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Review

Recruitment of stromal cells into tumour microenvironment promote the metastatic spread of breast cancer

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ABSTRACT

Currently, metastasis remains the primary cause of death of patients with breast cancer despite the important advances in the treatment of this disease. In the complex tumour microenvironment network, several malignant and non-malignant cell types as well as components of extracellular matrix cooperate in promoting the metastatic spread of breast carcinoma. Many components of the stromal compartment are recruited from distant sites to the tumour including mesenchymal stem cells, endothelial cells, macrophages and other immune cells whereas other cells such as fibroblasts are already present in both primary and secondary lesions. When these cells come into contact with cancer cells they are "educated" and acquire a pro-tumoural phenotype, which support all the steps of the metastatic cascade. In this Review, we highlight the role played by each stromal component in guiding cancer cells in their venture towards colonizing metastatic sites.

1. Introduction

Despite current advances in breast cancer (BC) diagnosis and therapy, tumour recurrence and metastases remain the primary cause of morbidity and mortality for this disease [1]. To date, multiple subtypes of breast carcinoma have been identified with peculiar molecular features that determinate different clinical outcomes. The main classification of BC is based on expression of the three predictive biomarkers: oestrogen receptor (ER), progesterone receptor (PR) and human epidermal growth factor receptor 2 (HER-2). ER+/PR+/ HER2+ breast carcinomas belong to the luminal A subtype that grows slowly and has the best prognosis whereas those with ER+/PR+/ HER2- are in the category of luminal B that grow slightly faster than luminal A and show a slightly worse prognosis. However, the most aggressive subgroup with a very poor prognosis is the basal-like that include triple-negative breast cancer lacking ER, PR and HER2 expression [1].

In the last decade, many studies highlighted the crucial role played by tumour microenvironment (TME) and its components in modulating cancer behaviour such as resistance to therapies as well as relapse and metastasis [2]. TME comprises an extracellular matrix (ECM) formed by collagen, proteoglycans, laminin, and fibronectin that provides an active supportive structure for cancer and stromal cells [3]. In this contest, malignant and non-malignant cells interact tightly and cooperate in the realization of all the hallmarks of cancer including metastatic spread (Fig. 1). The stromal cells that mainly populate the breast TME are fibroblasts, mesenchymal stem cells (MSCs), adipocytes and immune cells such as T cells, natural killers and macrophages [4]. In addition, endothelial cells and perycites are also present of and are involved in blood and lymphatic vessel formation. Numerous biomolecules produced and released by cancer cells like cytokines, chemokines and growth factors intervene in the recruitment of stromal cells to the TME (Fig. 2). Noteworthy, many findings reported the involvement and active aid of stromal cells to cancer cells in all steps of metastatic spread including ECM remodelling, migration, invasion, intravasation, survival in circulation, extravasation and colonization of distant sites [5] (Fig. 3).

It is well known that cancer cells of several solid tumours are induced by stromal cells to undergo the epithelial-mesenchymal transition (EMT) thus acquiring a more aggressive phenotype necessary to carry out all the processes described above [6].

In this review, we focus on new research regarding the influence of MSCs, cancer-associated fibroblast (CAFs), cancer-associated adipocytes (CAAs), tumour-associated macrophages (TAMs) and other immune-cells in breast cancer metastasis.

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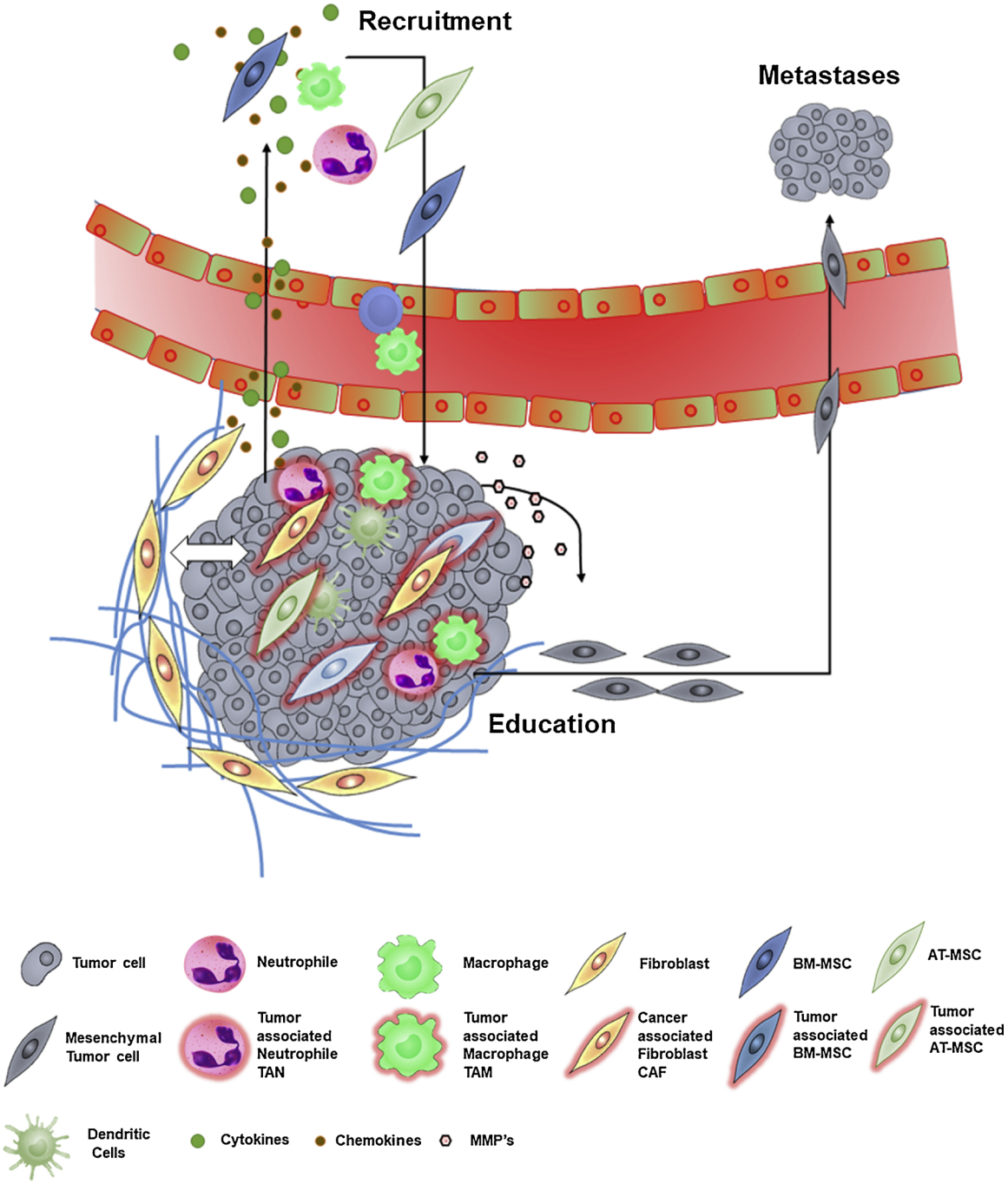

Fig. 1. Schematic illustration of each step involved in the metastatic spread of breast cancer.
Stromal components such as BM-MSCs, AT-MSCs, macrophages and neutrophils are recruited to TME and here "educated" to evolve in to tumour-associated stromal cells by means bidirectional communication with tumour cells. Tumour associated stromal influence the TME ultimately leading to the metastatic spread of breast cancer cells.

2. Tumour stroma cells

2.1. Mesenchymal stem cells

The main features of MSCs are: self-renewal, ability to differentiate in different cell types as well as to migrate towards injured tissue including tumours considered "the wounds that never heal". MSCs are recruited into breast cancer TME mainly from bone marrow (BM-MSCs) and adipose tissue (AT-MSCs) in response to different factors including microvesicles and exosomes released by cancer cells [7]. There, they are able to promote a pro-metastatic phenotype of BC cells by means of bi-directional communication through gap junction, nanotubes, receptors and or biomolecules [8]. Although the behaviour of BM-MSCs and AT-MSCs is very similar they differ in a few characteristics. AT-MSCs show higher stability in culture, proliferation and retain more efficiently their differentiation potential respect to BM-MSCs [8]. Moreover, in TME BM-MSCs mainly differentiate in CAFs whereas AT-MSCs transform into vascular and fibro-vascular stromal cells [8].

Many findings have reported that BM-MSCs homing towards breast TME and their activity is mediated by different signal pathways that involve hypoxia-inducible factors (HIFs), transforming growth factor β (TGF-β) and chemokine (C-C motif) ligand 5 (CCL5) [9].

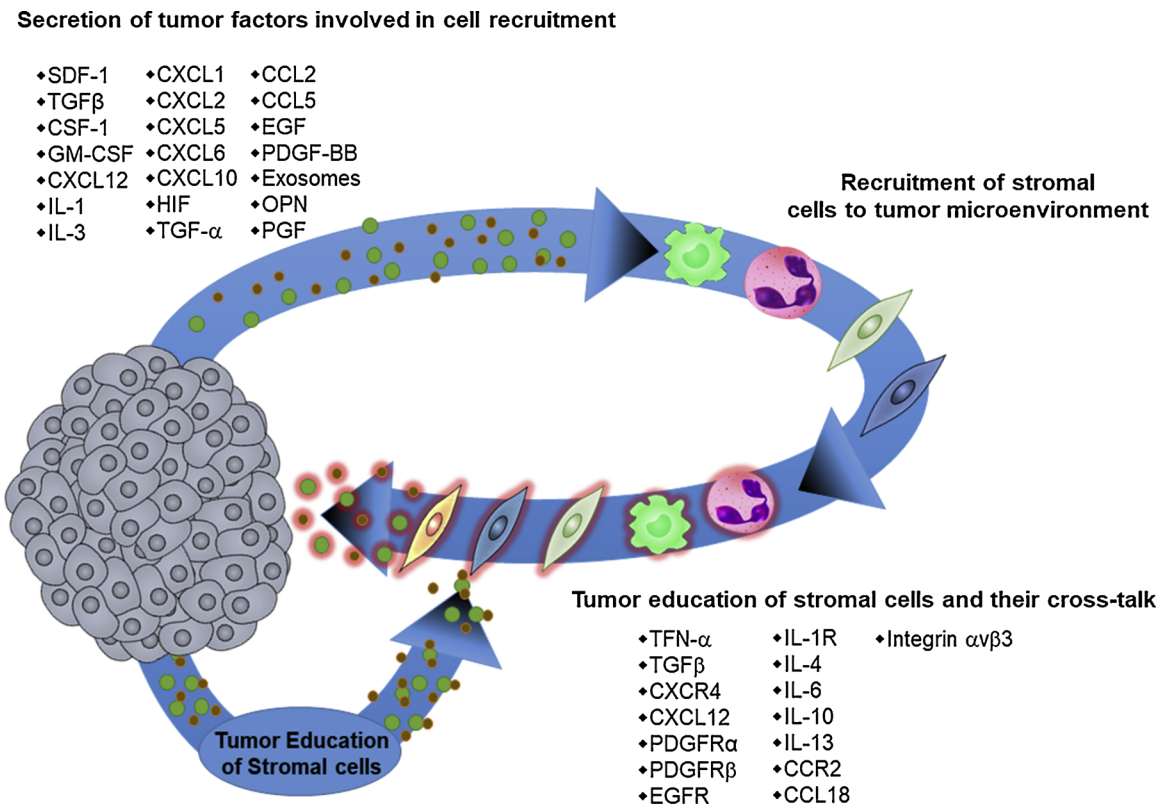

Fig. 2. Recruitment and tumour education of stromal components.
Many factors such as chemokines and cytokines are secreted from the primary tumour and are involved in the recruitment of stromal cells from distance sites for examples (MSCs, macrophages and neutrophils). These cells home towards the lesion and upon arrival together with resident cells such as fibroblasts are educated by cancer cells and can change their naïve gene signature and acquire pro-metastatic functions.

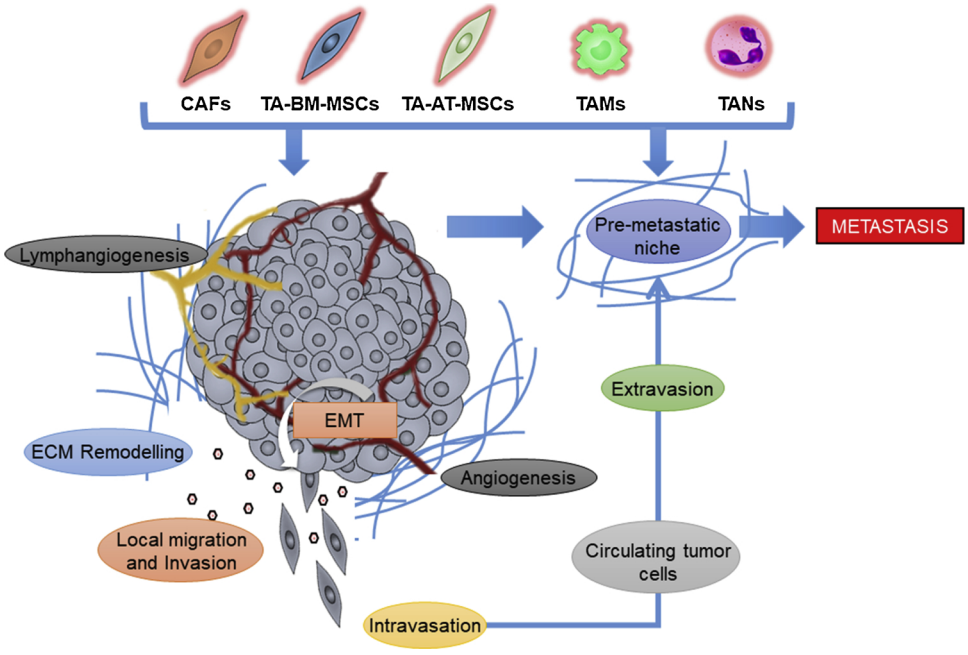

Fig. 3. Role Played by Tumour-Educated Stromal Cells in the Metastatic Cascade.
Here we illustrate the contribution of tumour educated stromal cells in the metastatic spread of breast cancer. These cells upon arriving into the TME in response to factors released by cancer cells acquired a new phenotype capable of supporting each step of the metastatic process. They induce EMT program in cancer cells that obtain a more invasive mesenchymal-like phenotype; promote the formation of new blood and lymphatic vessels (neo-angiogenesis and neo-lymphangiogenesis); ECM remodelling; intravasation; survival of CTCs; extravasation and finally contribute in the preparation of the pre-metastatic niche and support breast cancer colonization of metastatic sites.

Recently, Camorani et al reported the crucial role played by platelet derived growth factor receptor β (PDGFRβ) in BM-MSC recruitment into TME of triple negative breast cancer (TNBC) and their involvement in promoting tumour cell invasion and metastases [10]. When MDA-MB231 cells were co-injected with BM-MSCs into mammary fat pad of severely immunocompromised NOD scid gamma (NSG) mice an enhancement of lung metastatic foci was observed. Noteworthy, the treatment of BM-MSCs with a specific PDGFRβ aptamer reduced both their recruitment in subcutaneous xenografts as well as their support in lung metastasis formation. Moreover, a study showed that BRCA1-IRIS (aka IRIS, for In-frame Reading of BRCA1 Intron 11 Splice variant) TNBC cells recruited BM-MSCs through IL-6 production and in turn it activated in MSCs STAT3, AKT, and ERK/MAPK signalling to enhance their proliferation, migration and survival [11]. The inhibition of IL-6

signalling by neutralizing antibodies decreased BM-MSC migration. Interestingly, the cross-talk between cancer cells and BM-MSCs caused death rather than growth when IRIS was silenced in TNBC cells [11]. It has been observed that aberrant expression of microRNA-199a induced by BM-MSCs in breast cancer repressed the expression of forkhead transcription factor FOXP2 thus promoting cancer stem cell propagation and metastasis [12]. An interesting study showed that the fusion of MSCs from umbilical cord tissue (UC-MSCs) with breast cancer cells led a hybrid cell population with increasing ability to metastasize in *vivo* [13]. Recently, the same authors demonstrated that long-term co-cultures of MDA-MB-231 cells with (UC-MSCs) caused the formation of tumour spheroids and an altered expression of crucial factors involved in metastatic spread such as urokinase-type plasminogen activator (uPA), plasminogen activator inhibitor-1 (PAI-1) and TGF-β1 expression [14]. Furthermore, Bliss SA et al. reported that tumour cells were able to trigger BM-MSCs to produce exosomes containing miR-222/223 involved in promoting dormancy and metastatic resurgence of breast cancer cells in bone marrow [15]. When MSCs arrive in TME they are educated (TE-MSCs) by cancer cells and acquire a supportive pro-tumourigenic behaviour (Fig. 2). The main feature that differentiates TE-MSCs from naïve MSCs are the different factors contained in their secretome. Recently, Yu PF et al reported in a breast cancer model that MSCs educated and activated by tumour necrosis factor α (TNFα) strongly expressed CXCR2 ligands respect to naïve MSCs. Thanks to this, they recruited $CXCR2^+$ neutrophils into the tumour and they in turn promoted metastases [16].

Many studies have shown that when naïve BM-MSCs are co-cultured with TE-MSCs they acquire pro-tumourigenic properties thus demonstrating that not only tumour cells but also TE-MSCs themselves can educate naïve MSCs [17,18].

Interestingly, the secretomes produced by invasive MDA-MB-231 cells but not those released from MCF-7 cells were able to convert BM-MSCs into TE-MSCs indicating that overall tumours with a more aggressive phenotype can reprogram these cells [19]. Despite bone marrow, the other important source of MSCs for breast cancer is adipose tissue [20]. An interesting study by Muehlberg FL et al reported that AT-MSCs, when induced by breast cancer cells to release stromal cell-derived factor-1, are able to enhance their metastatic spread [21]. Furthermore, the authors demonstrated that AT-MSCs differentiated into endothelial cells and were incorporated into tumour vessels. Noteworthy, human adipose stem cells derived from healthy donors were able to enhance MDA-MB-231 cells invasion *in vitro* as well as lung, liver and spleen metastases *in vivo* [22,23]. Recent findings have shown that AT-MSCs from obese individuals (obAT-MSCs) overexpressing leptin promoted metastasis of both ER + breast cancer xenografts and TNBCs patient-derived xenograft (PDX) model [24,25]. The silencing of leptin gene expression in obAT-MSCs inhibited their metastatic contribution [24,25]. Both BM-MSCs and AT-MSCs can differentiate into adipocytes that provide metabolic substrates to cancer cells and increase their invasive capability [26].

Although many findings report the crucial role of MSCs from different sources in promoting breast cancer progression, there are some studies that demonstrate otherwise, probably due to changes in cancer cell secretome as well as different experimental settings. Noteworthy, Ryan et showed for the first time that IRIS-deficient TNBC cells were able to convert the activity of naïve MSCs from pro-tumourigenic to anti-tumourigenic [11]. When AT-MSCs were cultured at high-density in glucose deprivation, secreting type I interferon β (IFN-β) reduced MCF-7 growth through STAT1 phosphorylation [27]. Similarly, a recent study reported that microvesicles derived from AT-MSCs showed a cytotoxic effect on MCF-7 cells causing an increase of pro-apoptotic related gene expression and a decrease of anti-apoptotic genes in cancer cells [28]. Importantly, when MDA-MB-231 and T47D cells were co-cultured with an immortalized MSC line RCB2157, a different behaviour of these cells *versus* breast cancer cells was observed [29]. Media collected from 24 h co-cultures showed an increased MMP2 release from MSCs with a pro-metastatic effect. Conversely, a longer co-culture time (5 days) induced MSC tissue inhibitor of metalloproteinase-1 and 2 (TIMP-1 and TIMP-2) production changing MSC activity into anti-metastatic [29].

2.2. Cancer-associated fibroblasts

The most representative and heterogeneous population of stromal cells in breast cancer TME are cancer-associated fibroblast (CAFs). Nowadays, it is well known their crucial contribution given in cancer progression and metastatic process [30].

Despite resident fibroblast, numerous studies report that the main sources of CAFs are both AT-MSCs and BM-MSCs [31,32]. In addition, it has been observed that endothelial cells, pericytes as well as cancer cells and cancer stem cells can trans-differentiate into CAFs. This transformation is mediated by the exchange of multiple factors through different types of communications that each cell makes with each other. The main biomolecules involved in CAF establishment are often the same that in turn CAFs secrete to promote metastatic spread. Among them, the most prominent are TGF-β1, CXCL12, PDGF and IL-6. To date, although specific markers for CAFs have not been identified it is well known that their activation is correlated with high levels of alpha smooth muscle actin (αSMA), fibroblast activation protein (FAP), fibroblast specific protein 1 (FSP1 also reported as S1000A4) as well as platelets-derived growth factor receptor α and β (PDGFR α/β) [33]. Recently, Bush and colleagues suggested that in the TME there is a hierarchical differentiation of fibroblasts comprising diverse activation states with different molecular profiles and functions [34]. An interesting study reported that osteopontin was able to cause MSC trans-differentiation in CAFs through integrin-dependent expression of TGF-β1 and the block of this signal reverted the process and reduced metastases *in vivo* [35]. Similarly, the transformation of AT-MSCs into CAFs was hampered blocking TGFβ1 with a neutralizing antibody or with a TGFβ-1 receptor kinase inhibitor SB431542 [36]. Notably, it has been shown that aggressive breast carcinomas promote a metastatic phenotype by recruiting stromal fibroblasts and converting them into CAFs through Wnt7a potentiation of TGF-β receptor activity [37]. Recent findings by Li et al reported that ATP produced by breast cancer cells was able to activate fibroblasts which in turn through S100A4 secretion induced cancer cells motility [38].

2.3. Cancer-associated adipocytes

Recently, many findings highlight the crucial role played in breast cancer biology by tumour surrounding adipose tissue and in particular by adipocytes that represent the predominant cell population [39]. Currently, it is well recognized that obesity is correlated with higher risk in developing invasive breast cancer in postmenopausal women [40]. Noteworthy, in this cancer an inflamed adipose tissue is present where adipocytes secrete several cytokines known as adipokines including leptin, adiponectin, IL-6, TNF-α and HGF [39,41]. The adipocytes involved in breast cancer progression are activated in TME and show different features from adipocytes present in normal adipose tissues. These cancer-associated adipocytes are smaller for their reduced lipid content and show a modified phenotype that is able to produce high levels of pro-inflammatory cytokines in particular IL-6 that has been shown to promote a more aggressive behaviour of breast cancer cells [42]. Recently, He et al observed that both IL-6 and leptin produced by adipocytes increasing lysyl hydroxylase-2 (PLOD2) levels promoted breast cancer invasion *in vitro* and *in vivo* [43]. Remarkably, plasminogen activator inhibitor-1 (PAI-1) released by breast cancer cells is required to activate PLOD2 in CAAs resulting in collagen remodelling and metastasis formation [44]. Furthermore, in a recent very interesting study Kolb et al showed that obesity causes an increase of tumour-infiltrating macrophages that secreting IL-1β activate adipocytes to secrete angiopoietin-like 4 (ANGPTL4) that in turn promotes

angiogenesis in breast cancer [45].

In addition, it has been reported that in TNBC high levels of CCL5, also known as Regulated upon Activation Normal T-cell Expressed and Secreted (RANTES), in peritumoural adipose tissue correlated with metastasis and poorer patient overall survival [46]. Moreover, in this study the authors demonstrated that TNBC cells co-cultured with adipocytes showed increased invasiveness that was hampered by CCL5 inhibition [46].

2.4. Tumour-associated macrophages

In breast cancer, macrophages constitute over 50% of the number of cells within the tumour. They derive from monocytes recruited in TME by several molecules released in the blood by cancer cells and other stromal cells including monocyte colony stimulating factor 1 (CSF-1), granulocyte-macrophage (GM)-CSF, IL-3 and CCL2 [47]. Importantly, macrophage infiltration and high levels of CCL2 are associated with poor prognosis and metastatic disease in human breast cancer [48]. First, CSF-1 induce monocyte transformation into highly plastic non-polarized (M0) macrophages, then if they are stimulated by the type 1 T helper cell (Th1) cytokines such as interferon-γ (IFN-γ) they change their phenotype into "activated" M1-like macrophages. This phenotype shows anti-tumour capacity by releasing pro-inflammatory cytokines such as interleukin IL-2 and TNFα [49]. Conversely, if (M0) macrophages are stimulated by the type 2 T helper cell (Th2) cytokines such as IL-4, IL-10 and IL-13 they trans-differentiate in "alternatively activated" macrophages known as M2-like macrophages with pro-tumour characteristics [50]. In breast cancer, M2-like tumour-associated macrophages (TAMs) are the most numerous population among all immune-cells. Under different stimuli, they can differentiate in three subtypes: M2a, M2b and M2c macrophages that take part in all steps of metastatic cascade [50]. The most important signalling pathways involved in pro-metastatic behaviour of TAMs are activated above all by CCL18 and CCL2. For the first time, Chen and colleagues observed that TAMs expressing very high levels of CCL18, which was detected in blood or in tumour stroma of breast cancer patients, correlated with metastasis and poor overall survival [51]. Interestingly, in the same study they identified CCL18 receptor in a membrane-associated phosphatidylinositol transfer protein 3 (PITPNM3) and demonstrated that the interaction between this receptor with its ligand induced breast cancer cell migration and invasion *in vitro* and *in vivo*. Recently, the same authors discovered a small molecular compound, named SMC-21598, which tightly binds CCL18 inhibited lung metastasis formation but did not affect xenograft growth [52]. Furthermore, it has been shown that this chemokine synergizing with vascular endothelial growth factor (VEGF), promoted endothelial cell migration and angiogenesis and the silencing of PITPNM3inhibited these events [53]. Some isoforms of integrins, a family of adhesion receptors, are involved in aggressiveness of TNBC [54,55]. A recent very interesting finding reported that the overexpression of β4 integrin and its TGF-β1-dependent clustering on TAMs retained them in lymphatic endothelium in a TNBC murine model. Moreover, TGF-β1 released by TAMs activating RhoA signalling promoted contraction and hyperpermeability of lymphatic vessels thus facilitating metastasis [56]. A noteworthy study by Cassetta L et al (2019) highlighted on different transcriptomes of TAMs respect to monocytes and resident macrophages and identified a characteristic TAM signature that correlates with shorter disease-specific survival [57]. In addition, during cross-talk between cancer cells and TAMs an auto-regulatory loop was demonstrated where TNF-α produced by both cell types up-regulated SIGLEC1 and CCL8 expression on TAMs and CCL8 in turn stimulated cancer cell invasion, monocyte recruitment and CSF1 release from tumour cells [57]. Interestingly, Chen et al. showed that the infiltration of TAMs overexpressing cytochrome P450 (CYP) 4A was positively associated with pre-metastatic niche formation, metastasis as well as poor prognosis in breast cancer patients [58].

2.5. Other immune-cells recruited in TME

Despite TAMs other immune-cells such as neutrophils, dendritic cells, natural killer (NK) cells, T cells and B cells are recruited and modified by cancer cells to support their progression and metastatic cascade [59].

Tumour-associated neutrophils (TANs) have been shown to be a conspicuous population in the TME even if their role must be even better clarified. Similarly to TAMs, in TANs is known a subpopulation with antitumour activity (with N1-like phenotype) including mature high-density neutrophils (HDNs) and a pro-tumourigenic subtype (with N2 profile) containing mature low-density neutrophils (LDNs). The recruitment of neutrophils in TME is mainly regulated by chemokine receptor CXCR2. Interestingly, Yu et al demonstrated that TNFα-activated MSCs releasing CXCL1, CXCL2 and CXCL5 efficiently recruited $CXCR2^+$ neutrophils into tumour [16]. They in turn caused an up-regulation of metastasis-related genes in tumour cells. Notably, the inhibition of this cross-talk between different stromal cells inhibited neutrophil recruitment and blocked metastases formation [16]. Recently, a very impressive study by Szczerba and colleagues reported that circulating tumour cells (CTCs), which are well-known precursors of metastasis could associate with neutrophils in breast cancer [60]. The formation of these clusters involves VCAM1 and cause a change in the transcriptomic profile of CTCs as well as an enhancement of their metastatic potential [60].

Tumour-infiltrating lymphocytes (TILs) present in breast TME include CD4+ helper cells, immunosuppressive CD4+FOXP3+ regulatory T-cells (Tregs) and CD8+ cytotoxic T-cells (CTLs), each subtype shows different activity respect to metastatic spread of cancer cells. Many studies have demonstrated the crucial role played by Tregs into promoting invasion, metastasis and poor prognosis [61–63]. Furthermore, it has been elucidated that the recruitment of Tregs is under control of several chemokines including CCL2, CCL5 and CXCL12 produced by malignant and non-malignant cells present in TME. In addition, it has been reported that CXCL12 released by claudin low breast cancer cells caused recruitment of Tregs highly expressing programmed cell death protein-1 (PD-1) and their depletion increased the effect of immune checkpoint blockade antibodies [64]. Several studies suggest the strict and inverse connexion between Tregs and CTLs cells in promoting breast cancer metastatic phenotype. Interestingly, low-aggressive breast TM40D cancer cell line induced to overexpress cyclooxygenase 2 (COX2) and prostaglandin E2 (PGE2) showed an increase of Tregs recruitment into the primary tumour as well as CD8 + T cell apoptosis; both these two events were associated with a greater rate of bone metastasis [65].

NK cells are a component of the innate immune response and are responsible for the rapid recognition and elimination of cancer cells [66]. Moreover, Bidwell et al. observed that the silencing of Irf7 pathways reduced CTLs and NK cells causing an enhancement of bone metastasis while not affecting the growth of the primary tumour [67]. It has been observed that $CCR4^+$Tregs promoted lung metastasis in a murine 4T1 breast cancer model by inducing NK cell death through β-galactoside-binding protein [68]. In a genetically engineered mouse (GEM) model of breast cancer caused by the mammary epithelial expression of polyoma virus middle T (PyMT) antigen, the accumulation of TH17 cells into tumour increased MDSCs recruitment through IL-17 of which in turn was responsible for lung metastasis establishment [69]. Noteworthy, Zhang and collaborators demonstrated that gene delivery of TIPE2 (tumour necrosis factor-alpha-induced protein 8-like 2) inhibited breast cancer metastasis in lungs causing CD8+ T and NK cell cytotoxic activity [70]. A recent study showed that in breast cancer high levels of Morgana, positively correlated with NF-κB target gene expression, drove metastasis by decreasing NK cell recruitment in primary tumour and increasing neutrophil infiltration [71]. Furthermore, given the crucial role played by JAK/STAT pathway in development, maturation, and activation of NK-cells it has been demonstrated, that

the use of JAK inhibitors despite blocking STAT activation in tumour cells, enhanced metastatic burden in preclinical models of breast cancer by decreasing NK-cell-mediated anti-tumour immunity [72].

For the first time, Olkhanud et al showed that regulatory CD25 + B cells (tBregs), generated from normal B cells in response to cancer cell–produced factors, were able to induce metastases in a murine model of breast cancer stimulating the conversion of Tregs from non-Tregs by utilizing TGFβ [73]. Notably, Bregs depletion using a B220-specific antibody reduced lung metastatic foci due to a poor Treg transformation [73].

3. Role played by stromal cells in each step of metastatic cascade

3.1. Epithelial-mesenchymal transition programme

In the last decade, many studies have highlighted on the active role played by all stromal cells in inducing epithelial-mesenchymal-transition programme (EMT) that eliciting profound morphological and functional changes in epithelium cancer cells triggers a mesenchymal-like phenotype with a higher invasive potential [74,75]. A cascade of molecular events characterizes the transformation of cancer epithelial cells into mesenchymal cells. First, the destabilization of the epithelial cell–cell junctions, loss of apical–basal polarity, reorganization of the cytoskeletal architecture and an increased ability to migrate, invade as well as degrade ECM [6].

The mechanisms underlying these occurrences are correlated with a reconfiguration of gene expression signatures. In particular, the down-regulation of the most important epithelial marker E-cadherin and parallel up-regulation of mesenchymal markers: vimentin, N-cadherin and fibronectin. The activation and/or inhibition of gene expression involved in EMT are under control of multiple transcription factors including the zinc-finger proteins Snai1 (Snail) and Snai2 (Slug) (10–12), the basic helix-loop-helix protein Twist1 (Twist), and the zinc-finger, E-box-binding proteins Zeb1 and Sip1 (Zeb2), the forkhead box proteins FOXC1 and FOXC2 [6]. Interestingly, when the EMT core signature was compared with signatures that define breast cancer subtypes a close association was found overall with the claudin-low and metaplastic breast cancer subtypes [76].

Many factors released by stromal cells are involved into orchestrate EMT programme. MSCs secreted in TME cytokines such as IL1 and IL6 [77], chemokines including CCL5, CXCL1, CXCL5, CXCL7 CXCL8 and CXCL12 [78,79], growth factors (TGF-β, FGF, Hepatocyte growth factor HGF and epidermal growth factor, EGF) [80,81] as well as hypoxia inducible factors and reactive oxygen species. Many studied have reported the involvement of miRNAs in the network controlling EMT programme. In breast cancer cells miRNA-9 activated by MYC/MYCN down-regulate E-cadherin expression causing an increase in cell motility and invasiveness as well as β-catenin signalling. These effects contribute to an elevated expression of VEGFA that in turn induce tumour-associated angiogenesis and metastases *in vivo* [82]. Recently, a very interesting study showed that the orphan chemokine CXCL14, known to be a poor prognosis factor in breast cancer, released in particular by fibroblast in TME, through the engagement of atypical chemokine receptors 2 (ACKR), promoted EMT and metastasis *in vivo* [83]. Interestingly, Wu S et al observed that when human adipose derived stem cells were co-cultured with MCF7 breast cancer cells their paracrine effects inducing TGF-β/Smad and PI3K/AKT pathways activated EMT programme as well as cancer cells invasiveness [84]. To date, it is well established that TGFβ is a potent driver of EMT [85]. Different breast cancer cell lines including MCF-7, T47D and MDA-MB-231, grown with conditioned medium obtained by CAFs isolated from invasive breast cancer tissues trans-differentiate under TGFβ stimulation in a more aggressive phenotype characterized by EMT activation, enhanced cell-extracellular matrix adhesion, migration and invasion [86]. In addition, it has been reported that TGF-β1 secreted by CAFs is able to trigger EMT/metastatic processes increasing HOTAIR expression. Notably, Wen S and colleagues found that CAFs released IL32 that specifically bound to integrin β3 of breast cancer cells through the RGD motif thus activating p38 MAPK signalling. In turn, the activation of this pathway enhanced EMT markers expression such as vimentin, N-cadherin, and fibronectin and promoted tumour cell invasion [87]. Recently, a study from our group demonstrated that a novel peptide targeting αvβ3 integrin was able to revert EMT and hamper the aggressiveness of TNBC cells [54].

Furthermore, Kumar S et al demonstrated that PMN MDSCs were recruited in TNBC responding to CXCL2 and CCL22 under control of transcription factor ΔNP63 and contributed to increase EMT gene signatures making them more invasive and metastatic [88]. In another study, the same group showed that in 4T1 TNBC xenografts the genetic ablation of Crk by CRISPR-Cas9 suppressed EMT and programmed death ligand-1 (PD-L1) expression on tumour cells and enhanced anti-tumour immune cell populations in primary tumour causing a significant reduction in tumour growth and lung metastasis [89]. In addition, it has been reported in the same murine model that CXCR2+ MDSCs through IL-6 induced cancer cell EMT and promoted T cell exhaustion [90]. Furthermore, when adipocytes were co-cultured with MCF-7 they caused a down-regulation of E-cadherin expression while increased N-cadherin levels in these cells [91]. In addition, soluble factors secreted by activated T cells such as TNF-α, IL-6, and TGF-β were able to induce the expression of EMT-related genes and promote metastasis in inflammatory breast cancer [92]. Interestingly, TAM infiltration has been associated with EMT and low E-cadherin expression levels in TNBC demonstrating their involvement in inducing the activation of this programme [93]. In particular, some findings have reported an increased expression of EMT markers such as TWIST1 and MMPs in breast tumours with high immune infiltration in the TME [94]. Santisteban M and collaborators found that CD8 T cells in breast animal models induced EMT causing the formation of tumours with breast cancer stem cells characteristics [95].

3.2. Angiogenesis and lymphangiogenesis

Each type of stromal cell recruited in breast TME in concert with malignant cells play a pivotal role into promoting angiogenesis process where new blood vessels supplying oxygen and nutrients are needed to support tumour growth and metastatic spread. Vascular endothelial growth factor A (VEGFA) is is the leader in orchestrating the vessel network in the tumour [96].

In 2005, Orimo A with his collaborators demonstrated that CAFs extracted from human breast carcinomas secreting SDF1 induced the growth of admixed breast carcinoma cells *in vivo* and promoted angiogenesis through the recruitment of endothelial progenitor cells [97]. CAFs are the main source of many pro-angiogenic factors including VEGFA, PDGFC, FGF2, osteopontin and secreted frizzled-related protein 2 (SFRP2) [98]. Of note, it has been observed that hypoxia inducing the expression of HIF-1α and GPER (G-protein oestrogen receptor) in CAFs caused release of VEGFA and promoted tube formation in Human Umbilical Vein Endothelial Cells (HUVECs) [99]. Similarly, the same group in a following study demonstrated that IGF1/IGF1R axis through activation of ERK1/2 and AKT induced in CAFs and breast cancer cells the expression of HIF-1α and its targets GPER and VEGF. Furthermore, they established that the activation of both these factors is required for VEGF-induced human vascular endothelial cell tube formation [100]. Recently, an interesting study by Kugeratski FG showed that secretome of hypoxic human mammary CAFs, analysed using a mass spectrometry-based proteomic approach, and contained high levels of HIAR (hypoxia-induced angiogenesis regulator) regulator of VEGFA secretion [101]. Notably, miR-205/YAP1 (Yes associated protein) signalling axis induced trans-differentiation of normal breast fibroblasts into CAFs that in turn releasing IL11 and IL15 were able to stimulate tubule formation and sprouting of HUVECs [102].

TAMs are major contributors in angiogenesis process [47]. Their key

role in the angiogenic switch has been well elucidated by an excellent study carried out by Lin and collaborators in which they demonstrated that depletion of CSF-1 in mammary tumours caused inhibition of macrophage maturation and infiltration as well as vessel formation [103]. Conversely, CSF-1 overexpression triggered macrophages accumulation in hyperplastic lesions that supporting a very early and important angiogenic switch primed a more aggressive phenotype [104]. Another important factor released by TAMs involved in angiogenesis and tumour progression in breast cancer is CCL18. Silencing of the putative receptor of this chemokine in HUVECs hampered their ability to form vessels [105]. It has been reported that TAMs express very high levels of the WNT family ligand WNT7B. This in turn, causes in a MMTV-PyMT model of luminal breast cancer angiogenic switch as well as metastasis [106]. Interestingly, the deletion of the transcription factor Ets2 in TAMs decreased angiogenesis and lung metastases in different breast cancer murine models [107]. Similar findings revealed the ability of Ets2 in fibroblasts to promote blood vessel formation in the absence of tumour cells [108]. The pro-angiogenic factor angiopoietin 2 (ANG2) not only stimulated EC but also macrophages expressing its receptor TIE2 to actively participate in the formation of vessel networks in breast cancer [109,110]. Interestingly, the deletion of HIF1α in $CD8^+$ T cells reduced not only their tumour infiltration but also altered tumour vascularization [111]. Recently, Tian L et al suggested that type 1 T helper (T_H1) cells play a crucial role in vessel normalization [112].

Like angiogenesis the lymphangiogenesis is a process that determinate the formation of new lymphatic vessels starting from the remodelling of existing lymphatics and it is an important step in cancer metastasis. In particular, breast cancer cells through lymphatic vessels metastasize to adjacent lymph nodes [113]. Recently, it has been observed that Lysyl Oxidase-like protein 2 (LOXL2) significantly promoted tube formation by activating lymphatic endothelial cell invasion and induced CAFs to secrete high level of pro-lymphangiogenic factors VEGF-C and SDF-1α. In an orthotopic breast cancer model LOXL2 increased lymphatic vessel density and lymph node metastasis without affecting growth of primary tumour [114].

3.3. ECM remodelling

ECM contributes actively in supporting metastatic spread of breast cancer cells through the cross-talk between stromal cells and tumour cells. Predominantly CAFs are involved in the aberrant ECM remodelling where at the same time they produce components of ECM such as collagen, tenascin C and fibronectin as well as they secrete proteolytic enzymes such as metalloproteinases (MMPs) that intervene in ECM degradation [115]. The balance between these two process primes cancer cells migration and invasion.

The principal ECM constituent deposited by CAFs is fibrillary collagen type I and its accumulation causes an increase of stiffness strictly correlated to progression towards a more invasive phenotype [3,116]. Notably, during transformation of ductal carcinoma *in situ* to invasive ductal carcinoma there is an enhancement of the collagen fibers crosslinking as well as their thickening and linearization [117]. Interestingly, more aggressive breast cancer subtypes such as HER2+ and TNBC present a major accumulation of collagen and matrix rigidity respect to less malignant subgroups [117]. Furthermore, the organization of these ECM fibrils perpendicularly to the tumour boundary create the tracks that cancer cells have to follow for invading and metastasizing to adjacent tissues and vessels [117]. In a recent study, Jones CE et collaborators reported that phosphatase and tensin homolog (PTEN) depletion in fibroblasts promotes collagen deposition and its remodelling perpendicular to the tumour edge in a breast cancer murine model [118]. The fibrillar collagen receptor discoidin domain 2 (DDR2), a distinctive receptor tyrosine kinase activated by fibrillar collagens has been shown to be involved to mediate breast cancer metastasis but not tumour growth [119].

Furthermore, increased matrix stiffness down-regulating PTEN levels *via* miR-18a enhanced ability of breast cancer to migrate and invade [120]. It has been observed that CAFs are stimulated to produce collagen I and IV and fibronectin through a mechanism involving both TGF-β and MMP2. Indeed, shRNA-mediated MMP2 knockdown reduced the CAF release of ECM fibers and prevented breast cancer metastasis in the lung [121]. Similarly, Wang TN et al observed that thrombospondin-1 (TSP-1) released by fibroblast inducing MMP9 expression in breast cancer cells promoted their ability to invade the ECM [122]. Loss of COX-2 expression in cells reduced the number of CAFs, their collagen I production, and metastatic potential *in vivo* [123]. Of note, studies by Barcus et al showed that in collagen dense (Col1a1tm1Jae/+) mice, collagen fibers ran in parallel with the infiltrating tumour cells and were perpendicular to the bulk of the tumour and this behaviour was correlated with increased circulating tumour cells and the number and size of lung metastases [124]. Fibronectin is another prominent ECM protein produced by CAFs that contributes to breast cancer progression. Interestingly, its polymerization and organization into ECM is a prerequisite for the deposition of collagen-I [125]. Their synergistical interplay is mediated by MMPs and is correlated with invasive phenotype [125]. In addition, the multimeric extracellular glycoprotein Tenascin-C (TNC) component of breast cancer ECM, resulted overexpressed in CAFs when they were exposed to conditioned medium from the human breast cancer lines containing TGFβ1 [126]. Normal mammary gland present low or absent levels of TNC whereas in stroma of ductal carcinomas it is overexpressed. Furthermore, high levels of TNC correlate with tumour stage, lymph node metastasis, TAM infiltration, and predicts poor overall survival [127]. Notably, in addition to the high expression of the fully truncated TNC, others two isoforms, one containing exon 16 (TN16) and one containing both exons 14 and 16 (TN14/16), resulted significantly associated with the invasive phenotype [128]. These TNC isoforms promoted breast cancer invasion through up-regulation of MMP-13 and TIMP-3 [129]. Similar to TNC, tenascin-W was found in the breast tumour stroma and serum of patients. Fibroblasts adhered to TNW through integrin β1 and promoted the migration of breast cancer cells towards a fibronectin substratum *in vitro* [130]. The CAFs role in remodelling ECM is strictly connected with their production of multiple isoforms of MMPs such as MMP-1, MMP-7, MMP-9, MMP- 11, MMP-12, and MMP-14 [131]. Breast cancer cells can induce stromal MMP-9 expression *via* the release of TGF-β1 and TNF-α which leads to an enhanced migration [132]. These enzymes not only degrade ECM components but also promote metastatic spread activating EMT program [133]. It has been reported that CAFs increase ECM stiffening through transcription factor YAP1 that regulates the expression of several cytoskeletal regulators required for cancer cell invasion. At the same time *via* a positive-regulatory loop, matrix remodelling further enhances YAP activation [134]. Interestingly, caveolin-1 (Cav1) expression in breast CAFs stimulates contraction, matrix alignment and stiffening p190RhoGAP-mediated thus directing carcinoma cell migration and invasion [135]. Furthermore, matrix stiffness is regulated by lysyl oxidase (LOX) that is overexpressed in stromal cells stimulated by hypoxia, TGF-β1, and miR-200 [136,137]. High levels of LOX increased collagen crosslinking as well as migration and metastasis of breast cancer cells [138]. Interestingly, it has been observed that loss of stromal LOX did not alter primary tumour growth but did decrease metastasis in a PyMT mouse model [139]. In addition to their functions in ECM remodeling [140], MMPs can also alter tumour motility directly by cleaving E-cadherin and inducing EMT [141,142]. TAMs actively cooperate with CAFs in endorsing tumour cell invasion taking a part in ECM remodeling. It has been observed that TAMs secreted the protein acidic and rich in cysteine (SPARC) involved in regulating collagen IV deposition and cell-ECM interaction through integrin αvβ5 that in turn mediated migration of invasive cells [143]. Tumour cells move along with TAMs on fibrillar collagen 1 structures toward blood vessels where TAMs assist tumour cell intravasation as shown by intravital imaging of xenografted tumours [144–146]. Similarly, CAFs can create fissures in

the matrix or basement membrane to guide collective invasion of the tumour cells, which are linked, by cell–cell junctions [147,148]. The migration of TAMs together with breast cancer can be initiated by heregulin and CXCL12 and required a paracrine CSF-1-EGF loop [149]. Time-lapse imaging showed that tumour cells migrated toward the vessel-associated macrophages and that their intravasation only occurred when perivascular macrophages were present [145]. Recently, Linde N et al reported in a mouse model of $HER2^+$ breast cancer, that depletion of macrophages reduced early cancer cells dissemination as well as metastatic burden [150]. Breast cancer cell intravasation and dissemination takes place through tumour microenvironment of metastasis (TMEM) that are structures composed of tumour cells expressing the actin-regulatory protein Mammalian-enabled (MENA), perivascular macrophages and endothelial cells [151]. The presence of TMEM has been observed in mouse and human mammary carcinomas, and its density is associated with metastatic outcome in breast cancer patients [152]. In TMEM sites, cancer cells may intravasate and disseminate to secondary sites through endothelial cell transient dissociation of junctions, TMEM-bound macrophages expressing TIE2 *via* VEGF-A control vascular permeability [153].

3.4. Pre-metastatic niche

A crucial step in the establishment of metastases includes the formation of a pre-metastatic niche where recruited stroma cells contribute to create a favourable microenvironment permitting cancer cell seeding. They to successfully germ in a distant site need to find nutrients, an ECM that can support their attachment and stromal cells that help them with paracrine signal to survive and proliferate in the new environment [154]. In 2005 for the first time, Kaplan and colleagues introduced the concept of pre-metastatic niche because in their studies they observed that VEGFR1-positive bone marrow–derived cells (BMDCs) were recruited to future sites of metastasis before the tumour cells. There BMDCs led the arrival and attachment of circulating cancer cells through SDF-1/CXCR4 axis [155]. Pre-metastatic niche establishment not only involves the recruitment of foreign cells but also the reprogramming of the resident stromal cells to facilitate metastatic growth. However, the primary tumour secretes soluble molecules (TDSFs) and extracellular vesicles (EVs) including exosomes containing proteins, mRNAs, microRNAs, small RNAs, and/or DNA fragments needed to create a suitable environment for the survival of metastatic cells [156]. It is worth noting that Liu Y et al identified different steps in the preparation of metastatic sites. In the “priming phase”, the factors released by primitive tumour remodelling ECM and reprogramming stromal cells to create an immature pre-metastatic niche; follows a “licensing phase” where BMDCs and regulatory/suppressive immune cells are mobilized and recruited thus establishing a mature pre-metastatic niche for potential seeding and colonization of CTCs; in the third phase denominated “initiation phase”, CTCs arrive to fertile pre-metastatic niche and start to grow and colonize; some of them survive whereas others enter dormancy, in this phase there is the formation of micrometastases. The last phase is “the progression phase”, during which metastatic tumour cells expand and progress leading to macrometastases [156]. When breast cancer cells spread from the primary tumour, they exhibit a propensity to metastasize to specific sites such as the bone, lung, liver and brain [157]. Among early constituents of the pre-metastatic niche, there are ECM proteins such as fibronectin [155], TNC, Periostin (POSTN) and Versican (VCAN). It has been reported that breast cancer cells that infiltrate the lungs support their own metastasis-initiating ability by expressing TNC [158]. Similarly, S100A4 + fibroblasts are able to promote lung metastases releasing TNC and VEGFA [159]. Interestingly Malanchi et al showed that lung infiltrating breast cancer cells induced POSTN expression by stromal cells to initiate their colonization of pre-metastatic niche. Furthermore, they observed that blocking POSTN function prevented lung metastasis [160]. Notably, it has been elucidated that microvasculature of lung, bone marrow and brain metastases constitute a niche of dormant tumour cells that can be activated by POSTN and TGF-β1 produced from endothelial tip cells [161]. The production of VCAN in metastatic lungs mouse models of spontaneous breast cancer was mainly due to $CD11b^+Ly6C^{high}$monocytic fraction of the myeloid cells and its high levels was found within the metastatic lung of patients with breast cancer. In addition, it has been demonstrated that VCAN stimulated mesenchymal to epithelial transition of metastatic tumour cells by attenuating phospho-Smad2 levels [162]. It has been identified in a murine model of breast cancer that MDAMB231 cells induced recruitment of $CD11b^+$ cells in pre-metastatic lung releasing LOX which crosslinking collagen IV favour their adherence [163]. Similar findings showed that hypoxic breast cancer cells at the same time increased CD11b + cells accumulation and reduced cytotoxic NK cells in the lungs [164]. The factors produced by primary tumour recruit bone-marrow derived monocytes and activate tissue-resident macrophages during the different phases of pre-metastatic site formation. In turn, they trans-differentiate in metastasis-associated macrophages (MAMs) and support breast cancer cell arrival, extravasation as well as survival protecting them from immune cell attack [165]. Indeed, it has been observed that MAMs in murine models of breast cancer were able to suppress $CD8^+$ T cells [166]. Notably, it has been reported that osteoclasts, the bone-resident macrophages, play a key role in the formation of bone pre-metastatic niche. Indeed, ER- mammary tumour cells through LOX release induced osteoclastogenesis promoting osteolytic lesions that prepared a permissive environment for breast cancer cells [167]. Monteiro et al reported that also CD4 + T cells were able to induce osteoclastogenesis and promoted bone metastasis from breast cancer cells secreting RANKL (receptor activator of nuclear factor-kB ligand) [168]. Interaction between MAMs and endothelial cells help circulating cancer cells adhesion and transmigration into the metastatic sites thus increasing their metastatic potential [169]. In a breast cancer murine model, infiltrating neutrophils into the pre-metastatic lung hampered the development of lung metastases [170]. Their depletion, using a specific Ly6G + antibody, increased the lung metastatic burden whereas direct injection of TAN dramatically decreased lung foci [170]. Conversely, Szczerba BM et al, in a very interesting recent study, showed that CTCs isolated from patients with breast cancer and from breast mouse models resulted associated with neutrophils in clusters and this link was crucial for CTCs survival in a hostile environment of blood flow [60].

4. Conclusions

The recent findings have elucidated the importance of communication between breast cancer cells and tumour “educated” stromal cells recruited from other tissues or already resident in TME in promoting metastatic process. Many studies reported that the molecules released by both cancer and non-cancer cells in TME and in circulation during the different phases of tumour progression are often the same for example TGFβ, CXCL12, CCL2, IL-6, IL-3, CSF-1, GM-CSF. Currently, numerous researches are focusing on shedding light on the crucial role played by components of tumour stroma that when in the TME can change their naïve gene signature and acquire a more pro-metastatic function. A clearer understanding of the underlying mechanisms involved in the cross-talk between stromal and cancer cells in the microenvironment of the primary tumour and metastases could allow the implementation of novel strategies to block these interactions thus improving the effects of anti-cancer therapy in breast cancer.

Declaration of competing interest

The authors declare that there are no conflicts of interest

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