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*From Cell Biology to Tissue Engineering*

***Review***

# The role of tumor-associated macrophage in breast cancer biology

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**Summary.** Breast cancer is the most commonly diagnosed malignant tumor in women worldwide and contributes significantly as the primary cause of female cancer related mortality. Hence, research is focused on discovering new and effective treatment targets. The breast tumor microenvironment (TME) comprising of recruited host stromal cells and tumor cells, has recently emerged as an important player in tumor progression, with the potential for future treatment. The TME comprises immune system elements (such as macrophages and lymphocytes), cells composing blood vessel, fibroblast, myofibroblast, mesenchymal stem cells, adipocytes and extracellular matrix (ECM). Among these cells, tumor-associated macrophages (TAM) are the prominent components of TME in breast cancers. Macrophages exhibit a high plasticity in response to various external signals and participate in innate and adoptive immune responses to control numerous factors of TME. Depending on the microenvironmental signal present, macrophages are polarized into two distinct phenotypes, the classically activated (M1) or the alternative activated (M2) macrophages. Tumor-associated macrophages (TAMs) closely resemble the M2-polarized. Clinicopathological studies have suggested that TAM accumulation in tumors correlates with a poor clinical outcome. In human breast carcinomas, high TAM density correlates with poor prognosis. Over the years, studies into the role of TAMs in breast cancer progression have identified TAMs to be capable of inducing angiogenesis, remodelling the tumor extracellular matrix to aid invasion, modelling breast cancer cells to evade host immune system and recruiting immunosuppressive leukocytes to the tumor microenvironment. Along with these functions, the potential role for TAMs in activation of breast cancer stem cells (CSC) has also emerged. Thus, TAMs in breast cancer can enhance cancer cell invasion by degrading the ECM, stimulate tumor vascularization and angiogenesis and suppress the anti-tumor functions of cytotoxic T cells resulting in poor prognosis for patients. These observations make TAMs an attractive target for therapeutic intervention by targeting various aspects of their function. This review discusses the mechanisms responsible for TAM recruitment and highlights the roles of TAMs in regulating tumor angiogenesis, invasion, metastasis, immunosuppression, and chemotherapeutic resistance. Finally, the potential for TAM-targeted therapy as a promising novel strategy is also discussed.

**Key words:** Breast cancer, Tumor-associated macrophage, Tumor microenvironment

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## Introduction

Breast cancer is the most commonly diagnosed malignant tumor for women around the globe, in which it contributes as the primary cause for female cancer related mortality (Benson and Jatoi, 2012). Whereas a significant drop in the mortality rate of breast cancer has been observed recently due to the development of tissue diagnostic programs and medications. Limitations in improvements still occur in certain breast cancer types, especially triple negative breast cancer (TNBC); which

have no effective solutions except surgical measures (Ismail-Khan and Bui, 2010). Thus, continuous attempts are being made to discover effective treatment targets, one of which is the most promising, tumor micro-environment (TME). With the progression of cancer research, the prominence of tumor micro-environment (TME) has also risen. TME is a non-transformed element that is not a tumor cell, but is located within the tumor region. It includes immune system elements (such as macrophages and lymphocytes), cells composing blood vessel, fibroblast, myofibroblast, mesenchymal stem cells, adipocytes and extracellular matrix (ECM) (Burugu et al., 2017). The importance of TME in breast cancer has been emphasized by extensive research done on how it contributes to tumor formation, progression from ductal cell carcinoma *in situ* (DCIS) to invasive carcinoma, and metastasis (Hu et al., 2008; Mao et al., 2013), while the tumor-stroma ratio and stroma type has also been shown to be closely associated with the recurrence, distance metastasis, and survival of breast cancer (de Kruijf et al., 2011; Qian et al., 2011). Among the cells that consist breast cancer TME, tumor-associated macrophages (TAM) are the predominant component of tumor mass, in some cases comprising over 50% (Lewis and Pollard, 2006). Due to the high plasticity macrophages exhibit, it can efficiently respond to various external signals and participate in innate and adoptive immune responses to control numerous factors of TME. Further research on the correlation between TAM and malignant tumors has revealed potential in TAM not only as an important biomarker in cancer diagnosis and prognosis, but also a significant candidate of targeted therapy. This review discusses the various roles of TAM in breast cancer and its clinical implications as an anti-cancer therapy.

## The origin and categories of tumor-associated macrophage

Generally, tissue macrophages can be categorized into bone marrow derived 'recruited macrophages' and primitive yolk sac precursor derived 'tissue resident macrophages' (Yang and Zhang, 2017). While both types are found as TAM, the proliferation of tissue-resident macrophages in the HER-2 type breast cancer model has been found to contribute to the TAM pool (Van Overmeire et al., 2014). However, it has not yet been verified how the differently originated TAM affect the cancer progression stage.

As a key molecule and effector, macrophages are activated by various immune responses. Such macrophage activation can be classified into M1 (classic) and M2 (alternative) activation (Martinez et al., 2008 ; Cassetta et al., 2011). During M1 activation, IFN-γ and lipopolysaccharide or tumor necrosis factors are involved to bring about a Th1 response, which is related to type I inflammation, primarily participating in intracellular pathogen killing and antitumor immunity (Montes et al., 2013; Xu et al., 2016). M2 activation is further classified into M2a, M2b, M2c, and M2d (Zhang et al., 2010; Lu et al., 2013). M2a activation involves IL-4 and IL-13, leading to a Th2 response regarding type II inflammation for the killing and encapsulation of parasites (Nelson et al., 2012). On the other hand, M2b activation utilizes the immune complex and toll-like receptor ligand for Th2 activation and immunoregulation (Ohama et al., 2015). M2c activation is activated by IL-10 and is involved in immunoregulation, matrix deposition, and tissue remodeling (Lu et al., 2013). M2d activation is known to enhance the induction and growth of tumor cell mass through angiogenesis by the stimulation of IL-6 (Cao et al., 2015). Typical markers for M1 macrophages are CD64, IDO, SOCS1, CXCL10, while for M2 macrophages, there are MRC1, TGM2, CD23, CCL22 (Martinez and Gordon, 2014).

## Macrophage polarization in breast cancer

Traditionally, M1 macrophage has an anti-tumor activity, thus accurately recognizing cancer cells and destroying them by phagocytosis and cytotoxicity (Lamagna et al., 2006; Solinas et al., 2009). In contrast, M2 macrophage is known to accelerate tissue repair and tissue growth (Mantovani et al., 2004). Hence, the increase of M1 macrophage in cancer is associated with less tumor aggressiveness, whereas the increase of M2 macrophage stimulates tumor growth and leads to poor prognosis (Komohara et al., 2014). Through various processes within the TME, macrophages go through cell polarization under the influence of numerous hormones, cytokines, and apoptotic cells (Pollard, 2004; Mosser and Edwards, 2008). Research on macrophage polarization is progressing, though some aspects are yet incomplete or controversial. Since TAM is both protumoral and antitumoral, it is possible to say that the polarization determines its function. It has been reported that if TAM acquires a M2-like phenotype by the effect of T-cells, cancer cells, or other interacting cell types within the TME (Mantovani et al., 2002; Fukuda et al., 2012), tumor progression occurs through angiogenesis, tissue remodeling, and adaptive immunity suppression (Mantovani et al., 2002, 2009).

In the case of breast cancer, a high level of IL-10, a non-M1 macrophage hallmark, has been observed in-vivo (Guiducci et al., 2005; Weigert et al., 2009). Furthermore, gene profiling data has revealed that breast cancer TAM has a M2-like nature (Ojalvo et al., 2009; Pucci et al., 2009; Movahedi et al., 2010). One of the reported mechanisms that induce this is the chemicals secreted from the breast cancer cell (Sousa et al., 2015). Especially the basal-like breast cancer cell is reported to be an inducer of M2 phenotype (Stewart et al., 2012). Another suggested mechanism is the regulation by miRNA. The miR-146a has been reported to induce M2 macrophage phenotype (Stewart et al., 2012). Such M2-like polarization has even been seen in some breast cancer brain metastasis (Rippaus et al., 2016). However, gene profiling data from other research has shown M1-

associated genes expressed more frequently in breast cancer (Van Ginderachter et al., 2006). Hence, it is most likely that various subgroups of TAMs are present in breast cancers, thus TAM exhibit intratumoral heterogeneity. With populations of distinct functions existing in various regions of the tumor. TAM of M1-like phenotype is a migratory TAM located at the perivascular area with a pro-metastatic feature and characteristics of CD206 (-)/Dextran (-)/MHC II high. On the other hand, TAM of M2-like phenotype is a sessile TAM located at the invasive border and hypoxia area with a pro-angiogenic feature and characteristics of CD206 (+)/Dextran (+)/MHCII low (Laoui et al., 2011).

## The roles of tumor-associated macrophage on breast cancer

Since TAM affects breast cancer cells through diverse mechanisms, it influences the outcome of therapy and the overall progress of breast cancer, from initiation and progression to metastasis (Table 1).

### *Breast cancer progression*

One of the primary mechanisms of breast cancer progression by TAM is angiogenesis. Recent research showed the increased expression of VGEF and HIF-2α

**Table 1.** The roles of tumor-associated macrophage on breast cancer.

| Mechanism | Important findings | References |
|---|---|---|
| Breast cancer progression | | |
| Angiogenesis | Increased expression of VGEF and HIF-2α in TAM | Leek et al., 2002; Lewis et al., 2000 |
| | Mean vessel density correlated with macrophage index | Leek et al., 1996 |
| | Injection of breast cancer with macrophages into mice induce vessel formation | Bingle et al., 2006 |
| | Transcripts related to tumor angiogenesis: increased in TAM from mammary tumor | Ojalvo et al., 2009 |
| Extracellular matrix remodeling | TAMs adjacent to IDC and DCIS : higher urokinase receptor (uPAR) expression | Hildenbrand et al., 1999 |
| | Macrophages involved in collagen type I synthesis during mammary tumorigenesis | Ingman et al., 2006 |
| Breast cancer metastasis | | |
| Intravasation | Perivascular macrophages involved in mammary cancer cell intravasation | Wyckoff et al., 2007 |
| | Positive-feedback interaction between EGF from TAM and CSF1from breast cancer cell | Goswami et al., 2005; Wyckoff et al., 2007 |
| | CSF1-EGF-crosstalk induce invadopodia in cancer cell and podosome in TAM | Condeelis and Pollard, 2006 |
| | Integrin clustering by the secretion of CCL18 | Chen et al., 2011 |
| The seeding of cancer cells to a metastatic site | Close connection between macrophages and the seeding of tumor cells | Qian et al., 2009 |
| | Create an adherent scaffold that arrests and seed tumor cells | Cox et al., 2015; Erler et al., 2009 |
| Immune alteration | | |
| Inhibition of antitumor T-cell responses | Control of CD8+T-cell activation and proliferation by IL-10 originated from TAM | DeNardo et al., 2011; Ruffell et al., 2014 |
| | TAM secretes Arg1 which reduces the level of L-arginine | Bronte and Zanovello, 2005 |
| | TAM located in murine mammary tumor restricts T-cell response by Arg1 and iNOS | Doedens et al., 2010 |
| Recruitment of immuno-suppressive leukocytes | TAMs recruit immunosuppressive cells, such as inflammatory monocytes | Huang et al., 2007; Zhu et al., 2014 |
| Inhibition of tumoricidal function | Macrophages from breast cancer has less expression of IL-12 and iNOS | Dinapoli et al., 1996; Handel-Fernandez et al., 1997 |
| | Macrophages from breast cancer has less expression of MHC class II | Zhang et al., 2015 |
| Drug resistance | | |
| IL-10/STAT3/Bcl-2 signaling pathway | Secretion of IL-10 by TAMs results in the upregulation of bcl-2 and STAT3 | Yang et al., 2015a |
| Abnormal vascularization of the tumor | Downregulation of pro-angiogenic factors from TAMs enhance chemotherapy delivery to tumors | Bolat et al., 2006; Tsutsui et al., 2005 |
| Cancer stem cell | | |
| IL-6 mediated signaling | TAM closely related with maintenance of CSCs like properties | Sainz et al., 2016 |
| | M2 macrophages promoted tumor sphere formation *in vitro* | Ward et al., 2015 |
| Maintenance of CSC niche | Direct binding of breast CSC with TAM promote CSC maintenance | Lu et al., 2014 |

IDC, invasive ductal carcinoma, DCIS, ductal carcinoma *in situ*, CSC, cancer stem cell.

in TAM when breast cancer is in hypoxic condition (Lewis et al., 2000; Leek et al., 2002). The mean vessel density and macrophage index has been found to have a correlation for breast cancer, while VEGF and macrophage infiltration are also associated with each other (Leek et al., 1996). In one study, the injection of breast cancer sphenoids with constituting macrophages into mice caused vessel formation by VGEF overexpression (Bingle et al., 2006). Also, in a gene expression study, transcripts related to tumor angiogenesis mediator secretion increased more in TAM from late stage mammary tumor than in macrophages from the spleen in mice (Ojalvo et al., 2009).

Another mechanism of TAM that causes breast cancer progression is tumor extracellular matrix remodeling. In certain types of breast cancer, such as invasive ductal carcinoma (IDC) and DCIS, it has been reported that TAMs adjacent to the inflammatory area of cancer cells have a higher urokinase receptor (uPAR) expression compared to normal breast tissue macrophages (Hildenbrand et al., 1999). uPAR couples with urokinase-type plasminogen activator (uPA) and bring on matrix remodeling and cell movement by plasminogen-dependent proteolysis (Rabbani and Xing, 1998). In addition to the experimental finding that cancer cells move 10 times faster when moving along collagen fiber compared to when it is not (Wyckoff et al., 2007), it has also been found that when macrophages are removed during formation of mammary tumor in mouse models, collagen type I synthesis decreases, and when macrophages are restored, the decrease is also recovered (Ingman et al., 2006).

### *Breast cancer metastasis*

Since TAM is involved in breast cancer progression, it inevitably that they also affect tumor metastasis. While a correlations between TAM and lymph node metastasis has been shown in breast cancer (Bolat et al., 2006). More recently, TAM, especially CD68+ TAMs were found more frequent in lymph nodes with metastasis compared to those without (Yang et al., 2015b) and the number of TAMs and VEGF-C+ TAMs were higher in the case of breast cancer with lymph node metastases (Ding et al., 2012). TAM is not only related to lymph node metastasis but also distant metastasis. It has been reported that the higher the rate of infiltrating TAM in TNBC, the risk of distant metastasis also increases (Yuan et al., 2014). When TAM is exposed to apoptotic MCF-7 cells, metastasis rises accordingly (Zhou et al., 2015).

Likewise, TAM contributes to one of the most important processes of metastasis, intravasation. Tested with animal models, perivascular macrophages are found to be involved in mammary cancer cell intravasation (Wyckoff et al., 2007). During this process, there is a positive-feedback interaction between EGF and CSF1, which are secreted from TAM and cancer cells respectively (Goswami et al., 2005; Wyckoff et al., 2007). CSF1 secreted from the breast cancer cell recruit macrophages from circulation to become TAM. This results in increased EGF expression within TAM, which binds with breast cancer cells that express EGFR to accelerate the survival and growth of breast cancer (Goswami et al., 2005). Such CSF1-EGF-crosstalk brings about invadopodia formation in mammary cancer cell and podosome formation in TMA, leading to the acceleration of intravasation by ECM break down (Condeelis and Pollard, 2006). One last mechanism that induces intravasation in breast cancer is integrin clustering by the secretion of CCL18 (Chen et al., 2011).

TAM provokes not only intravasation but also the seeding of cancer cells to a metastatic site. An animal model using breast cancer revealed a close connection between macrophages and the seeding and growth of tumor cells (Qian et al., 2009). Such macrophages involved in this process generally have the characteristics of VEGFR1 (+)/CCR2 (+)/CX3CR1 (+)/Tie2 (-)/CXCR4 (-) (Pucci et al., 2009; Qian et al., 2009). Lysyl oxidase (LOX) secreted from hypoxic breast cancer cells links macrophages and collagen type IV in bone marrow and lung to create an adherent scaffold that arrests circulatory tumor cells (Erler et al., 2009; Cox et al., 2015). Furthermore, lung metastasis from breast cancer has been reported to result from CD11b positive macrophage recruitment by CCL2 (Gil-Bernabe et al., 2012).

### *Immune alteration*

TAM is further involved in tumor immune evasion in breast cancer. The initial mechanism is inhibition of antitumor T-cell responses by secreting anti-inflammatory cytokines. A breast cancer mouse model presented the control of CD8+T-cell activation and proliferation by IL-10 originated from TAM (DeNardo et al., 2011; Ruffell et al., 2014), which also inhibits IL-12 secretion from dendritic cells and eventually constrains CD8+T-cell response (Ruffell et al., 2014). Further verifying this mechanism, the anti-tumor activity was brought back from the CD8+T-cell when TAM was removed from the mouse model (DeNardo et al., 2011; Ruffell et al., 2014). On top of the effect of cytokines, another T-cell inhibition mechanism by TAM is L-arginine metabolism. TAM secretes Arg1, which hydrolyzes L-arginine to urea and L-ornithine. This reduces the level of L-arginine and directly inhibits the function of effector T-cells (Bronte and Zanovello, 2005). The increase of Arg1 has been observed in both TAMs of an early stage mammary tumor mouse model (Wynn et al., 2013), and circulatory myeloid cells of breast cancer patients (de Boniface et al., 2012). TAM raises the expression of iNOS, which metabolizes L-arginine as well. It has been reported that TAM located in the hypoxic area of murine mammary tumor restricts T-cell response by Arg1 and iNOS (Doedens et al., 2010).

Another potential mechanism of TAM involvement

in tumor immune evasion is the recruitment of immunosuppressive leukocytes. TAMs recruit immunosuppressive cells, such as inflammatory monocytes, to the tumor region by using the CCL22/CCR2 and CSF1/CSF1R signaling axis (Huang et al., 2007; Zhu et al., 2014). Validation is provided by research on exceptionally high serum CCL22 level in breast cancer patients, and how a close linear correlation is formed between serum CCL22 level and the stage of breast cancer (Jafarzadeh et al., 2015). The last mechanism for tumor immune evasion by TAM is the inhibition of tumoricidal function. Loss of the original functions of macrophages, such as cytotoxicity and proinflammatory signaling, becomes a major obstacle in immunologically constraining the tumor. It has been reported that macrophages extracted from mice with breast cancer have less expression of IL-12 and iNOS, which both are crucial molecules in destroying cancer cells (Dinapoli et al., 1996; Handel-Fernandez et al., 1997). In the case of macrophages in mammary tumor, the expression of MHC class II decreases significantly and reduces antigen presentation and immune reaction (Zhang et al., 2015).

### *Drug resistance*

The response of breast cancer cells to therapy does not depend solely on the molecular characteristics of the tumor cells or the genetic aberrations driving the tumor development. The tumor microenvironment is known to modulate the efficiency of therapy based on the cellular components and the various factors secreted into the microenvironment (Whiteside, 2008). The potential role of TAMs as regulator of therapeutic response in breast cancer has emerged. TAMs modulation of tumor response to chemotherapy is based on the predominant TAMs population in the tumor microenvironment (Whiteside, 2008; Chanmee et al., 2014). A high M2 macrophage population is associated with therapeutic resistance. The antitumor activity of docetaxel is associated with depletion of M2 TAMs and activation/expansion of M1 macrophages, implicating TAMs in therapeutic response in breast cancer (Kodumudi et al., 2010). Another group reported TAM induced chemotherapeutic resistance is proposed to occur through the IL-10/STAT3/Bcl-2 signaling pathway in breast cancer (Yang et al., 2015a). Yang et al demonstrated that secretion of IL-10 by TAMs results in the upregulation of bcl-2 and STAT3 gene expression (Yang et al., 2015a). In a mouse model of breast cancer, exposure to chemotherapy resulted in the secretion of CSF-1 that recruits CSF-1 receptor expressing monocytes and macrophages into the tumor site. Treatment with chemotherapy and recombinant anti-CSF-1 antibody decreased recruitment of TAMs, with a decrease in tumor progression, lung metastasis and vessel density (Paulus et al., 2006). Node-positive breast cancer patients with high macrophage, high CD4+, but low CD8 T-cell signature who underwent intense chemotherapy subsequently had reduced recurrence-free survival compared to patients with low macrophage, low CD4 and high CD8 T cell signature (DeNardo et al., 2009). TAMs have also been associated with resistance to tamoxifen in postmenopausal breast cancer patients (Xuan et al., 2014).

There is presently enormous interest to characterize the subset of TAMs that contribute to chemotherapy resistance and to elucidate the potential mechanisms by which TAMs associated drug resistance in breast cancer occurs. It has been proposed that TAMs present in the microenvironment may release "chemoprotective" factors such as cathepsins B and S which protect cancer cells from the direct cytotoxic effects of several chemotherapeutic agents (Shree et al., 2011). TAMs induced drug resistance may also occur through the limitation of CD8+ cytotoxic T-cells recruitment (DeNardo et al., 2009). TAMs in breast cancer have also been linked to resistance to anti-angiogenic therapy, through the secretion of various factors (including basic fibroblast growth factor, chemokine CCL18, thymidine phosphorylase and VEGFA) that create a pro-inflammatory tumor microenvironment, enhance angiogenesis and suppress adaptive immunity (Komohara et al., 2014; Lin et al., 2015). The abnormal vascularization of the tumor by these factors suppresses chemotherapy and leads to breast cancer resistance. Downregulation of pro-angiogenic factors secreted by TAMs decreases tumor vessel density and enhances chemotherapy delivery to tumors (Tsutsui et al., 2005; Bolat et al., 2006).

### *Metabolic alterations*

The acidified and inflammatory microenvironment can direct macrophage polarization, instruct effector function and alter macrophage metabolism to adapt to the microenvironment. The polarization of TAMs into specific phenotypes depends on different micro-environmental stimuli. Certain stimuli alter metabolic signals that results in polarization into either M1 or M2 phenotypes, while other metabolic changes are required for activation of macrophages for function (Biswas et al., 2012; den Breems and Eftimie, 2015). Polarized M1 macrophages switch their metabolism towards increased glycolytic flux and reduced mitochondrial oxidative phosphorylation with lactate release, pentose phosphate pathway activation, protein and fatty acid synthesis and a decrease in oxygen consumption rate (Galván-Peña and O'Neill, 2014). The metabolic alterations enable M1 macrophages to produce acetyl CoA (AcCoA), lactate, succinate, and nitric oxide (NO) which are essential for their function (Galvan-Pena and O'Neill, 2014; Jha et al., 2015). This metabolic pattern is similar to what occurs in tumor cells and requires upregulation of genes involved in glucose uptake and glucose fermentation usually controlled by hypoxia inducible factor 1a (HIF-1a) (Burke et al., 2002). Alternatively, M2-macrophages have enhanced oxidative phosphorylation and fatty acid

oxidation, with no changes in the glycolytic flux (Galvan-Pena and O'Neill, 2014; Jha et al., 2015). Changes in expression of kinases and enzymes involved in glucose metabolism may be key regulators of macrophage polarization, influencing cytokine production and the expression of key surface receptors essential for their function (Freemerman et al., 2014; Torres et al., 2016).

A key distinction between M1 and M2 occurs in how arginine is metabolized. Ines Maria Corraliza et al showed that in M1 macrophages, arginine catabolism occurs by upregulating nitric oxide synthase (iNOS) producing citrulline and nitric oxide (Corraliza et al., 1995). Alternatively, in M2 macrophage arginine catabolism occur by arginase-1 (Arg1) and results in urea, polyamines, and ornithine production which are important for M2 macrophage function in wound healing (Corraliza et al., 1995). The polyamines secreted by M2 macrophage are linked to pro-tumor functions by promoting tumor cell proliferation. Differential metabolism of arginine is presently a reliable factor for characterizing M1 and M2 macrophages (Munder et al., 1998; Geelhaar-Karsch et al., 2013). Furthermore, lactic acid produced as a by-product of tumor cell metabolism induces the expression of VEGF and polarizes macrophages into M2-phenotype mediated by HIF-1a. Lactate induces the expression of arginase 1 in M2 macrophages that is used in arginine metabolism to promote tumor growth (Colegio et al., 2014, 2016).

Hypoxic is a key regulator of the altered metabolism seen in TAMs. TAMs localize significantly in hypoxic tumor regions and subsequently display alterations in several metabolic genes in order to adapt their metabolism to low oxygen tension, particularly up-regulation of HIF-1a (Kelly and O'Neill, 2015; Varesio et al., 2016). Thus, alteration of TAMs metabolism is influenced by a myriad of signals such as cytokines, hypoxia and growth factor. These findings offer an opportunity to use the metabolic status of macrophages for therapeutic targeting in cancers (Kelly and O'Neill, 2015; Varesio et al., 2016). Further studies into the metabolic characteristics of macrophages in cancer are required as it remains unknown if a change in TAM metabolism could influence their phenotype and thus affect cancer growth and metastasis.

### *Cancer stem cell*

As an essential component of cancer stem cell function, the tumor microenvironment and its constituting cells is being highlighted and correlation of high numbers of TAMS and clinical progression has been analyzed in several cancers. The paracrine signaling originating from TAMs mediate activation of cancer stem cell (CSC) and promote stem celllike features of CSCs (Sainz et al., 2016). TAMs seem to be associated with CSCs in every aspect of tumor progression in breast cancer, especially, in tumor associated inflammatory response in which TAMsperform crucial roles by IL-6 mediated signaling. Activation of IL-6 signaling by TAM is closely related with the maintenance of CSCs-like properties in the premalignant, primary tumor and metastatic tumor stage (Sainz et al., 2016). Activation of NF-kB, following Lin28-mediated repression of Let7 and IL-6 signaling was shown to promote the self-renewal of breast cancer CSCs (Iliopoulos et al., 2009) In ER+ positive breast cancer cell line, coculture with M2 macrophages promoted tumor sphere formation *in vitro* (Ward et al., 2015). TAMs are associated with Sox2 upregulation which is known to have a regulatory effect of CSCS in murine mouse breast tumor model (Yang et al., 2013). In addition, the maintenance of CSC niche is associated with TAMs. Using proteome profiling, Lu et al. showed the direct binding of breast CSC toTAM via EphA4, which promote the initiation of tumor formation and CSC maintenance. The binding activates NF-kB in cancer cells', stimulate the release of cytokines, which in turn promotes the maintenance of the stemness of CSCs (Lu et al., 2014).

### *Breast cancer prognosis*

TAMs enhance cancer cell invasion by secreting matrix metalloproteinases that degrade the ECM, and stimulate tumor vascularization and angiogenesis through the secretion of various proangiogenic factors and suppress the anti-tumor functions of cytotoxic T cells. Enhanced invasion, coupled with hypo-perfused, abnormal blood vessels and limited anti-tumor response contribute to therapeutic failure, ultimately affecting patient prognosis (Riabov et al., 2014). Thus, breast cancer epidermiological studies have reported a significant association between high infiltration of tumor by TAMs and poor clinical prognosis (Obeid et al., 2013). A direct correlation between high focal infiltration of TAMs and tumor cell invasion, increased vascularization and axillary lymph node involvement is well documented in breast cancer (Medrek et al., 2012).

A meta-analysis by Bingle et al, showed an association between increased macrophage density and poor prognosis in over 80% of breast cancer cases. Patients with higher TAMs density had a significantly worse relapse-free survival (RFS) and overall survival (OS) (Bingle et al., 2002). Leek et al also reported that increased angiogenesis correlated with increased TAMs infiltration and was inversely correlated with poor clinical prognosis in invasive breast cancer patients (Leek et al., 1996). The association been high TAMs and poor prognosis in breast cancer is linked to their potential to stimulate angiogenesis (Bolat et al., 2006). TAMs by secreting pro-angiogenic factors induce the formation of blood vessels. These poorly formed, leaky blood vessels limit delively of chemotherapeutic agents to the tumours, resulting in poor patient response (Leek et al., 1996; Lin et al., 2015; Mantovani and Locati, 2016). This hypothesis is supported by studies in invasive breast cancer, where increased macrophage

index has a positve correlation with high mean vessel density and an inverse relationship to relapse-free and overall survival (Leek et al., 1996; Ch'ng et al., 2011). Correlation between high macrophage infiltration and signs for poor prognosis such as high tumor grade, low estrogen and progesterone receptor status and high tumor mitotic activity has also been reported (Volodko et al., 1998). In macrophage deficient mice models, growth of transplantable tumors is markedly impaired and blockage of macrophage-CSF (M-CSF) function significantly suppresses tumor growth (Nowicki et al., 1996). VEGF-A expression in the mammary gland of the mice restores tumor progression in M-CSF-deficient mice. These experimental results support the hypothesis that TAMs contribution to poor prognosis may occur by stimulating tumor angiogenesis (Nowicki et al., 1996).

A recently developed stroma-derived prognostic predictor encompassing 163 stroma-expressed genes in human breast cancer associates macrophage-associated genes to the poor outcome sample cluster (Finak et al., 2008). Similarly, genes associated to leukocyte or macrophage infiltration (e.g., CD68) are part of molecular signatures attributed to poor prognosis in lymphomas and breast carcinoma (Paik et al., 2004). Using macrophage infiltration chemotactic factors as markers, an indirect relationship between TAMs and poor prognosis has been reported. Macrophage chemoattractants, such as CCL2, CCL5 and CSF-1 have been shown to be increased in breast cancer and expression of these factors correlates with poor prognosis (Gyorki and Lindeman, 2008).

## Future targets for breast cancer treatment

The evidence that infiltrating TAMs contribute to poor clinical prognosis and resistance to chemotherapy through immunosuppressive and tumor-promoting function, makes TAMs an attractive target for therapeutic intervention (Mantovani et al., 2011). Various approaches have been developed over the years and used in breast cancers and other forms of cancers. The success of these therapeutic approaches depends on understanding the biology of TAM. Recent research has made great strides in this aspect and offers the potential for the development of alternative strategies to target these cells or modulate their function (Mantovani et al., 2011). Several strategies targeting TAM have emerged (Table 2). Several such strategies have been successful in experimental settings and are now considered promising therapeutic approach in the clinic.

### *Inhibition of TAM recruitment*

If high levels of TAMs correlate with poor clinical prognosis, the basic therapeutic approach would be to inhibit macrophage recruitment to the tumor site or to directly deplete TAMs. The recruitment of circulation monocytes and their infiltration into tumor tissue is regulated by the secretion of macrophage chemo-attractant by both tumor cells and host cells in the tumor microenvironment (Chanmee et al., 2014).

The chemokine CCL2 is a macrophage chemotactic factor which recruits macrophages through the CCL2-CCR2 axis to the tumor site. Hence, targeting this axis offers the potential to limit monocyte/macrophage recruitment (Panni et al., 2013). This is demonstrated in studies where CCL-2 is targeted and inhibited with anti-CCL2 antibodies in breast cancer xenograft models. Blocking CCL-2 reduced tumor growth and dissemination in breast cancer models (Lu and Kang, 2009; Kitamura et al., 2015), similar results were observed in prostate and lung cancer (Loberg et al., 2007; Fridlender et al., 2011). Hence, antibodies against CCL2 or its cognate receptor have been developed and trialed in preclinical models of breast cancer (Sandhu et al., 2013; Crusz and Balkwill, 2015). Results from the study support the potential role of anti-CCL2 therapy in breast cancer. The monoclonal antibody therapy against CCL-2, carlumab (CNTO 888) has been recently developed. Brana et al showed that a combination of

**Table 2.** TAM targeted anti-cancer therapy.

| Action mechanism | Target | Drug | References |
|---|---|---|---|
| Inhibition of TAM Recruitment | CCL2-CCR2 axis | Carlumab (CNTO 888) | Brana et al., 2015 |
| | CSF-1 receptor | Agents that blocked CSFR1 | DeNardo et al., 2011 |
| Inhibition of TAM survival | IL4Rα or CD124 | RNA aptamer | Roth et al., 2012 |
| | TRAIL receptors | Trabectedin | Allavena et al., 2013 |
| | Apoptosis pathway | M2pep | Cieslewicz et al., 2013 |
| Inhibiting TAM polarization and differentiation | CSF1/ CSF1R signaling | RG7155 | Ries et al., 2014, 2015 |
| | Toll-like receptor (TLR) | Zoledronic acid | Gnant, 2011 |
| | NF-κB pathway | TLR agonists<br>Anti-CD40 mAbs<br>IL-10 mAbs | Panni et al., 2013 |
| | Immunomodulation | Thymosin-α<br>B-glucan | Garcia-Valtanen et al., 2017 |

carlumab with four other chemotherapy regimens for the treatment of patients with solid tumors was well tolerated (Brana et al., 2015).

DeNardo et al recently showed in genetic mouse model for breast cancer that chemotherapy with paclitaxel, increased expression of macrophage chemotactic factors CSF1, CCL8, and IL-34, and an increase in TAMs expressing CSF-1 receptor (DeNardo et al., 2011). Administration of agents that blocked CSFR1 combined with chemotherapy enhanced therapeutic activity, inhibited metastases and increased T cells in the tumors (DeNardo et al., 2011). Evidence from studies targeting CCL2 has resulted in the development of agents targeting other macrophage chemotactic factors such as CCL5 and CXCL12 in estrogen receptor positive breast cancer (Svensson et al., 2015). In colon cancer the antitumor agent dequalinium-14 has been shown to reduce macrophage motility, and inhibit macrophage infiltration (Timaner et al., 2015). Such therapies can be combined with agents that block macrophage differentiation for effective targeting of TAMs.

### *Inhibition of TAM survival*

In addition to blocking macrophage chemotactic factors, agents with potential to eliminate or kill macrophages are also being considered. These chemical or synthetic drugs are designed to directly induce apoptosis in TAMs leading to their death (De Palma and Lewis, 2013). To demostrate the efficiency of such an approach Roth et al using an RNA aptamer were able to block the murine or human IL-4 receptor-α (IL4Rα or CD124), preferentially target and eliminate TAMs (Roth et al., 2012). In tumor bearing mice this resulted in the elimination of TAMs, increased number of tumor-infiltrating T cells and a reduction in tumor growth (Roth et al., 2012). Trabectedin, a licensed and commercially available anticancer agent has been demonstrated to selectively deplete TAM population by inducing caspase 8-dependent apoptosis via TRAIL receptors (Allavena et al., 2013). However, trabectedin does not selectively affect TAMs but also affect monocyte/macrophage-mediated host defense, hence the development of TAM-specific agents is still required (Allavena et al., 2013). A unique peptide, M2pep carrying a pro-apototic peptide has been shown to selectively target and kill TAMs resulting in improved survival rates in tumor brearing mice (Cieslewicz et al., 2013). These molecules have also demonstrated potential to synergistic kill tumor cells, while also inhibiting differentiation of TAMs into the M2 phenotype (Cieslewicz et al., 2013). Agents with such dual effect are continuously being discovered. Cyclosporin A and trabectedin in addition to directly inhibiting tumor cell growth, also suppress activation of TAMs (Komohara et al., 2014). The advent of immunotherapy offers an alternative approach to eliminate tumor cells via host immune response. This is achieved directly via the main effectors of the immune system, such as macrophages. In a sorafenib-resistant tumor model, photoimmunotherapy targeting TAMs was found to inhibit tumor growth and metastasis (Zhang et al., 2016). Therefore, TAMs have become promising therapeutic targets for cancer treatment.

### *Inhibiting TAM polarization and differentiation*

The two main macrophage populations M1 and M2 perform different functions and are differentiated/ polarized by distinct signaling pathways. M2-polarized macrophages perform pro-angiogenic and immuno-suppressive functions while M1 macrophages perform anti-tumor functions (Panni et al., 2013). The inherent plasticity of macrophages means M2 macrophages can switch their phenotype based on environmental stimuli. Hence this ability can be exploited as a therapeutic strategy, where various agents are used to reprogram M2 macrophage to express a pro-immunity, anti-tumor (M1-like) phenotype (Panni et al., 2013).

A key target in this approach is CSF1/ CSF1R signaling. The CSF1/CSF1R signaling pathway promotes differentiation of myeloid progenitors into the heterogeneous population of mononuclear phagocytes (Mantovani et al., 2014). The pathway is also involved in altering macrophage polarization and promoting macrophage survival. High expression of CSF1 or CSF1R is associated with poor clinical prognosis in post-menopausal breast cancer (Tamimi et al., 2008) and the loss of CSF1 results in a low incidence, delayed tumor progression and significantly reduced metastasis in breast tumor models (Panni et al., 2013). A monoclonal antibody (RG7155) blocks dimerization of the CSF1 tyrosine kinase receptor and its activation. In a phase I clinical trial of patients with diffuse giant cell tumors, patients receiving RG7155 had a significant reduction in TAMs infiltration, a decrease in the CSF-1R+CD163+ macrophage population and an increase of the CD8/CD4 T cell ratio in tumor biopsies (Ries et al., 2014, 2015). Clinical trials for RG7155 are presently ongoing in breast cancer patients.

M1 macrophage phenotype is driven by lipopolysaccharide (LPS) and toll-like receptor agonists, hence activation of toll-like receptor (TLR) in macrophages reprograms macrophages to function as tumourcidal effectors (Biswas et al., 2012; den Breems and Eftimie, 2015). In breast cancers, the cancer drug zoledronic acid, has been used to reprogram M2 macrophages into M1 phenotype that inhibit carcinogenesis (Gnant, 2011). This approach of TAMs targeted therapythat reprogramTAMs to an M1 phenotype, blocks pro-tumorigenic effects of M2 macrophages resulting in vascular normalization and enhancedresponse to therapy. Various studies in breast cancer models have demostransted this, Sousa et al. showed that polarization of M2 to M1 phenotype suppressed mammary tumor growth and angiogenesis *in vivo* (Sousa et al., 2015).

The signal transduction pathways involved in M2 polarization can also be directly targeted, these signals include nuclear factor (NF)-κB, Stat3, Stat6, c-Myc, and interferon regulatory factor 4 (Komohara et al., 2014). Several agents have been used to activate the NF-κB pathway, including TLR agonists, anti-CD40 mAbs, and IL-10 mAbs resulting in polarization of TAMs to an antitumor phenotype (Panni et al., 2013). Regulation of STAT1 activity in murine carcinoma models can induce an antitumor phenotype in macrophages, through an increased production of IL-12. Various immunomodulation agents have recently emerged such as Thymosin-α and B-glucan that can polarize macrophages to the M1 phenotype (Garcia-Valtanen et al., 2017). The use of β-glucan is currently under investigation in a phase II multi cancer study (Weitberg, 2008).

## Conclusion

Breast cancer microenvironment consists of various stromal cells, of which TAMs are major components. The various populations of TAMs in the tumor microenvironment have distinct phenotypic and functional characteristics. Polarizations into specific subtype are influenced by the tumor microenvironment and by the interaction of TAM with tumors. TAMs affect the biology of breast cancer in various ways including cancer progression, metastasis, immune response, metabolism, therapeutic resistance, metabolism, cancer stem cell, and prognosis. Consideration of TAM as a target of breast cancer treatment may be a reasonable approach. Although several candidate agents are being tested in the preclinical and clinical trials, the potential limitation of this approach is the uncertainty of the predominant pathway of TAMs, given that the actionable pathways of TAMs seem very heterogeneous.

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