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Oncobiologia

Effect of RANK+ Breast Cancer Cells' Secretome in Monocytic Differentiation

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Resumo

A via RANKL - RANK é um regulador chave da remodelação óssea e fisiopatologia de metástases ósseas, assim como um importante mediador da carcinogénese mamária mediada por hormonas. Dados preliminares do nosso laboratório sugeriram que o meio condicionado (*conditioned media*, CM) de células de cancro da mama recetor de estrogénio positivas sem amplificação de HER2 (ER+HER2-) e com sobre expressão de RANK (RANK OE) poderia induzir a diferenciação de monócitos *in vitro*. Esta evidência é particularmente importante, uma vez que o RANKL é uma quimiocina que participa no recrutamento de monócitos e macrófagos com expressão de RANK. Além disso, os macrófagos associados ao tumor (*tumor associated macrophages*, TAMs), também derivados de monócitos, podem contribuir para a inibição ou crescimento tumoral, se M1 ou M2, respetivamente.

Este projeto derivou destas evidências e visou identificar as células diferenciadas da linhagem monocítica após exposição ao secretoma de células RANK OE, através da avaliação da presença de osteoclastos e/ou macrófagos M1/M2.

Um painel de linhas de células de cancro da mama, e os seus clones RANK OE, foi cultivado sob condições padrão e os CM recolhidos. As células monocíticas RAW264.7 foram cultivadas durante 5 dias em condições de diferenciação ± 25% CM, ou mantidas em co-cultura com as diferentes linhas celulares de cancro da mama durante 5 dias em condições de diferenciação, e a osteoclastogénese foi quantificada através da contagem de osteoclastos (céulas gigantes multinucleadas com coloração TRAcP 5b positiva) e da quantificação da TRAcP 5b secretada por ELISA. Finalmente, a presença de osteoclastos e macrófagos M1/M2 foi avaliada através da análise de expressão de marcadores específicos, *nfatc*, *iNOS* e *CD206*, respectivamente, por RT-qPCR.

Os nossos resultados indicam que o secretoma de células tumorais inibe a osteoclastogénese em comparação com a adição de RANKL exógeno (controlo positivo), quer com adição do CM quer em co-cultura. Nenhum dos CM ou células tumorais foi capaz de induzir osteoclastogénese na ausência de RANKL exógeno. Em ambos os ensaios, observámos células morfologicamente diferenciadas mas sem características de osteoclastos. Verificámos ainda que a sobre expressão de RANK aumenta o efeito inibitório, o que poderá estar

associado com a expressão de osteoprotegerina (OPG), já descrita nestas células por nós. Não fomos capazes de confirmar a presença de macrófagos M1/M2 por RT-qPCR, mas colocamos a hipótese de que estas células possam ser macrófagos ou células dendríticas, o que requer uma investigação mais aprofundada.

A caracterização das células diferenciadas, bem como do secretoma das diferentes linhas celulares do cancro da mama por multiplex cytokine profiling, poderá ser de grande importância para identificar factores putativos que afetam a diferenciação monocítica, e que podem contribuir para a progressão tumoral.

Palavras-chave: Via RANKL-RANK; Osteoclastogénese; Tumor Associated Macrophages

Abstract

The RANKL - RANK pathway is a key regulator of bone remodeling and pathophysiology of bone metastases and a major mediator of hormone-driven breast carcinogenesis. Preliminary data from our lab suggested that conditioned media (CM) from breast cancer cells that are estrogen receptor positive without HER2 amplification (ER+, HER2-) and with RANK overexpression (RANK OE) could induce the differentiation of monocytes in vitro. This evidence is particularly important since RANKL has shown to be a chemokine that participates in the recruitment of RANK-expressing monocytes and macrophages. Moreover, tumor-associated macrophages (TAMs), also derived from monocytes, can inhibit or stimulate tumor growth, if M1 or M2, respectively.

This project stems from these findings and aims to identify the cells differentiated from the monocytic lineage upon exposure to the secretome of RANK OE cells, by assessing the presence of osteoclasts and/or M1/M2 macrophages.

A panel of breast cancer cell lines, and their derived RANK OE clones, was cultured under standard conditions, and their CM were collected. RAW264.7 monocytic cells were cultured for 5 days under differentiation conditions ± 25% CM, or maintained in co-culture with the different breast cancer cell lines for 5 days in differentiation conditions, and osteoclastogenesis was quantified through the counting of osteoclasts (giant multinucleated cells with positive TRAcP 5b staining) and the quantification of TRAcP 5b secreted, by ELISA. Finally, the presence of osteoclasts and M1/M2 macrophages was assessed through the analysis of the expression of specific markers, *nfatc*, *iNOS* and *CD206*, respectively, by RT-qPCR.

Our results indicate that the secretome of cancer cells inhibits osteoclastogenesis in comparison to the addition of exogenous RANKL (positive control), whether with the addition of CM or in co-culture. In both assays, we have observed morphologically differentiated cells but that did not have characteristics of osteoclasts. Furthermore, we verified that the expression of RANK increases the inhibitory effect, which might be associated with the expression of osteoprotegerin (OPG), already described in these cells by us. We were not able to confirm the presence of M1/M2

macrophages by RT-qPCR, but we hypothesize that these cells may be macrophages or dendritic cells, which requires further investigation.

The characterization of these cells and of the secretome of the different breast cancer cell lines by multiplex cytokine profiling, could be of great importance in order to identify putative factors in monocytic differentiation, and which may contribute to tumor progression.

Key Words: RANKL-RANK pathway; Osteoclastogenesis; Tumor Associated Macrophages.

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Abbreviations

AP-1, Activator protein-1

BC, Breast Cancer

CAFs, Cancer-associated fibroblasts

CM, Conditioned Media

DALYs, Disability-adjusted life years

DC, Dendritic cells

ECM, Extracellular matrix

EMT, Epithelial-to-mesenchymal transition

ER, Estrogen Receptor

ERL, extracellular signal–regulated kinase

GES, Gene expression signature

GH, Growth hormone

HER2, Human Epidermal Growth Factor Receptor 2

HR, Hormone Receptor

ICIs, Immune-checkpoint inhibitors

IFNγ, Interferon gamma

IGF1, Insulin-like growth factor 1

IKK, Inhibitor of NF-kB kinase

IL-1, Interleukin-1

iNOS, Nitric oxide synthase

JNK, c-Jun N-terminal kinase

LECs, Luminal epithelial cells

MAMs, Metastasis associated macrophages

MaSCs, Mammary stem cells

MDSC, Myeloid-derived suppressor cells

MMPs, Matrix metalloproteinases

MECs, Myoepithelial cells

OE, Overexpressing

OPG, Osteoprotegerin

PEG2, Prostaglandin E2

PR, Progesterone Receptor

PTHrP, Parathyroid hormone-related protein

RANK, Receptor activator of the nuclear factor-κb

RANKL, Receptor activator of the nuclear factor-κB ligand

ROS, Reactive oxygen species

TAMs, Tumor associated macrophages

TLR4, Toll-like receptor 4

TME, Tumor microenvironment

TNF, Tumor necrosis factor

TNFR, Tumor necrosis factor receptor

Treg, Regulatory T cells

VEGF, Vascular endothelial growth factor

1. Introduction

1.1. Breast Cancer: Epidemiology, Etiology and Classification

Breast cancer (BC) is the most commonly diagnosed cancer and the leading cause of cancer death in women (Bray et al., 2018). In fact, in 2020 there were 2.3 million women diagnosed with BC and 685 000 deaths globally. There have been improvements in survival, thanks to early detection programs and different modalities of treatment, but it remains the cancer with the most lost disability-adjusted life years (DALYs) in women (WHO/B. Anderson, 2021).

There are several risk factors associated with development of BC, such as aging, family history (specifically those associated with mutations in the *BRCA*1 and *BRCA*2 genes), reproductive factors (early menarche, late menopause, late age at first pregnancy and low parity), exposure to estrogen (both endogenous or exogenous) and lifestyle (excessive alcohol consumption and dietary fat intake, as well as smoking) (Sun et al., 2017). On the other hand, breastfeeding and physical activity may be protective factors (Sun et al., 2017).

Since most deaths are associated with advanced disease (Weigelt et al., 2005), early detection is the corner stone for advanced BC prevention (Sun et al., 2017). Screening programs have been developed to detect BC in early stages, in order to prevent invasive disease. Currently, there are evidence-based recommendations for mammography screening, particularly between women aged 50 - 69 years and with conditional recommendations for women in other age groups, considering their individual risk factors (Paluch-Shimon et al., 2020).

The diagnosis of BC is based on a combination of clinical examination and imaging methods, and later confirmed by pathological assessment (Paluch-Shimon et al., 2020). Once diagnosed, it is important to stage the disease according to the TNM system. An accurate pathological staging is achieved by evaluating the histological type of the tumor, basement membrane invasion, multicentricity and focality, limphovascular invasion, cell's proliferation rate and grade. Since BC can be a local or systemic disease, it is mandatory to evaluate the involvement of lymph nodes or/and

distant organs. Involvement of axillar lymph nodes and distant organs are considered poor prognosis factors (Weigelt et al., 2005); being the most common sites of metastasis from BC the lymph nodes, bone, lung, liver and brain (Kim, 2021). However, BC is a very heterogeneous disease with different sub-types presenting different etiology, prognosis and metastization patterns.

In order to understand the heterogeneity of BC, several molecular studies have disclosed a series of biomarkers, which contribute to better defining therapeutic targets and prognosis, allowing for a more individualized approach. The current molecular classification of BC used in the clinics is based on three criteria: Hormone Receptor (HR) status (estrogen receptor (ER) and progesterone receptor (PR)), human epidermal growth factor receptor 2 (HER2) status and Ki-67 (Tsang & Tse, 2020). Taking in to account these biomarkers, BC can be further categorized in a molecular subtype, with implications in treatment and prognosis (Figure 1) (Prat et al., 2015).

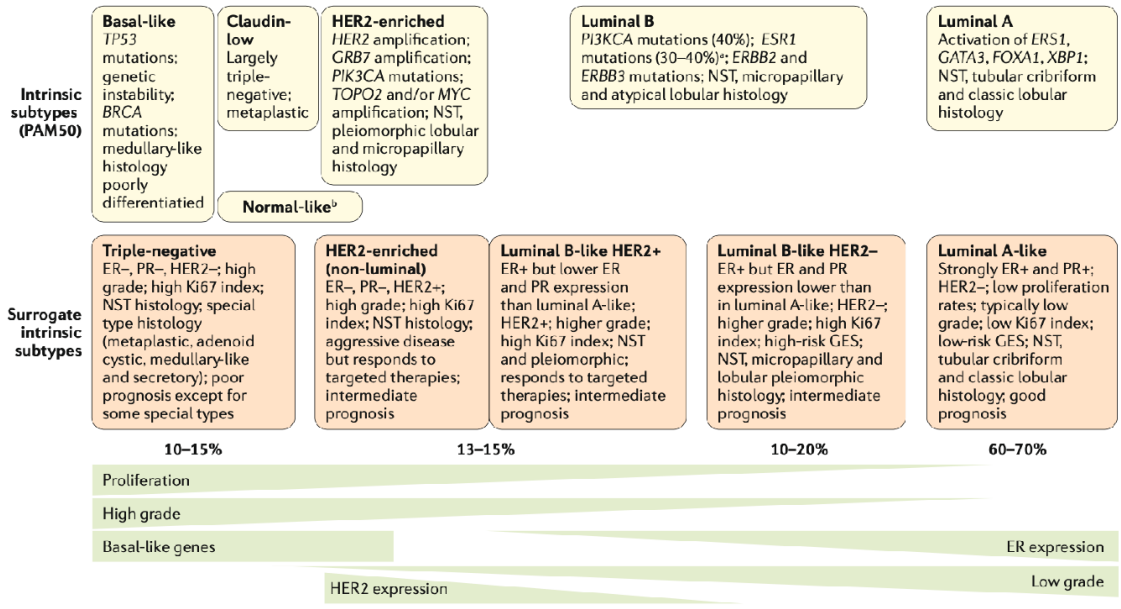

Figure 1. Histological and Molecular Characteristics of Breast Cancer [adapted from (Harbeck et al., 2019)]. The intrinsic subtypes of Perou and Sorlie (Perou et al., 2000) are based on a 50-gene expression signature (PAM50). The surrogate intrinsic subtypes are typically used in the clinics and are based on histology and immunohistochemistry expression of key proteins: estrogen receptor (ER), progesterone receptor (PR), human epidermal growth factor receptor 2 (HER2) and the proliferation marker Ki67. Tumours expressing ER and/or PR are termed "hormone receptor- positive"; tumours not expressing ER, PR and HER2 are called "triple- negative". The relative placement of the boxes aligns with the characteristics (for example, proliferation and grade in green. GES, gene expression signature. [a]ESR1 mutations induced by aromatase inhibitor targeted therapy. [b]Artefact; expression of normal breast components due to low tumour cellularity.

1.2. RANKL-RANK Pathway in bone physiology and bone metastases

The receptor activator of the nuclear factor-κB (RANK) is a member of the superfamily of the tumor necrosis factor receptor (TNFR), activated upon binding of the RANK ligand (RANKL), promoting proliferation, survival, and cell differentiation (Infante et al., 2019).

RANK is highly expressed on the surface of osteoclast precursors and mature osteoclasts, and in the surface of dendritic cells (DC), whereas RANKL is expressed in numerous tissues, including bone and bone marrow and lymphoid tissues (Infante et al., 2019).

In the bone, RANKL expressed by osteoblasts and stromal stem cells binds to RANK on the surface of osteoclast precursors, inducing osteoclastogenesis and osteoclast activation, which will lead to an increase in bone resorption (Boyce & Xing, 2007a). There are multiple downstream pathways involved in the RANKL-RANK signal transduction cascade, inhibitor of NF-kB kinase (IKK)/NF-kB, c-Jun N-terminal kinase (JNK)/activator protein-1 (AP-1), calcineurin/NFATc1, Src, MKK6/p38/MITF, and extracellular signal–regulated kinase [ERK] (Boyce & Xing, 2007a; Liu & Zhang, 2015). Osteoprotegerin (OPG), secreted by osteoblasts and osteogenic stromal cells (Boyce & Xing, 2007a) acts as a soluble decoy receptor for RANKL, blocking the pathway (Liu & Zhang, 2015).

The RANKL-RANK pathway is also known for its role in the pathophysiology of bone metastasis (Figure 2) (Casimiro et al., 2016). These can be osteolytic or osteoblastic, according to the primary tumor cells, tumor's microenvironment (TME) and the effect of the secreted factors. For instance, in BC, tumor-derived osteoclastogenic factors, such as interleukin-1 (IL-1), interleukin-6, parathyroid hormone-related protein (PTHrP), prostaglandin E2 (PEG2), and tumor necrosis factor (TNF) lead to an increase in bone resorption by up-regulation of RANKL expression in the bone microenvironment. In the case of osteoblastic bone metastases, like in prostate cancer, the TME leads to an increase in osteoclastogenesis but also of the activation of

osteoblasts (via the ET-1 – ETR – Wnt signaling pathway), leading to the accumulation of new bone (Casimiro et al., 2016).

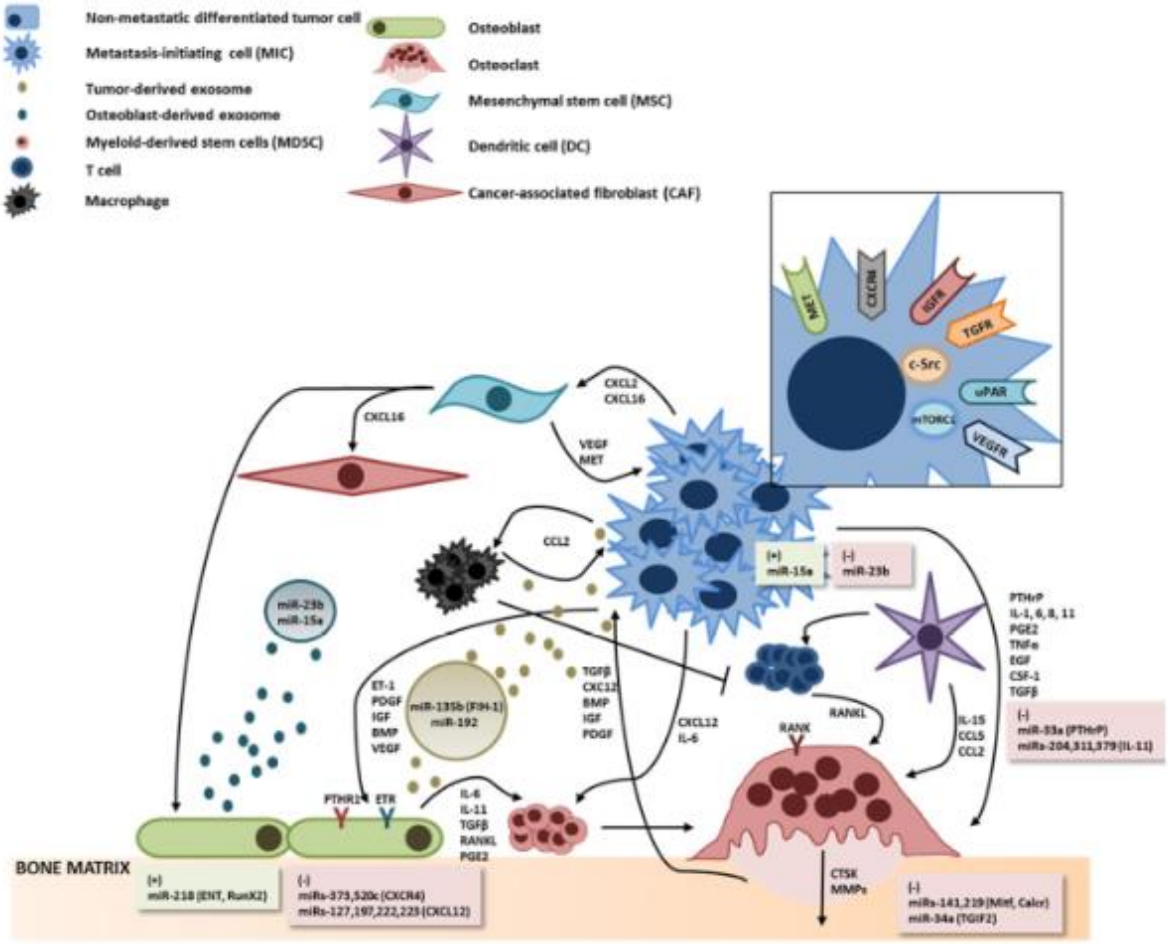

Figure 2. "Vicious Cycle of Bone Metastasis" [adapted from (Casimiro et al., 2016)]. The "vicious-cycle of bone metastases" results from the complex interaction between tumor cells, bone forming osteoblasts, bone resorbing osteoclasts, and a variety of cells from the bone microenvironment and immune system, like cancer-associated fibroblasts (CAFs), mesenchymal stem cells (MSCs), dendritic cells (DCs), T cells and macrophages (Ursini-Siegel & Siegel, 2016). Osteoblasts are activated by tumor-derived parathyroid hormone-related protein (PTHrP), leading to increased production of receptor activator of RANKL. RANKL binds to RANK expressed on hematopoietic osteoclast precursors, leading to osteoclastogenesis and bone resorption. Bone matrix-stored minerals and growth factors are released and activated, further feeding the tumor cell growth. In osteoblastic bone metastases, like in prostate cancer, osteoblasts activity is stimulated by several growth factors like basic fibroblast growth factor (bFGF), bone morphogenetic proteins (BMPs), endothelin 1 (ET-1), transforming growth factor beta (TGFβ) and insulin-like growth factor 1 (IGF-1), and deposition of disorganized new bone matrix is exacerbated (Vignani et al., 2016). miRNAs can act as master regulators of gene expression, having a positive (+) or negative (−) effect on specific genes that will control multiple aspects of bone metastasis formation (Croset et al., 2015). CCL-2,Ccl2 chemokine (C–C motif) ligand 2; CTSK, cathepsin K; CSF-1, colony stimulating factor 1; CXCL12, C–X–C motif chemokine12; CXCR4, C–X–C chemokine receptor type 4; EGF, epidermal growth factor; ENT, equilibrate nucleoside transporter 1; ETR, endothelin receptor; IGFR, insulin-like growth factor 1 (IGF-1) receptor; IL, interleukin; MET, hepatocyte growth factor receptor; MMP, matrix metalloproteinases; mTORC1, mammalian target of rapamycin complex 1; PDGF, platelet-derived growth factor; PGE2, prostaglandin E2; RDx, radixin; TGIF2, TGF beta induced factor homeobox2; TGFR, transforming growth

factor beta receptor II; uPAR, urokinase receptor; VEGF, vascular endothelial growth factor; VEGFR, vascular endothelial growth factor receptor.

1.3. RANKL-RANK Pathway and Breast Cancer

Although RANKL-RANK pathway is mostly known for its role as key regulator of bone remodeling (Dougall et al., 1999) and pathophysiology of bone metastasis (Casimiro et al., 2016), it is also an important mediator of mammary gland morphogenesis (Asselin-Labat et al., 2010; Wang et al., 2013) and progesterone induced breast carcinogenesis (Wang et al., 2013).

Mammary Gland Development and Carcinogenesis

The mammary gland develops through several distinct stages, under the regulation of hormones, such as growth hormone (GH), estrogen, insulin-like growth factor 1 (IGF1), progesterone and prolactin, ending with the formation of complex lobolo-alveolar structures (Macias & Hinck, 2012). There are two main cell types that compose the mammary epithelium: basal and luminal. The basal epithelium is composed by myoepithelial cells (MECs) that generate the outer layer of the gland, and that, upon contraction, facilitate the extraction of breast milk. The luminal epithelium represents the inner layer of luminal epithelial cells (LECs), forming ducts and secretory alveoli and is composed by cells defined by their HR status (Macias & Hinck, 2012). Evidence has surfaced that the RANKL-RANK pathway has an important role in the mammary epithelium branching, alveologenesis and proliferation, as well as mammary stem cells (MaSCs) expansion (Beleut et al., 2010). In fact, mice lacking RANKL or RANK, fail to undergo alveologenesis during pregnancy (Macias & Hinck, 2012). On the other hand, overexpression of RANKL and its receptor is strongly mitogenic in MECs (Beleut et al., 2010) and its ectopic expression in the mammary epithelium has been shown to elicit ductal side branching and alveologenesis (Beleut et al., 2010).

Progesterone Signaling

Both RANKL and progesterone act on stages of the mammary lactation morphogenesis. Progesterone leads to an upregulation of RANKL in PR+ Luminal cells, and RANKL acts in a paracrine manner on ER and PR negative cells, generating pro-growth response to progesterone. This has been demonstrated by recent studies that illustrate RANKL's role as a modulator of progesterone signaling (Kiesel & Kohl, 2016).

It has been documented a connection between progesterone function on the mammary gland and BC, which may be connected to RANKL dependent proliferation of the mammary epithelium (Figure 3) (Kiesel & Kohl, 2016). RANKL seems to provide growth and survival advantage to damaged MECs and mammary tumor cells showing an overexpression of RANK demonstrate interferences in acinar formation and impair the development of functioning growth arrest in DNA-damaged cells. In addition, RANKL, through the upregulation of Snail, promotes epithelial-tomesenchymal transition (EMT) within breast tumors, which is one of the initial steps in carcinogenesis (Kiesel & Kohl, 2016).

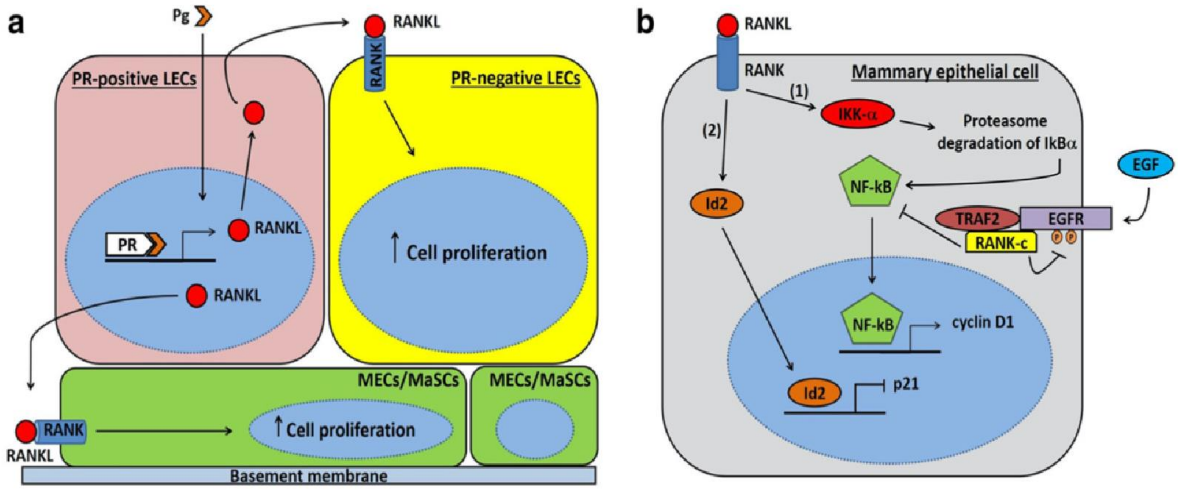

Figure 3. PR/RANKL pathway and downstream RANK-mediated signaling in mammary epithelial cells [(Infante et al., 2019)]. **A.** Progesterone binds to its receptor in PR-positive breast luminal cell, leading to an increase in RANKL protein levels mainly through stabilization of its mRNA. Then, RANKL binds to its cognate receptor RANK expressed on the surface of the neighbouring PR-negative breast luminal cell, activating downstream signalling pathways that promote cell proliferation. Basal cells (MECs and MaSCs, drawn in green at the bottom of the figure) constitutively express RANK on their surface, but they lack PR. RANKL produced by PR-positive breast luminal cells further up-regulates RANK expression on MECs and MaSCs surface, and activates RANK-downstream signalling pathways

promoting cell proliferation, expansion and survival. **B.** RANK-IKK-α-NF-kB-cyclin D1 pathway (1), and RANK-Id2-p21 pathway (2) represent the two main signalling pathways activated by RANK in mammary epithelial cells. IKK-α catalyses phosphorylation, ubiquitination and proteasome degradation of IkBα, leading to its dissociation from NF-kB, which then migrates to the nucleus and induces cyclin D1transcription. On the other hand, Id2 translocates into the nucleus and reduces expression of the cell cycle inhibitor p21. Altogether, these molecular events result in increased proliferation and survival of mammary epithelial cells. RANK-c is a RANK isoform derived from alternative splicing of RANK gene, which has been identified in breast cancer cell lines and breast tumors. It acts as a dominant negative regulator of RANK-dependent NF-kB activation, inhibiting the NF-kB-mediated cell survival effect and correlating with lower cell motility and proliferative index. RANK-c may exert its function through the intracellular interaction with other key molecules, such as TRAF2 and EGFR. Notably, RANK-c has also been shown to act as a negative regulator of EGFR signalling, inhibiting EGFR phosphorylation after EGF ligand stimulation. Abbreviations: EGF, Epidermal growth factor; EGFR, Epidermal growth factor receptor; Id2, inhibitor of DNA binding protein 2; IkBα, inhibitor of kappa Bα; IKK-α, inhibitor-kB kinase-α; LECs, luminal epithelial cells; MaSCs, mammary stem cells; MECs, myoepithelial cells; NF-kB, nuclear factor-kB; Pg, natural or synthetic progesterone; PR, progesterone receptor; RANK, receptor activator of NF-kB; RANKL, receptor activator of NF-kB-ligand; TRAF2, TNF receptor-associated factor-2.

Since RANKL expression seems to be dependent on serum progesterone levels, and progesterone is associated with low OPG levels in serum and tissue, which is an endogenous inhibitor of RANK (Kiesel & Kohl, 2016), we can see that there is a close interaction between the RANKL-RANK pathway and progesterone in mammary tissues.

1.4. Tumor Microenvironment (TME)

The TME is the cellular and physical environment in which cancer cells reside (Arneth, 2020). The TME is composed by surrounding immune cells, blood vessels, extracellular matrix (ECM), fibroblasts, lymphocyte, and signaling molecules. The TME and the interactions between the cells there present affect cancer development and progression. The nonmalignant cells often stimulate tumor cell proliferation, thus having pro-tumorigenic functions. These will allow malignant cells to spread and invade healthy tissues, the lympathic and the circulatory systems (Balkwill et al., 2012).

The ECM in the TME is composed by macromolecules (glycoproteins, collagens and enzymes) and active tissue that influence cell adhesion, proliferation and communication, as well as cellular growth factors (Arneth, 2020).

The TME includes three main cellular components (Arneth, 2020; Balkwill et al., 2012): endothelial cells, fibroblasts and immune cells. The endothelial cells play an important role in protecting the tumor from the immune system and in the tumor

development itself, by helping to bring nutritional support to the tumor. Fibroblasts are crucial in the migration process of the tumor cells to the bloodstream, leading to systemic metastasis, and are also important during angiogenesis. Myofibroblasts are abundant in many TMEs and are also called cancer-associated fibroblasts (CAFs) (Balkwill et al., 2012). The immune cells are present in several types, such as granulocytes, lymphocytes and macrophages, which are important for the inflammatory reactions associated with the tumor. Of these, the most abundant are the macrophages (Arneth, 2020).

Tumor Associated Macrophages (TAM's)

Macrophages are cells of the innate immune system, which arise from the differentiation of monocytes, upon recruit by chemotactic signals (Cassetta & Pollard, 2020). Macrophages have important roles such as phagocytosis, and in development, homeostasis, and tissue repair.

According to factors in the TME, macrophages can be activated into: classically activated M1 macrophages, and alternatively activated M2 macrophages (Cassetta & Pollard, 2020; Tariq et al., 2017). M1 macrophages are induced by TH1-type cytokines (like interferon gamma (IFNγ) through Toll-like receptor 4 (TLR4)), and usually present with pro-inflammatory and anti-tumoral phenotypes (Cassetta & Pollard, 2020; Franklin et al., 2014). They characteristically express nitric oxide synthase (iNOS), reactive oxygen species (ROS) and cytokine IL-12 (Pan et al., 2020). M2 macrophages are induced by Th2-type cytokines IL-4/IL-12, presenting with anti-inflammatory and pro-tumoral phenotypes (Cassetta & Pollard, 2020). They express a large number of scavenger receptors, related to the high expression of IL-10, IL-1b, vascular endothelial growth factor (VEGF), matrix metalloproteinases (MMPs) and CD206 (Pan et al., 2020).

Tumor associated macrophages, or TAMs, are macrophages recruited from circulating monocytes to tumors (Cassetta & Pollard, 2020) that participate and influence the formation of the TME (Pan et al., 2020). In metastatic tumors, TAMs have different

phenotypes and functions and are often called metastasis-associated macrophages (MAMs) (Cassetta & Pollard, 2020).

Most TAMs originate from bone-marrow derived monocytes (Cassetta & Pollard, 2020; Pan et al., 2020) and the major recruitment factor is the chemokine CCL2 produced by tumor cells. Tumor growth can also induce the differentiation of CCR2+ monocytes into TAMs (Franklin et al., 2014). Recent studies regarding the differences between breast and endometrial TAMs suggest that different niches of cells can activate TAMs in a tumor and tissue-specific way (Cassetta & Pollard, 2020). The high infiltration of TAMs in human solid tumors as well as the expression of macrophage growth factors or chemoattractants (for instance, CSF1 and CCL2) in tumor or in the circulation is associated with poor clinical outcomes and poor prognosis (Cassetta & Pollard, 2020).

So, one might ask, "What specifically are the roles of TAMs in tumors?" (Figure 4). TAMs interact with a wide range of cell types, including fibroblasts, endothelial cells and other immune cells, as well as secreted factors, within the TME (Cassetta & Pollard, 2020), promoting tumor growth, invasion, metastasis, and drug resistance (DeNardo & Ruffell, 2019; Pan et al., 2020). In fact, TAMs can contribute to tumor progression by promoting angiogenesis through the secretion of pro-angiogenic factors like VEGF, CXCL8 and CXCL12, and by stimulating lymphangiogenesis (Cassetta & Pollard, 2020). They also have an important role in tumor's immunity (Pan et al., 2020), mediating immunosuppression by inhibiting T-cell functions and recruiting regulatory T cells (Treg) to the TME (DeNardo & Ruffell, 2019; Pan et al., 2020). In addition, studies also indicate that they have an important role in the remodeling of the ECM and may induce stem-cell-like properties.

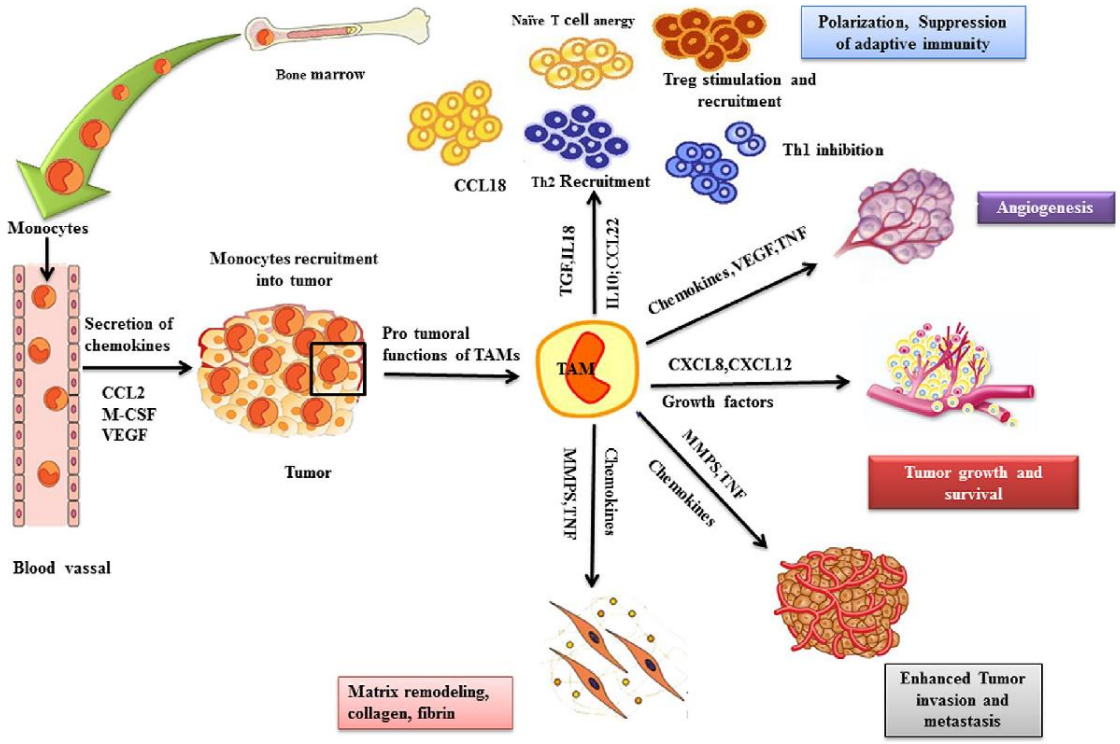

Figure 4. Overview of TAMs in tumor progression, invasion, matrix remodeling, and metastasis [(Tariq et al., 2017)]. Circulating monocytes are recruited into the tumor tissue due to the effect of growth factors and chemokines like CCL2, M-CSF, and VEGF. In tumor microenvironment, tumor-derived factors initiate the polarization of monocytes to tumor-associated macrophages (TAMs). Tumor-molded macrophages resemble M2-like-polarized cells. Furthermore, TAMs secrete growth factors which lead to the expression of molecules that support to increase the tumor cell growth and survival, regulate matrix remodeling, tumor invasion, angiogenesis, and metastasis. TAMs secrete factors that induce local immune suppression by recruiting and stimulating the Tregs and Th2 lymphocytes, which in turn block the Th1 cells, and induce naïve T cells anergy. TAMs stimulate adaptive immune responses to secrete factors like TGF, IL18, IL10, and CCL22 that enhance local immune suppression, T-regulatory stimulation and recruitment of cells or suppress Th1 cell responses directly. CCL2, C-C motif chemokine ligand 2; CCL22, C-C motif chemokine ligand 22; CXCL8, C-X-C motif chemokine ligand 8; CXCL12, C-X-C motif chemokine ligand 12; IL18, interleukin 18; IL10, interleukin 10; M-CSF, macrophage colony-stimulating factor; TGF, tumor growth factor; TNF, tumor necrosis factor; VEGF, vascular endothelial growth factor.

RANK expression in Monocytes and Macrophages

We know that RANK has an important role in the immune system. As a matter of fact, RANK and RANKL were initially described in the context of T cell–DC interactions (Ahern et al., 2018) and the RANKL-RANK pathway seems to be a regulator of interactions between T cells and DCs.

RANK is expressed on immature DC, immunosuppressive M2-type macrophages and myeloid-derived suppressor cells (MDSC). RANKL is expressed in CD8+ and CD4+ T

cells and has shown to be a chemoattractant to RANK-expressing monocytes and macrophages (Ahern et al., 2018).

Given the fact that various cell types present in the TME, such as macrophages, MDSC and DC, express RANK and/or RANKL, the RANKL-RANK pathway may influence a tumor's immunity. In fact, some studies suggest that denosumab (a monoclonal antibody anti-RANKL) can improve the efficacy of immune-checkpoint inhibitors (ICIs), which means that RANKL-RANK signaling might suppress anti-tumor immunity in the TME (Ahern et al., 2018).

In non-cancer settings, studies suggest that RANKL-induced activation of RANK on DCs promotes survival and a tolerogenic role, as well as plasticity in myeloid precursors. However, in human cancers, the role of RANK-expressing DCs is not well described (Ahern et al., 2018).

Preliminary data from our lab suggested that conditioned media (CM) from RANK overexpressing (OE) ER+HER2- BC cells could induce the differentiation of monocytes *in vitro*, which is particularly important, since RANKL has shown to be a chemoattractant for RANK-expressing monocytes and macrophages (Gomes et al., 2020). This project stems from these findings and aims to determine if RANK OE cells' secretome has the potential to induce an immunosuppressive TME.

2. Objectives

Based on the hypothesis that the BC cells secretome may influence the differentiation of monocytes, the specific objective of this project was to identify the cells differentiated from the monocytic lineage upon exposure to RANK OE BC cell's secretome (focusing on osteoclast and/or M1/M2 macrophages).

3. Materials and Methods

3.1. Cell lines and cell culture

Human breast carcinoma cell lines MCF-7GFP+Luc+ and MDA-MB-231GFP+Luc+ (from now on designated by MCF-7 and MDA-MB-231, respectively) were provided by Dr. Sérgio Dias (Instituto de Medicina Molecular, Lisbon, Portugal); T47D and BT474 were provided by Dr. Phyllippe Clézardin (INSERM, Lyon, France) and monocytic murine cell line RAW264.7 was provided by Dr. Robert Maki (University of Pennsylvania, Philadelphia, USA). RANK overexpressing MDA-MB-231, MCF-7, T47D and BT474 cells (RANK OE cells) were previously derived in our Lab by lentiviral transduction as previously described (103).

Parental or RANK OE cells lines were cultured in Dulbecco's Modified Eagle's Medium (DMEM, Gibco) supplemented with 10% (v/v) fetal bovine serum (FBS, Gibco) and 1% (v/v) Penicillin/Streptomycin (Pen/Strep, 10,000 U/mL Penicillin, 10,000 µg/mL Streptomycin, Gibco). In the case of MCF-7 and T47D parental or RANK OE cell lines medium was also supplemented with 0,01 mg/mL insulin (Gibco).

RAW 264.7 cell line was cultured in DMEM supplemented with 10% (v/v) heat inactivated FBS (Gibco) and 1% Pen/Strep (Gibco).

Cells were maintained at 37°C with 5% CO_2, used at low passage number, and regularly tested for Mycoplasma contamination by qPCR.

3.2. CM Preparation

CM was collected from cell lines cultured under standard conditions at 80% of cellular confluence and centrifuged at 1000g for 5 minutes. Supernatants were divided into aliquots and stored at -80°C.

3.3. Osteoclastogenesis Assay

To induce the differentiation of RAW264.7 cells into osteoclasts, cells grown under standard conditions were plated in 96-well plates at a density of 950 cells/well in alpha Minimal Essential Medium (αMEM, Gibco) supplemented with 10% (v/v) heat inactivated FBS (Gibco), 1% Pen/Strep (Gibco), 1 µg/ml RANKL (#11000457, Amgen Inc.). To test the influence of CM, RAW264.7 cells were differentiated with or without 25% CM (v/v) (obtained as described above). Medium was changed at day 3 and osteoclastogenesis was assessed at day 5.

3.4. Monocyte and BC cells co-culture System

For co-culture assays, RAW264.7 cells were plated in 24-well-plates at a density of 1x10^5/well in differentiation medium (as described above). MDA-MB-231, MCF-7 parental or respective RANK OE cells were seeded on Bio-One ThinCert™ CellCoat™ 24 Well Cell Culture Inserts with 8µm pores (Greiner), at a density of 5x10^3 cells per insert, in DMEM medium (Gibco), supplemented with 10% FBS (Gibco) and 1% Pen/Strep (Gibco) and placed on top of RAW264.7 cells. Medium was changed at day 3 and osteoclastogenesis was assessed at day 5.

3.5. TRAcP 5b Staining and ELISA assay

Tartrate-resistant acid phosphatase 5b (TRAcP 5b) is an enzyme highly expressed by osteoclasts, macrophages and DC, and used as a marker of osteoclasts. Therefore, osteoclastogenesis was assessed by cytochemical staining of TRAcP 5b (Sigma-Adrich), according to the manufacturer's instructions, followed by the counting of the number of multinucleated TRAPcP 5b-positive cells.

TRAcP5b was quantified in the supernatant following osteoclastogenesis assay, using the MouseTRAP (TRAcP 5b) ELISA kit (SB-TR103, IDS), according to the manufacturers' instructions.

3.6. RT-qPCR

For RT-qPCR analysis of mRNA expression, RAW26.7 cells from the co-culture assay were lysed at day 5 of the protocol and total RNA was extracted using the NZY Total RNA Isolation kit (Nzytech), according to manufacturer's instructions. Total RNA was quantified in a NanoDrop spectrophotometer (Thermo Ficsher Scientific) and treated with RQ1 RNase-free DNase I (#M6101, Promega) for 30 min at 37°C, according to manufacturer's instructions. DNase I-treated RNA was reverse transcribed using the NZY M-MuLV First-Strand cDNA Synthesis kit (Nzytech) and Oligo (dT)20 primer, and cDNAs were amplified by real-time PCR using NZY qPCR Green Master Mix (Nzytech). Specific primers included mouse *iNOS* (Fw: 5´-TCAGCTACGCCTTCAACACC-3'; Rv: 5'-TTCCCAAATGTGCTTGTCACC-3'), mouse *CD206* (Fw: 5'-CATTCCCTCAGCAAGCGATG-3´; Rv: 5'-GGGTTCCATCACTCCACTCA-3´), mouse *Nfatc1* (FW: 5'-GGAGCGGAGAAACTTTGCG-3'; Rv: 5'-GTGACACTAGGGGACACATAACT-3') and mouse β-actin (Fw: 5'-GGCTGTATTCCCCTCCATCG-3'; Rv: 5'-CCAGTTGGTAAC AATGCCATGT-3'). Gene expression was normalized using the housekeeping gene *β-actin*, and relative mRNA expression was calculated using the 2^{-DCt} method.

4. Results

4.1. Effect of BC cell lines CM in osteoclast differentiation

To evaluate if CM of BC cell lines had an effect on osteoclastogenesis, RAW264.7 cells were cultured in differentiation conditions in the presence of different BC cell lines' CMs.

As observed by the number of TRAP+ multinucleated cells/well (Figure 5a,c), and secreted TRAcP 5b quantification (Figure 5b), osteoclastogenesis was induced by RANKL (control + group) compared with the negative control condition (control - group). None of the BC CM was able to induce osteoclastogenesis in the absence of exogenous RANKL (data not shown) and in all cases, addition of CM decreased osteoclastogenesis in comparison with RANKL alone (control +). The impairment of osteoclastogenesis was more pronounced in RANK OE CM from Luminal BC cell lines compared with the parental counterparts. On contrary, in the case of the TNBC MDA-MB-231 cell line, RANK OE CM increased osteoclastogenesis in comparison with the parental cell line.

Additionally, cells with a morphology suggestive of differentiation, other than osteoclasts, were observed in all CM conditions (Figure 5d, red arrows).

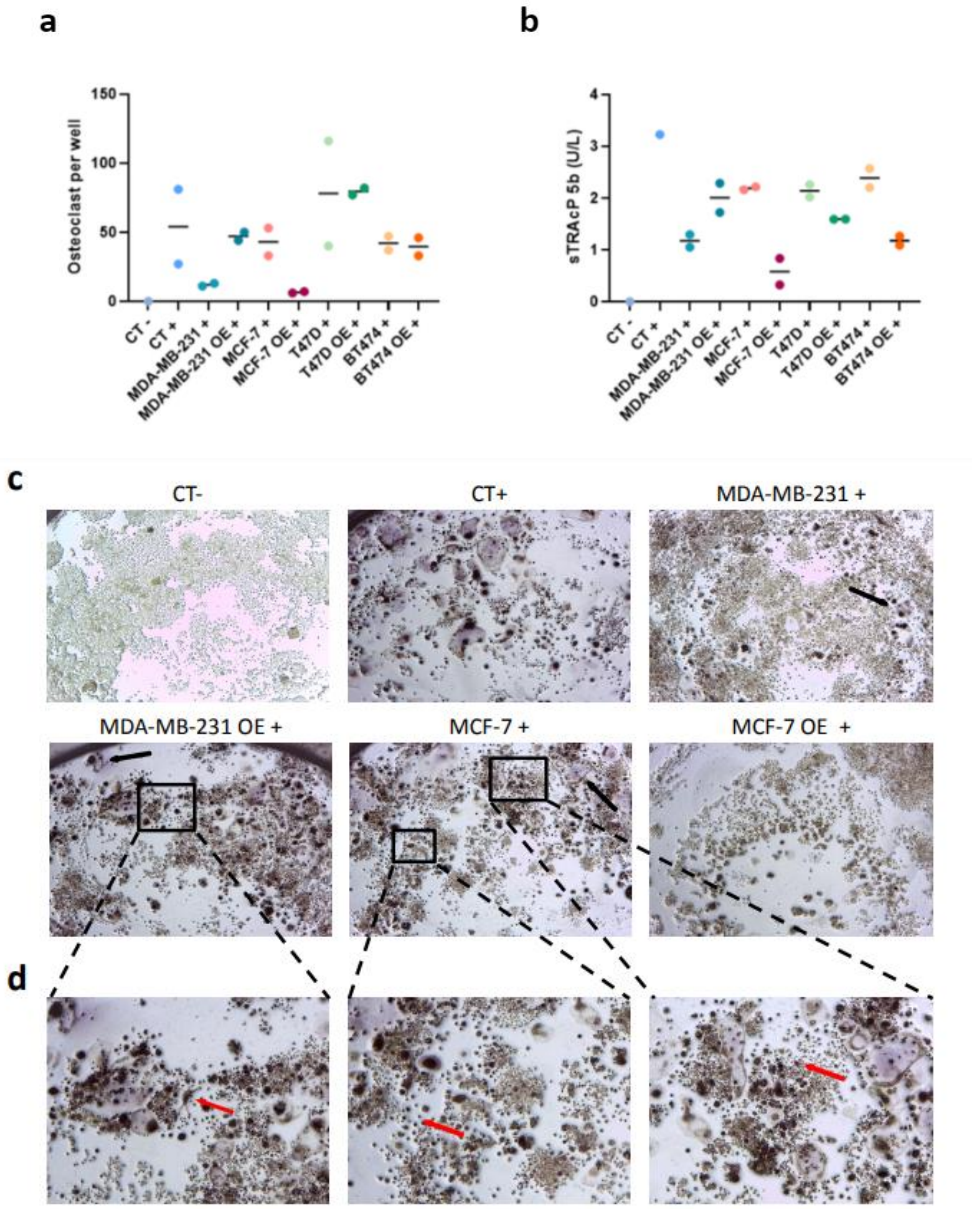

Figure 5. CM from Luminal BC RANK OE cells impairs osteoclastogenesis. RAW264.7 cells were cultured in differentiation medium, with 1 ng/mL RANKL alone or in combination with 25% BC cells CM, for 5 days. Osteoclastogenesis was quantified by (a) counting the number of TRAP+ multinucleated cells/well or (b) TRAcP5b quantification in the supernatant. (c) Representative images cytochemical TRAP staining, magnification 4x. Osteoclasts are indicated by black arrows (d) Representative images of non-osteoclasts cells with differentiation morphology (red arrows) magnification 10x.

4.2. Effect of BC cell lines co-culture in osteoclastogenesis

Considering that some cytokines may be lost during the conservation and defrosting process of CM, we decided to test our hypothesis using a co-culture system. For the co-culture assay, RAW264.7 cells were cultured in differentiation medium as mentioned above and MDA-MB-231, MCF-7 parental and RANK OE cells were plated in inserts placed above RAW264.7 cells.

As observed previously in the CM experiment, none of the BC cells was able to induce osteoclastogenesis in the absence of exogenous RANKL (Figure 6 a,b,c). In the co-cultured system, the impairment of osteoclastogenesis in the presence of BC cells versus RANKL alone was exacerbated, in comparison with CM experiments. Secreted TRAcP 5b (Figure 6 a) was only detected RANKL-stimulated cells, and RANK OE decreased osteoclastogenesis in comparison with parental cells. TRAP+ multinucleated cells were only observed in CT+ and in co-culture with MCF-7 (Figure 6 b,c).

Morphologically differentiated cells, but not osteoclasts, were again observed in the co-culture system (Figure 6d).

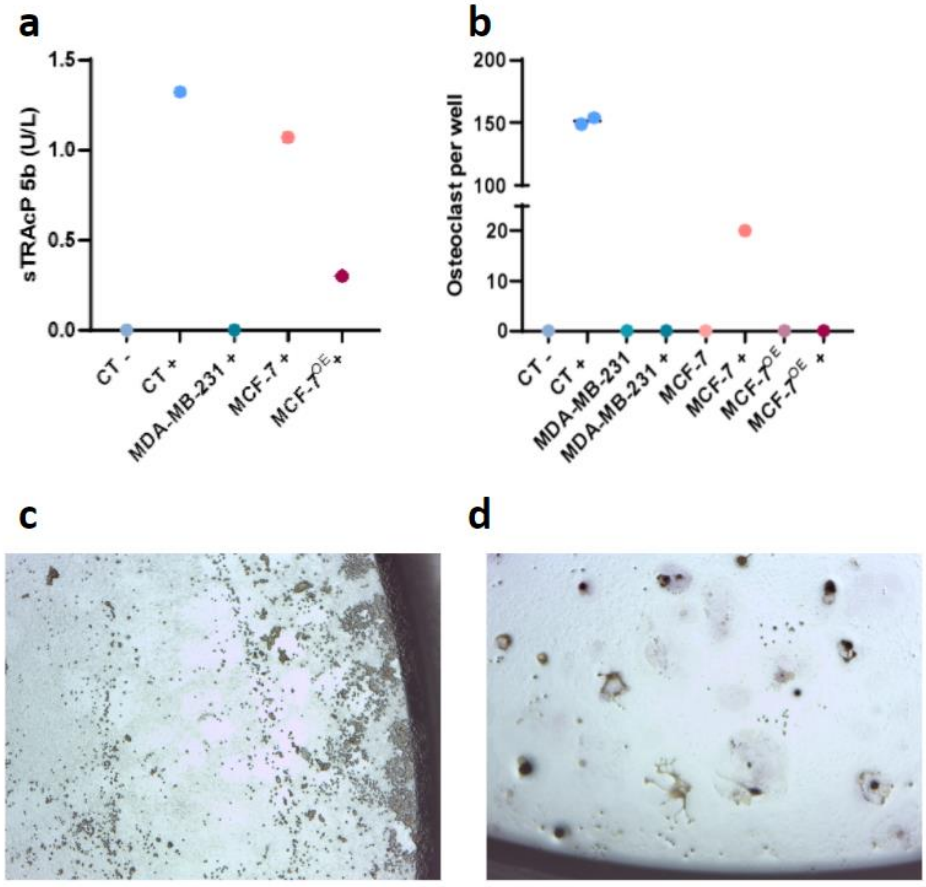

Figure 6. Co-Culture impairs osteoclastogenesis. RAW264.7 cells were cultured in differentiation medium, with 1ng/ml of RANKL alone or in co-culture with MDA-MB-231, MCF-7 Parental or RANK OE cells, for 5 days. Osteoclastogenesis was quantified by (a) TRAcP5b quantification in the supernatant (b) by counting the number of TRAPcP 5b positive multinucleated cells/well. (c) Picture representative of osteoclastogenesis of RAW264.7 cells in the presence of CM of MCF-7 OE magnification 10x. (d) Picture representative of morphologically differentiated cells in co-culture with MCF-7 PAR + RANKL cells, magnification 10x.

4.3. *CD206*, *iNOS* and *nfatc1* expression in RAW264.7 cells co-cultured with BC cells

Since we observed the presence of cells with different morphology suggestive of differentiation, but clearly not osteoclasts (TRAcP 5b negative non-multinucleated giant cells), we hypothesized that those cells could be macrophages, considering that the RAW264.7 is a monocytic cell line and thus can be differentiated into macrophages (Khabipov et al., 2019).

Therefore, we analyzed the expression of *nfatc1*, a key transcriptional factor of osteoclastogenesis, as well as two macrophage-specific markers, *CD206* (M2 macrophages) and *iNOS* (M1 macrophages).

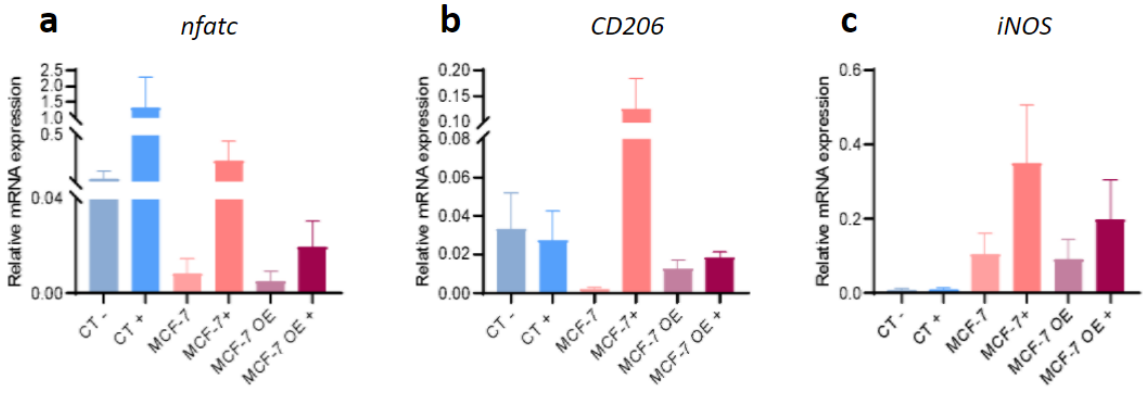

Figure 7: *nfatc1*, *CD206* and *iNOS* have increased expression in co-culture and exogenous RANKL conditions. Relative mRNA expression of *nfatc* (a), *CD206* (b) and *iNOS* (c) were analyzed by RT-qPCR. Gene expression was normalized using the housekeeping gene *β-actin*, and relative mRNA expression was calculated using the 2^{-DCt} method.

As expected, *nfatc1* expression was increased in the conditions where osteoclastogenesis was higher, previously assessed by the number of osteoclasts and secreted TRAPcP 5b (Figure 7a). *CD206* and *iNOS* were expressed in all conditions, without differences between negative and positive controls (without or with RANKL) (Figure 7b,c). The expression of both genes was increased when RAW264.7 were co-cultured with BC cells, and especially in the presence of exogenous RANKL (Figure 7b,c).

5. Discussion

Preliminary data from our lab suggested that the CM of BC cells lines, particularly of the luminal subtype (hormone responsive), such as MCF-7 cell line, with overexpression of RANK (RANK OE), could induce the differentiation of monocytes into osteoclasts and/or macrophages, *in vitro*. Therefore, to the main goal of this project was to test this hypothesis, and to address it we analyzed monocyte (murine RAW264.7 cell line) differentiation in the presence of BC cells CM or in co-culture with BC cells, always comparing with exogenous RANKL-induced osteoclastogenesis.

In the presence of BC cell lines CM, we observed an inhibition of osteoclastogenesis, in comparison with exogenous RANKL alone, which was higher in RANK OE cells. This effect was magnified in the co-culture system and was particularly accentuated when looking at the murine cells exposed to luminal BC cell line's CM. Considering this, we hypothesize that the luminal RANK OE cells may secret different amounts of cytokines, factors or molecules that lead to a more accentuated inhibition of osteoclastogenesis. One hypothesis that could explain this observation could be a higher secretion of OPG. Previous results from our Lab have demonstrated that MCF-7 and T47D cells express OPG, and that RANK OE derivatives have higher expression (Gomes et al., 2020).

OPG is secreted by osteoblasts, as well as other tissues, and acts by binding to RANKL, preventing its interaction with RANK and thus protecting excessive bone resorption (Boyce & Xing, 2007a). Therefore, in the presence of higher concentrations of OPG, less RANKL will be available to bind to RANK, and osteoclastogenesis decreases. (Boyce & Xing, 2007a, 2007b) The expression of OPG is regulated by many factors that induce expression of RANKL by osteoblasts in an inversely proportional manner, such that upregulation of RANKL is usually associated with downregulation of OPG, and vice-versa (Boyce & Xing, 2007b).

In fact, Poznak *et al* showed that MCF-7 cell lines had strong intracellular staining for OPG (van Poznak et al., 2006). It was also demonstrated that OPG is expressed in healthy breast tissue, but, moreover, its expression is correlated to ER/PR status in

human BC, with luminal BC having higher expression of OPG (van Poznak et al., 2006). However, the exact role of OPG in breast cancer tumorigenesis remains to be clarified (Infante et al., 2019).

To further investigate this hypothesis, it would be of interest in the future to quantify OPG in CM used in our studies and measure OPG expression in MDA-MB-231 BC cells, which did not impaired osteoclastogenesis.

Another hypothesis could be the secretion of osteoclastogenesis inhibitory cytokines, such as IL-33 (Ohori et al., 2020), by BC cells. In order to evaluate this hypothesis, it would be of interest to do a multiplex cytokine profiling of the CM of the different BC cell lines, and particularly compare the luminal BC cell lines with TNBC, in order to identify putative factors affecting monocyte differentiation.

As mentioned before, preliminary results from our lab suggested that the monocytic cell line RAW264.7 could differentiate in other cells than osteoclasts in the presence of BC cells' CM. The same was observed in this work, and since RAW264.7 monocytes can also differentiate in macrophages, we hypothesized that BC cells could potentiate macrophage differentiation. To test this hypothesis, expression of two macrophage gene markers, *CD206* and *iNOS* was analysed by RT-qPCR. *CD206* was expressed in all conditions, including the negative control without RANKL (unstimulated cells). RAW264.7 cells are considered to be macrophage-like cells (Kong et al., 2019), which may explain *CD206* expression. The expression of iNOS was increased in cells co-cultured with MCF-7 cells (Parental and RANK OE) in combination with RANKL, but not in the positive control where RANKL alone was used, which relative mRNA relative levels were similar to the negative control. This may suggest that co-culture induced a differentiation towards an M1 macrophage phenotype, especially in the presence of MCF-7 cells. Based on these results we are still not able to confirm or exclude our hypothesis, which requires further experiments. In the last years, several markers have been described to characterize macrophages, thus an extended panel of genes should be used to conduct this characterization.

Overall, we believe that the results obtained justify further research, to understand what other cells might being differentiated. In fact, considering that

macrophages are very sensitive to environmental conditions and that RAW264.7 are monocytic lineage cells, its phenotype might change with the passages and microenvironment. These cells may differentiate not only into osteoclasts but also M1 or M2 macrophages as well as DC, as seen in other studies (Garibaldi et al., 2017; Kong et al., 2019; Ma et al., 2015). Other types of cells were not assessed in our work.

In the future, and in order to characterize the differentiated cells, other techniques should be performed such as flow cytometry, where an extended panel of myeloid markers can be used to detect different cell populations, including monocytes, macrophages and DC.

6. Conclusions

In conclusion, our results demonstrate that human BC cell lines secretome impairs osteoclastogenesis, which can be exacerbated by RANK OE cells. Furthermore, our results suggest that BC cells' secretome may also promote the differentiation of monocytes into other cell types than osteoclasts, which remains to be further characterized. The characterization of these cells and of the secretome of the different BC cell lines by cytokine profiling, could be of great importance in further studies, helping us to better understand TME and tumoral behavior.

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