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Mini Review

Dendritic Cells as Double-Agents for Breast Tumor Pre-Metastatic Bone Disease Establishment

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ABSTRACT

Pre-metastatic niche formation at distant sites can be initiated by the primary tumor through "education" of non-tumoral cells present in the primary cancerous niche. Among other participants, immune cells and their secreted factors can boost the successful seeding of the distant disease. Accordingly, we showed that RANKL production by breast tumor-primed T cells is required for development of bone metastasis. Pro-osteoclastogenic tumor-specific RANKL$^+$ T cells were shown as messengers from the periphery to the bone marrow, where they alter bone turnover homeostasis in favour of osteoclasts and before tumor colonization. Pre-metastatic T cell-mediated osteolytic disease generates a rich environment that will allow further colonization of the bone cavity by the metastatic clones. Once initial seeding of the bone tissue is achieved, tumor cells can continue the osteolytic process on their own, feeding themselves through the vicious cycle established. More recently, we explored the contribution of dendritic cells for the maintenance of such tumor-specific T cells activity for bone marrow pre-metastatic niche formation. Indeed, dendritic cells can act as both an APC for RANKL$^+$ tumor-specific T cells activation and as an osteoclast-like cell, amplifying the pre-osteolytic phenomena. Here, we discuss the potential differentiation of DCs into OCs for bone pre osteolytic disease establishment, either directly or through the maintenance of RANKL$^+$ T cell inside the bone marrow. The understanding of the cellular and molecular interactions that build the bone pre-metastatic niche can be directed towards prevention and/or treatment of metastatic bone disease.

Keywords: Bone metastasis; Dendritic cells; T cells; Osteoclasts; Breast tumor; Pre-metastatic niche

ABBREVIATIONS

RANK: Receptor Activator of NF-κB; RANKL: Receptor Activator of NF-κB ligand; OPG: Osteoprotegerin; M-CSF: Macrophage Colony-Stimulating Factor; TRAP: Tartrate-Resistant Acid Phosphatase; NFATc1: Transcription Factor Nuclear Factor of Activated T Cells

INTRODUCTION

Metastasis is the leading cause of death in cancer patients. Bone is one frequent site for breast cancer metastasis with around 70% of incidence in patients with invasive disease, affecting both quality of life and survival rates [1,2]. Once breast cancer cells have spread to bone´s microenvironment it become incurable, causing bone destruction and complications secondary to bone metastasis such as bone pain, pathologic fractures, hypercalcemia and paralysis due to spinal cord compression [3]. Mundy´s vicious cycle hypothesis proposed that once in the bone, breast tumor cells dysregulate bone turnover homeostasis, that is controlled by the crosstalk between osteoclasts (OCs), bone-resorbing cells; osteoblasts (OBs), bone-forming cell; osteocytes, mature osteoblasts embedded in the bone matrix; and chondrocytes, via RANK-RANKL-OPG molecular system [4]. This dysregulation tips the balance in favor of osteoclasts leading to an intense release of growth factors stored in the mineralized matrix, which in turn stimulate tumor outgrowth, and give rise to a clinically significant osteolytic disease characterized by a constant loss of bone mass and haematological alterations [1,5].

Primary breast cancer, have been shown to ''prepare'' distant organs for tumor cell colonization even before their arrival [6-8], reinforcing the active participation of the metastatic tissue as first proposed by

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Stephen Paget in his "seed and soil" theory [6,9]. Immune cells such as macrophages (Mφs) [10,11], dendritic cells (DCs) [12], neutrophils [13] and T cells [11,14-16], are associated with the formation of those permissive and supportive microenvironments in secondary organ sites, termed "pre-metastatic niches", highlighting the importance of basic mechanisms responsible for tumor cells distant establishment [7,17]. Accordingly, it has been found that cells of the immune system acting as pro-tumor cells are enriched in the pre-metastatic niches and support cancer cell seeding via paracrine signaling and/or by suppressing anti-tumor immune cells [7,17-19].

Dendritic cells (DCs), the most potent antigen-presenting cells (APCs) known and osteoclasts share several features, as they both originate from myeloid progenitors [20-27]. DCs display a high developmental and functional plasticity depending on local factors and stimuli encountered during their differentiation and maturation, providing a multitude of necessary signals crucial for shaping the immune response [28]. Indeed, in the past 15 years, several studies showed that DCs plasticity can allow their differentiation into OCs multinucleated giant cells (DC-OCs) [29-31]. In this mini-review, we discuss DCs plasticity properties regarding bone marrow pre-metastatic niche formation [32].

T CELL-DEPENDENT FORMATION OF BREAST TUMOR PRE-METASTATIC BONE NICHE

Bone and hematopoietic immune cells share the same microenvironment in the bone marrow and interact with each other to cooperatively carry out the functional activities of osteoimmune system [4,33]. This interaction has been appreciated since pioneering studies on immune cell-derived OC-activating factors in the 1970s and 1980s [34–36]. It is well known that both systems share a variety of molecules, including cytokines, chemokines, hormones, receptors and transcription factors [4]. For the last 20 years, studies from the osteoimmunology field have revealed that immune cells exert a powerful impact on bone remodeling system mechanisms under pathological conditions [4,37-49], and new evidences have demonstrated that bone cells reciprocally regulate immune cells and hematopoiesis [38]. Indeed, several studies showed that there is a close relationship between the abnormal activation of pathogenic specific T cell subtypes (Th17 and exFoxP3Th17) and osteoclasts dysregulated activities [33,38,41] in the context of rheumatoid arthritis [4,38,41,42], periodontitis [43,44], osteoporosis [45,46] and bone metastases [15].

Using the 4T1 triple negative metastatic mouse model of breast carcinoma, we have previously demonstrated that RANKL tumor-specific $CD4^+$ Th17 T cells are the major players for pre-metastatic niche bone formation in the 4T1 breast tumor model [15]. In fact, osteolytic disease is observed before tumor cells colonize the bone cavity. This pre-metastatic osteolytic disease is mediated by RANKL, produced by specific-tumor T cells. Moreover, inhibition of RANKL production (using shRNA) in fresh tumor-primed T cells does not generate osteolytic disease and the associated pre-metastatic niche. Consequently, development of bone metastases is completely absent. Altogether, we proposed an extra step to Mundy's vicious cycle where initial bone consumption, mediated by pre-metastatic T cells, generates a rich microenvironment that will allow further colonization of the bone cavity by the metastatic clones [47]. Once the initial seeding of the bone tissue is achieved, tumor cells shall continue the osteolytic process on their own, feeding themselves through the vicious cycle established within the bone microenvironment [15].

Even though antigen-primed and memory T cells have been described to seed the bone marrow in different models [48–50], it is still unclear whether the large fraction of activated/memory T cell in the marrow is activated in lymph nodes or locally [48,49,51]. As pre-metastatic osteolytic disease happens much before metastatic colonization, it is not known how the tumor antigen would get to the bone marrow to be recognized by T cells. We envisage at least two non-exclusive possibilities: (i) cancer-derived exosomes could travel to the bone cavity, and provide tumor antigens to be processed and presented by local resident DCs [52,53] and/or (ii) DCs loaded with tumor antigens at the primary tumor or at the tumor draining lymph nodes, can migrate to the bone marrow where antigen presentation would take place [54,55]. However, regardless of priming, in breast tumor bone metastasis, the role of DCs has never been addressed.

DENDRITIC CELLS DEVELOPMENT INTO OSTEOCLASTS TYPE CELLS (DC-OCs)

Dendritic cells (DCs), the most potent antigen-presenting cells (APCs) are responsible for activation of naïve T cells and orchestration of tolerogenic and immunogenic responses [22]. DCs present antigens to T cells in the context of major histocompatibility (MHC) molecules, with additional input delivered in the form of costimulatory surface ligands and cytokines [27,56,57]. According to the nature of DCs stimuli, different specific T cells phenotypes would be achieved [27,56,57]. Many subsets of DCs with unique and specific functions, morphology, and localization have been described [58]. They display a high developmental and functional plasticity depending on local factors and stimuli encountered during their differentiation and maturation, providing a multitude of necessary signals for shaping the immune responses [27,56,57]. Plasticity can also allow DCs to develop into other cell types, among them OCs (DC-OC), what is not unexpected considering their same origin from common myelopoietic stem cell progenitors [29–31].

DCs and OCs are both affected by multiple shared immune factors in bone marrow microenvironment [29,59]. Many crucial cytokines for DCs immune physiology have been indicated to be equally important for OCs differentiation in the skeletal system [60–62]. Both OCs and DCs are activated through RANK-RANKL-OPG signaling pathway, which not only plays important roles in homeostatic bone remodeling [38,63] but is also essential for the development and function of primary and secondary lymphoid organs, as well as the mammary tissue [38,62,64–67]. Regarding the skeletal system, RANKL or RANK-deficient mice present with severe osteopetrosis due to an osteoclast deficiency and lack lymph nodes and Peyer´s patches as well [68–70]. In contrast, mice lacking OPG, the decoy receptor for RANKL, exhibit severe osteoporosis characterized by an intense trabecular and cortical bone porosity. Surprisingly, these animals also exhibit medial calcification of the aorta and renal arteries, suggesting that regulation of OPG signaling pathway play a role in the long observed association between osteoporosis and vascular calcification [71,72].

Regarding RANK-RANKL-OPG signaling pathway in osteoimmune system, effector T cells expressing RANKL promote DC survival and increase their longevity, via CD40 upregulation and leading

to RANK molecule overexpression on DCs [73,74]. In addition, RANK-RANKL system increases antigen-specific primary and memory T cell responses *in vivo* [75]. OPG, a CD40-regulated gene in B cells and DCs, also regulates B cell development and function regulating B cell maturation for efficient antibody responses [76]. Moreover, OPG, which can also be expressed by DCs, binds to TRAIL (TNF-related apoptosis-inducing ligand) produced by activated T cells [77]. This reciprocal action induces apoptosis of DCs, suggesting that OPG might also be a regulatory key factor of DC survival [29,77]. RANK is expressed by both cell types [29,59,78–80], DC and OC, and its activation is dependent on its ligation by RANKL present and/or secreted by immune and/or bone cells, in homeostatic and pathological conditions [61,69,73,81–84]. For OC differentiation and activation, RANKL-RANK must encounter on the surface of pre-OC. As a result, the intracellular signaling cascades lead to the induction of NFATc1 (transcription factor nuclear factor of activated T cells 1), the master regulator of osteoclastogenesis [85–87]. Considering the above, it is reasonable to think that RANK signaling on the surface of a DC, present inside the BM and close to OC niches, could differentiate into OCs.

Indeed, for the last 15 years, it has been reported that immature DCs can develop into OCs *in vitro*, when cultured with osteoclastogenic factors, M-CSF and RANKL or RA synovial fluids containing pro-osteoclastogenic cytokines [30,88,89]. The same phenomenon was also shown *in vivo* [90–92]. In humans, when multiple myeloma derived DCs were cultured with RANKL$^+$ plasma cells they differentiated into OCs [93]. Moreover, DCs derived from Langerhans cells Histiocytosis patients are capable to develop into OC type-cells when stimulated by IL-17A [94–96]. In addition, citrullinated proteins and RA specific anti-citrullinated protein antibodies deposit on DCs surface as immune complexes and promote differentiation toward the OC lineage, implicating DC-OCs in the bone consumption observed in RA [97]. Regardless the presence of DCs at bone resorptive sites during inflammatory conditions [29,69,98–103], their direct contribution to bone resorption, either as APCs, keeping osteoclastogenic Th17 T cells locally activated, or overcoming their own phenotype achieving OCs mature functional phenotype, has yet to be solved. In pathological conditions, it has been assumed that the increase in pro-inflammatory cytokines or the presence of bacterial antigens could provide a supportive environment for the development of DCs into OCs [30,90,91,103]. Indeed, it has been confirmed that multinucleated giant cells expressing markers of DCs and OCs are located next to the bone in inflammatory bone disease [103]. In bone metastasis, DC-OC differentiation was shown to be induced by RANKL, either recombinant or produced by specific-tumor T cells [32]. Although DC-OC and conventional OCs have similar morphological features and mineral matrix resorbing activity, their role regarding T cell activation is not the same, in bone pre-metastatic disease context [32].

DC-OCs AS POTENT PLAYERS IN THE CONTEXT OF BREAST TUMOR BONE METASTASIS

Several reports have indicated that IL-23 plays a critical role in inflammatory Th17 immunity establishment [104,105]. It does so by enhancing IL-17 production *in vitro* and *in vivo* through the expansion of already committed Th17 cells [106,107]. Indeed, two independent reports showed that systemic IL-23 [108] or IL-23 expressed by conventional OCs [109] drives severe arthritis causing a profound osteolytic phenotype mediated by direct activation of CD4$^+$ Th17 T cells. Additionally, circulating DCs expressing IL-23 are normally recruited to inflamed tissue, where they could either play an indirect role in osteoclastogenesis by stimulating T cells to express pro-osteoclastogenic cytokines; release of TRAP and cathepsin K by resident OCs; or by direct releasing of cathepsin K itself [69]. A still-open question is whether growth factors controlling homeostatic osteoclastogenesis are also involved in de novo inflammatory-induced osteoclastogenesis of potent DC-OCs, with bone resorption activity directly participating at the inflammatory disease site. It would be of great interest to determine whether the biological function of mature OCs derived from bona fide OCs precursors or derived from immature DCs differs, either in physiological or in pathological conditions.

In fact, one characteristic function of DCs is its efficiency to activate T cells [21] and shape the T cell fate [20,21,110], characteristics not necessarily shared with OCs. Interestingly, we recently showed that OCs derived from conventional splenic DCs, but not conventional BM derived OCs, are incredibly good in activating T cell proliferation and cytokine secretion (Figure 1) [32]. DC-OCs secrete high amounts of IL-23, which in turn boosts IL-17 and RANKL production by T cells, feeding the positive osteoclastogenic loop of adaptive T cell immunity. This positive loop, not shared with conventional OCs, has IL-23 as one limiting step since blocking IL-23 with monoclonal antibody inhibits T cell IL-17 and RANKL production. Of note, is the fact that conventional OCs do not stimulate T cell proliferation, nor IL-17 and RANKL production [32]. Immune interactions between T cells and DCs, in bone inflammatory disease scenarios, responsible for DCs development into OCs type cells, were previously investigated [90,91]. It was reported that DC-OCs can partially reverse a mice osteopetrotic phenotype *in vivo* because of the presence of inflammatory CD4$^+$ T cells that are able to maintain a high RANKL expression by bone marrow stromal cells [91]. Moreover, interactions *in vitro* between activated CD4$^+$ T cells and CD11c$^+$ DCs generate DC-OCs capable of inducing bone loss after adoptive transfer *in vivo* [90].

Concerning RANKL and M-CSF cytokines dependence to induce osteoclastogenesis from BM precursor cells or DCs, high levels of RANKL is required for DC-OC development *in vivo* and for the activity and survival of DCs [63,74]. In particular, the longevity of mature DCs pretreated with RANKL is greatly enhanced [75]. Moreover, RANKL augments the ability of DCs to stimulate T cell proliferation [82,111,112]. The resulting increase in DC survival is accompanied by a proportional increase in DC-mediated T cell proliferation. Therefore, we can suppose that RANKL enriched environment set up by osteoclastogenic CD3$^+$ T cells located inside the BM probably contributes to a higher DC survival ratio which in turn would support T cells activities in promoting the pre-metastatic niche formation [32].

Differentiated DCs can carry antigen from peripheral tissues via lymphatics to lymph nodes, and also travel from the peripheral tissue into the blood and to the spleen, liver, lungs and bone marrow, where they were better retained than in most other tissues, by microvascular P and E-selectin as well as VCAM-1 [49,54]. Moreover, by adoptive transfer experiments in mice, it is already known that bone marrow can prime naive T cells and recruit effector T cells, but it also serves as a site of preferential proliferation for CD4$^+$ and CD8$^+$ T cells [49]. Altogether, it becomes clear that DCs and T cells interact with each other and, importantly, with the tissue they are in, contributing to its homeostasis.

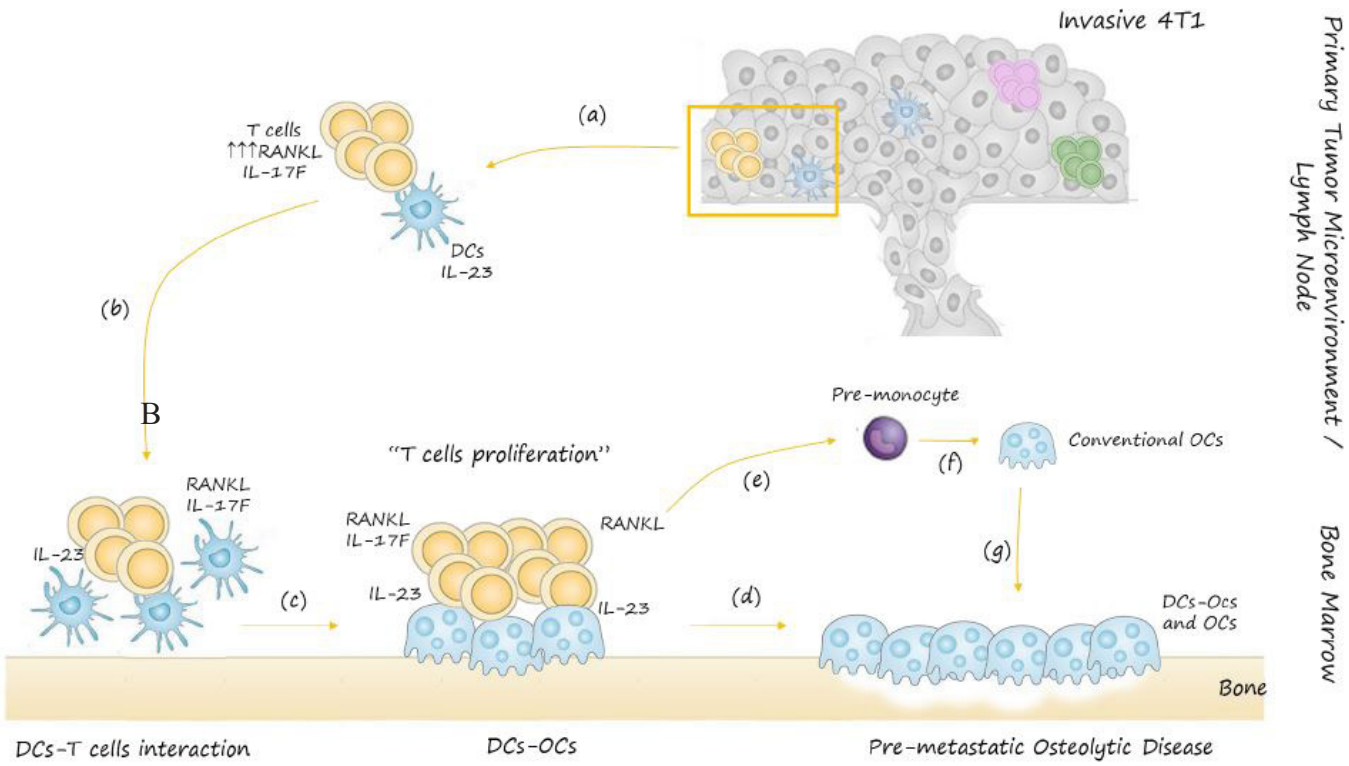

Figure 1: Dendritic cells and T cells partnership for bone marrow pre metastatic niche formation in 4T1 breast tumor bone metastases. (a) DCs and T cells activation in primary tumor microenvironment or draining lymph nodes. DCs loaded with tumor Ags at the primary tumor site or inside the draining lymph nodes, prime naïve $CD4^+$ T cells towards a Th17 phenotype, producing RANKL and IL-17F, which in turn migrate to bone marrow before bone tumor colonization. (b) T cells activation in bone marrow by migrating DCs or by resident DCs. Once immunogenic $IL\text{-}23^+$ DCs loaded with tumor Ags activate the maintenance of anti-tumor $RANKL^+$ $CD4^+$ Th17 T cells. BM resident DCs could also capture Ags from micro vesicles (for example) loaded with tumor Ags. (c) DC differentiation into OC (DC-OC). Upon T cell encounter DCs differentiate into true OC type-cells keeping their APC activity. (d) T cell expansion and keeping osteoclastic phenotype. Development of DC-OCs from BM tissue resident DCs or from migrating DCs, – both loaded with Ags, under the influence of RANKL along with other cytokines produced by tumor-specific T cells. Dysregulation of bone homeostasis by an intense activation of BM-OCs by RANKL and M-CSF under breast tumor pre metastatic osteolytic conditions induced by $RANKL^+$ $CD4^+$ Th17 T cells. Anti-tumor $RANKL^+$ $CD4^+$ Th17 T cells interaction with DC-OCs stimulate IL-23 production, which in turn will sustain $RANKL^+$ $CD4^+$ Th17 T cells phenotype. (e, f and g) More BM-OC differentiation with no further T cell stimulation. BM-OCs, after differentiation, is not capable to activate T cells in this 4T1 breast tumor pre-metastatic model. Establishment of a positive loop between activated T cells and DC-OCs. DC-OCs are as good as fresh DCs in presenting Ags to T cells, building a positive loop which can amplify the osteoclastogenic potential of T cell stimulation in the establishment of the pre metastatic niche. Whether both 4T1 derived DC-OCs and BM-OCs dwells at the same resorbing pit or are segregated into separate sub niches is unknown.

CONCLUSION

In conclusion, we showed that DC-OCs are excellent immunogenic APCs, different from OCs derived from macrophages or bone marrow conventional precursors. As so, DC-OCs will boost the T cell response unbalancing the bone remodeling system towards osteolysis. We can say that DCs are partners for $RANKL^+$ Th17 cells in the context of bone pre-metastatic osteolytic disease as both, an OC-like cells, with osteolytic capacity which keeps its excellence as antigen presenting cell.

PERSPECTIVES

We consider that our study has introduced DC-OCs as tumor-specific T cells partners for the formation and/or maintenance of breast tumor bone marrow pre-metastatic niche. Moreover, the set of our studies are revealing the cellular and molecular dynamics interaction for pre-metastatic niche formation. This complex network can be used either as prognostic tools and/or biomarkers of pre-metastatic bone niche for breast cancer patients or even as therapeutic targets. Multiple questions remain and need to be investigated to translate our current knowledge toward clinical impact.

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CONFLICT OF INTERESTS

The authors state that they have no conflict of interest.

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