggest also that the monitoring of bone metastases during the treatment with anti-PD-1 could be useful to evaluate the prognosis, since a bone response within 2 months from the beginning of the

monotherapy with anti-PD-1 was indicative of a better PFS (Nakata et al., 2020). In another study by Schmid et al., anti-PD-1 treatment failed, and patients reported metastatic progression (Schmid et al., 2018). In two case reports, anti-PDL-1 treatment resulted effective in control bone metastases (Asano et al., 2021). Recently, a retrospective study on NSCLC patients with bone metastases, contemporary treated with bone-targeted therapy and anti-PD-L1, showed an increased survival (Qiang et al., 2022). Advanced NSCLC patients with bone metastases should receive the ICI early, in order to improve the tumor microenvironment of the metastatic sites, therefore enhancing efficacy and prolonging survival. Indeed, it has been demonstrated that the anti-PD-L1 was more effective in monotherapy both when used in first- or second-line therapy for patients with advanced NSCLC (Peters et al., 2018).

7. Conclusions

Due to the success of the new anti-cancer treatments, NSCLC patients' survival has been prolonged. Unfortunately, increased survival may also be accompanied with an increased chance to develop bone metastases. MICs, a subset of CSCs particularly endowed of metastasis initiating capabilities, have been recognized as drug resistant, tumorigenic and able to seed secondary organ, such as bone. Understanding how MICs reach, survive and grow in bone is mandatory to identify potential targets able to inhibit bone metastasis formation.

NSCLC MICs can exploit SDF-1 gradient to reach bone where they can find a favorable microenvironment, composed of bone cells, HSCs, dense network of capillaries and hypoxia condition, which may all concur in facilitating MIC seeding and colonization of the bones. Among the several strategies proposed to prevent CSC-induced metastasis, preventing the chemokine axis governing CSC dissemination to bone metastasis might impair metastasis formation. Moreover, the combination of anti-resorptive agents with ICI drugs, as well as ICI monotherapy, should be effective in preventing/controlling NSCLC CSC-derived bone metastasis especially whether administered early in the history of the disease, thus modifying the timing of administration so far adopted. Indeed, helping the immune system to exit the immunosuppressive state induced by CSCs could avoid their outgrowth that ends with overt metastatization, overall resulting in long-lasting efficacy in treatment of patients with NSCLC.

Declaration of competing interest

None.

Acknowledgements

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