Int. J. Dev. Biol. 55: 861-867
doi: 10.1387/ijdb.113371dl

THE INTERNATIONAL JOURNAL OF DEVELOPMENTAL BIOLOGY
www.intjdevbiol.com

Tumor-associated macrophages in breast cancer: distinct subsets, distinct functions

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ABSTRACT Macrophages display remarkable plasticity, allowing these cells to adapt to changing microenvironments and perform functions as diverse as tissue development and homeostasis, inflammation, pathogen clearance and wound healing. Macrophage activation can be triggered by Th1 cytokines and pathogen-associated or endogenous danger signals, leading to the formation of classically activated or M1 macrophages. On the other hand, anti-inflammatory mediators, including IL-4, IL-10, TGF-β and M-CSF, induce diverse anti-inflammatory types of macrophages, known under the generic term M2. In human breast carcinomas, tumor-associated macrophage (TAM) density correlates with poor prognosis. In mouse models of breast cancer, eliminating macrophages from the tumor site, either via genetic or therapeutic means, results in retarded tumor progression. Over the years, multiple signals from the mammary tumor microenvironment have been reported to influence the TAM phenotype and TAM have been propagated as anti-inflammatory M2-like cells. Recent developments point to the existence of at least two distinct TAM subpopulations in mammary tumors, based on a differential expression of markers such as CD206 or MHC II and different *in vivo* behaviour: perivascular, migratory TAM which are less M2-like, and sessile TAM found at tumor-stroma borders and/or hypoxic regions that resemble more M2-like or "trophic" macrophages. Hence, a further refinement of the molecular and functional heterogeneity of TAM is an avenue for further research, with a potential impact on the usefulness of these cells as therapeutic targets.

KEY WORDS: *tumor-associated macrophage, mammary carcinoma, breast cancer, tumor microenvironment, M2*

Introduction

For immunologists, macrophages are best known as central players in the innate immune system with an exceptional capacity to recognize, engulf and destroy pathogens. For developmental biologists however, macrophages are mainly seen as trophic cells that are instrumental for tissue remodelling during morphogenetic processes. Both functions represent different sides of the same coin, and illustrate the plasticity and polyvalency of this cell type. During postnatal mammary gland development, macrophages are recruited to the terminal end buds (TEBs) where they fulfil a non-redundant role in mammary ductal outgrowth. Indeed, TEB formation, their outgrowth in the mammary fat pad and duct branching are all impaired in mice lacking functional macrophages (Gouon-Evans *et al.,* 2000). In recent years, it has become increasingly clear that the basic mechanisms behind breast tumor progression show similarities to the process of tissue reorganization in the developing mammary gland (Pollard 2009). As a matter of fact, all solid tumors can be considered as organ-like structures in which a

Abbreviations used in this paper: AP-1, activator protein-1; C/EBP, CCAAT/enhancer binding protein; DC-SIGN, dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin; EGF, epidermal growth factor; Gas6, growth arrest-specific 6; GM-CSF, granulocyte macrophage-colony stimulating factor; HIF-1, hypoxia inducible factor-1; HRG, histidine-rich glycoprotein; Hsp27, heat shock protein 27; IFN, interferon; IL, interleukin; IL-4Rα, IL-4 receptor α; iNOS, inducible nitric oxide synthase; JNK, c-jun N-terminal kinase; M1, classically activated macrophage; M2, alternatively activated macrophage; M-CSF, macrophage-colony stimulating factor (= CSF-1, colony-stimulating factor 1); MDSC, myeloid-derived suppressor cell; MMR, macrophage mannose receptor; NF-κB, nuclear factor κB; PlGF, placental growth factor; S1P, sphingosine-1-phosphate; STAT3, signal transducer and activator of transcription 3; TADC, tumor-associated dendritic cell; TAM, tumor-associated macrophage; TEM, tie2-expressing monocyte; TGF, transforming growth factor; TMEM, tumor microenvironment of metastasis; TNF, tumor necrosis factor; TP, thymidine phosphorylase; VEGF, vascular endothelial growth factor.

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Final, author-corrected PDF published online: 29 November 2011

ISSN: Online 1696-3547, Print 0214-6282

complex bidirectional interplay exists between transformed and non-transformed cells, the latter of which contain many macrophages. Originally strictly thought of as mediators of anti-tumor immunity, macrophages are now also known as potential contributors to tumor progression. As such, they can increase the survival and proliferative capacity of cancer cells, promote cancer cell motility, invasiveness and intravasation, drive angiogenesis, and mediate immunosuppression and extracellular matrix reorganization (Mantovani *et al.,* 2002; Qian and Pollard 2010). This review mainly focuses on recent developments illustrating the heterogeneity of tumor-associated macrophages (TAM) at the primary tumor site, most studies of which were performed in mouse models of breast carcinoma formation and progression.

Clinical relevance of tumor-associated macrophages in breast cancer

Studying mechanisms of TAM-mediated tumor promotion in mouse models of breast carcinoma should be backed-up by clinical data illustrating the importance of this cell type in breast cancer patients. Several studies, mainly focussing on large groups of invasive ductal breast carcinomas, revealed a significant positive correlation between an increased level of macrophage infiltration and expression of typical monocyte/macrophage chemoattractants, such as CCL2 (also known as MCP-1), CCL5 (also known as RANTES) and CSF-1 (also known as M-CSF), in the tumor (Goede *et al.,* 1999; Ueno *et al.,* 2000; Lin *et al.,* 2002). Both cancer cells and tumor-infiltrating cells are potential producers of these chemokines (Goede *et al.,* 1999). A higher TAM density is typically associated with a high vascular density, suggesting an angiogenic activity of TAM in human tumors (Leek *et al.,* 1996; Tsutsui *et al.,* 2005). VEGF could be a potential mediator of TAM-driven angiogenesis as its expression is positively correlated with TAM levels (Tsutsui *et al.,* 2005). Of importance, intensive macrophage infiltration is associated with known poor prognostic signs such as high tumor grade, low estrogen and progesterone receptor status and high tumor mitotic activity (Volodko *et al.,* 1998). A similar link between the presence of a CSF-1 gene signature in the tumor, CCL2, CCL5 or VEGF levels, and other bad prognosticators reinforces the conception of TAM as contributors to breast tumor malignancy (Beck *et al.,* 2009; Ueno *et al.,* 2000). This is ultimately illustrated by a strong relationship between increased macrophage counts and reduced relapse-free and overall survival as an independent prognostic variable in invasive breast carcinoma patients (Leek *et al.,* 1996). Corroborating these data, a recently developed stroma-derived prognostic predictor - encompassing 163 stroma-expressed genes in human breast cancer - positions macrophage-associated genes in the poor outcome sample cluster (Finak *et al.,* 2008). Overall, these studies provide statistically sound data linking the presence of TAM with bad prognosis in breast cancer patients, but provide little insight in the molecular mechanisms accounting for this phenomenon.

These significant, but still only correlative data in patients have now been supported by experimental evidence using mouse models. In M-CSF-deficient mice (*Csf1op/Cs^{f1}op*), which lack mature macrophages, growth of transplantable tumors is markedly impaired (Nowicki *et al.,* 1996). In addition, blocking M-CSF function via antisense oligonucleotides and siRNA, or inhibiting M-CSF receptor signaling, significantly suppresses tumor growth (Aharinejad *et al.,* 2004, Priceman *et al.,* 2010). These studies were performed with transplantable tumors, and are hence relatively far from the human situation. However, transgenic mouse models such as MMTV-PyMT, expressing the polyoma middle T oncogene under control of the mouse mammary tumor virus promoter, spontaneously develop mammary tumors in stages comparable to the human situation. Mammary tumor progression in this MMTV-PyMT model is altered in a M-CSF null background, with no effect on tumor growth but a delay in the development to invasive, metastatic carcinomas (Lin *et al.,* 2001). Of note, transgenic VEGF-A expression in the mammary gland restores tumor progression in M-CSF-deficient MMTV-PyMT mice, through stimulating tumor angiogenesis, leukocyte infiltration and cancer cell invasion (Lin *et al.,* 2007). It should be noted however, that the levels of VEGF produced by TAM determine whether this protein has pro- or antitumoral activity. Indeed, TAM might produce very high VEGF levels leading to a very dense, but also dysfunctional vessel network. Deletion of VEGF specifically in TAM might cause vascular normalization and actually accelerate tumor progression (Stockmann *et al.,* 2008). Irrespective of the mechanism, these data, obtained in genetically modified mice, clearly illustrate a non-redundant role for monocytes/macrophages in tumor progression. Finally, this conclusion is reinforced by experiments aimed at depleting TAM from mouse mammary tumors. DNA vaccination against legumain, a member of the asparaginyl endopeptidase family overexpressed by TAM, results in immune elimination of TAM and strongly reduced breast tumor growth and metastasis (Luo *et al.,* 2006). Similarly, depletion of TAM in transgenic MMTV-HER-2 mice by an attenuated strain of *Shigella flexneri*, which induces apoptosis specifically in macrophages, resulted in a block of tumor growth and even tumor regression (Galmbacher *et al.,* 2010).

Macrophage activation states

An important concept in the study of macrophages, including TAM, is the remarkable plasticity of these cells. Macrophages are implicated in functions as diverse as tissue development and homeostasis, wound-healing, inflammation and immunity. To accommodate for this, these cells are able to adopt diverse activation states depending on the stimuli they receive. A popular classification system for macrophage activation, that has gained considerable success over the past years, is the M1/M2 dichotomy. Classically activated (or M1) macrophages are induced by Th1 cytokines, such as IFN-γ and TNF-α, and/or by recognition of pathogen–associated molecular patterns or endogenous danger signals. This type of macrophage is extensively studied and plays a pivotal role in propagating inflammation and pathogen clearance. More recently, it became clear that macrophage physiology is also significantly altered by the prototypical Th2 cytokines IL-4 and IL-13, inducing so-called *bona fide* alternatively activated macrophages or M2 (Martinez *et al.,* 2009). Hence, the M1/M2 nomenclature reflects the status of macrophages functioning during ongoing polarized T helper (Th1 versus Th2) responses. In addition, a wide array of anti-inflammatory cues, such as IL-10, TGF-β, glucocorticoids, immune complexes and apoptotic cells, are known to influence the macrophage phenotype leading to different macrophage classification systems by different authors (Mantovani *et al.,* 2004; Mosser *et al.,* 2008; Martinez *et al.,* 2009). A common denominator of all these non-M1 macrophages is their ability to dampen Th1

cytokine-driven inflammation, to coordinate adaptive immune responses and to contribute to wound healing. On the other hand, IL-4/IL-13-induced M2 are implicated in Th2-driven pathologies, such as helminth infections and asthma (Martinez *et al.,* 2009). Though the M1/M2 concept provides a useful working scheme, it should be realized that any form of classification underscores the complexity of the *in vivo* situation, where macrophages are exposed to a mixture of stimuli and will adopt mixed functional profiles. This is illustrated by the consensus gene signature for *in vivo* induced M2 in different pathologies, which not only contains genes that are strictly IL-4/IL-13-inducible such as E-cadherin (Van den Bossche *et al.,* 2009), but equally so genes that are not inducible *in vitro* by any of the known M2 inducing stimuli (Hassanzadeh Ghassabeh *et al.,* 2006).

Remarkably, in the human system, M-CSF and GM-CSF are used to differentiate more M2-like or M1-like macrophages from peripheral blood monocytes, respectively (Puig-Kröger *et al.,* 2009). M-CSF-generated macrophages do not secrete the pro-inflammatory cytokines IL-12 and IL-23, but instead produce high levels of IL-10. Of note, this type of macrophage might resemble most the 'trophic', developmental type of macrophage (Pollard 2009). Considering the association of a M-CSF response signature with worse prognosis in breast carcinoma patients (Beck *et al.,* 2009), trophic macrophages might also be present in the breast tumor microenvironment and function as important promotors of tumor progression. Exemplifying this, the surface markers folate receptor β and DC-SIGN are found to be expressed on $CD14^+CD68^+$ TAM from human breast adenocarcinomas, and are mainly regulated by cancer cell-derived M-CSF (Puig-Kröger *et al.,* 2009; Dominguez-Soto *et al.,* 2011). Cross-linking DC-SIGN on these TAM by cancer cells results in increased expression of IL-10, further amplifying the anti-inflammatory phenotype of the TAM (Dominguez-Soto *et al.,* 2011). Interestingly, while M-CSF-stimulated macrophages might help cancer growth, GM-CSF treatment of mouse mammary tumors inhibits tumor growth and metastasis by invoking an antitumoral program in TAM (Eubank *et al.,* 2009). Hence, a picture emerges whereby M2-like macrophages are protumoral, and M1-like cells exert antitumoral activity.

Tumor-associated macrophage activation states in breast cancer

The concept that TAM are mainly M2 activated, or even M2 'polarized', has been around for almost a decade (Mantovani *et al.,* 2002), and is corroborated by the expression pattern of at least some of the TAM markers. For example, high production of IL-10 and low production of IL-12 is seen as a hallmark of all non-M1 macrophages, and is also applicable to most TAM populations in different cancer types. Also in breast cancer, high *in vivo* IL-10 production has been reported and is linked to reduced immune responsiveness (Guiducci *et al.,* 2005; Weigert *et al.,* 2009; Puig-Kröger *et al.,* 2009). A skewing of the L-arginine metabolism towards higher arginase-1-mediated and lower iNOS-mediated L-arginine consumption is another typical feature of anti-inflammatory macrophages, at least in mice. High arginase enzyme activity has been reported in TAM from mouse breast cancer models (Movahedi *et al.,* 2010). In addition, TAM from breast tumor-bearing mice showed a reduced tumoricidal capacity due to deficient expression and function of the transcription factors NF-κB and C/EBP, resulting in impaired iNOS gene expression and NO production (Torroella-Kouri *et al.,* 2005). The predominant M2-like nature of breast tumor TAM has recently been supported by gene profiling data illustrating an immunoregulatory phenotype for these cells in different models (Ojalvo *et al.,* 2009; Pucci *et al.,* 2009; Movahedi *et al.,* 2010). However, the picture of TAM being more M2-like is definitely not black and white, and TAM have been reported to express more M1-associated marker genes as well (Van Ginderachter *et al.,* 2006a, Movahedi *et al.,* 2010). This is probably linked to the existence of distinct TAM subpopulations with specialized functions, as will be discussed in the next chapter of this review, cautioning against overinterpretation of data based on total TAM populations.

Several microenvironmental stimuli were shown to influence the TAM phenotype in mammary tumors. In MMTV-PyMT tumors, IL-4 produced by tumor-infiltrating $CD4^+$ Th2 cells skews the TAM into a metastasis-promoting population producing high levels of Epidermal Growth Factor (EGF). Consequently, the absence of $CD4^+$ T cells or IL-4Rα signalling in PyMT mice results in reduced pulmonary metastasis, without any effects on tumor latency, primary tumor growth and tumor angiogenesis (DeNardo *et al.,* 2009). Also cathepsins, induced by IL-4 in the local tumor microenvironment, appear to contribute to the metastasis-promoting phenotype of PyMT TAM (Gocheva *et al.,* 2010). The importance of a more M2-like TAM polarization for tumor growth and metastasis is recently further highlighted by the effects of the serum protein histidine-rich glycoprotein (HRG). Under normal circumstances, HRG is rapidly degraded in the tumor microenvironment, but its forced expression switches M2 to M1 through downregulation of placental growth factor (PlGF). This event promotes antitumor immune responses and vessel normalization, thereby preventing metastasis and enhancing the effect of chemotherapy (Rolny *et al.,* 2011).

Besides an interaction with tumor-infiltrating lymphocytes, TAM are expected to crosstalk with cancer cells. M-CSF is often produced by breast cancer cells and, as mentioned earlier, is able to regulate the expression of functionally important TAM markers such as folate receptor β, DC-SIGN and EGF (Goswami *et al.,* 2005; Puig-Kröger *et al.,* 2009; Dominguez-Soto *et al.,* 2011). In accordance with a role for M-CSF, the Ets2 transcription factor, which is a direct effector of M-CSF signalling pathways, drives a transcriptional program in PyMT breast tumor TAM that promotes lung metastases formation (Zabuawala *et al.,* 2010). Mechanistically, Ets2 represses a gene program that includes several inhibitors of angiogenesis, thus its ablation leads to decreased angiogenesis and decreased tumor growth. Besides M-CSF, human breast cancer cells secrete high amounts of Heat shock protein 27 (Hsp27), which accumulates to extremely elevated levels in the tumor interstitial fluid (Banerjee *et al.,* 2011). Hsp27 causes the differentiation of monocytes to macrophages with an immune-tolerizing phenotype in vitro, and this type of macrophage is also found in human breast tumors. Hsp27-differentiated macrophages induce unresponsiveness in T cells and are strongly proangiogenic (Banerjee *et al.,* 2011). In addition, dying cancer cells secrete sphingosine-1-phosphate (S1P) and TGF-β that polarize macrophages towards an anti-inflammatory phenotype (Herr *et al.,* 2009; Weigert *et al.,* 2009). A knockdown of sphingosine kinase 2, the enzyme responsible for S1P production, in human MCF-7 breast cancer cells strongly impairs tumor xenograft growth associated with a deficiency in anti-inflammatory TAM generation (Weigert *et al.,* 2009). Interestingly, S1P and TGF-β induce HIF-1 activity, which has been shown to contribute

to protumoral TAM functions (Herr *et al.,* 2009). Indeed, myeloid cell-specific HIF-1α deletion in the MMTV-PyMT background does not affect VEGF levels or vascularization, but reduces iNOS and arginase-1 expression in TAM, resulting in the loss of T-cell suppressive capacity (Doedens *et al.,* 2010). Fra-1, a member of the AP-1 family of transcription factors, is also overexpressed in TAM from mouse breast tumors and appears to be important for the release of proangiogenic factors and the induction of 4T1 migration and invasion (Luo *et al.,* 2009). As a matter of fact, Fra-1 functions upstream of the well-known protumoral transcription factor STAT3 by stimulating IL-6 production, which in turn activates STAT3 (Luo *et al.,* 2009). Hence, an entire transcriptional network governed by several transcription factors seems to be at work to skew macrophages at the tumor site into a protumoral phenotype. It should be realized however that the early skewing towards typical TAM functions, such as proangiogenic activity, already takes place at the earlier stages of myelomonocytic differentiation in the blood and bone marrow of tumor bearers, under the influence of cancer cell secreted factors such as VEGF, CXCL12 and PlGF (Hiratsuka *et al.,* 2011; Laurent *et al.,* 2011).

Finally, TAM from mouse breast tumors employ different mechanisms to alter the behaviour of cancer cells. Through the secretion of TNF-α, TAM induce NF-κB and JNK activity in breast cancer cells resulting in enhanced invasiveness (Hagemann *et al.,* 2005). The mitogen Gas6 on the other hand increases cancer cell proliferation, and is strongly induced in tumor-conditioned macrophages (Loges *et al.,* 2010). In addition, the interaction between invasive cancer cells and the extracellular matrix is facilitated by macrophage-derived SPARC, also known as osteonectin (Sangaletti *et al.,* 2008). An overview of the mechanisms involved in the bidirectional interaction between breast tumor TAM and their environment, including cancer cells and T cells, is represented in Fig. 1.

Heterogeneity of tumor-associated macrophages in established breast tumors

Breast tumors are not only populated by tumor-associated macrophages, but also by other members of the mononuclear phagocyte system, including dendritric cells (TADC), Tie-2-expressing monocytes (TEM) and Myeloid-derived suppressor cells (MDSC). TEM elimination *in vivo* results in severely reduced tumor neovascularization, suggesting that these cells play a non-redundant proangiogenic role in tumors (De Palma *et al.,* 2005). MDSC is a definition describing a function rather than a lineage of myeloid cells, and encompasses immature $CD11b^{+}Ly6C^{hi}Ly6G^{neg}$ monocytic (MO-MDSC) and $CD11b^{+}Ly6C^{int}Ly6G^{hi}$ granulocytic (PMN-MDSC) cells with a common immunosuppressive capacity, albeit through different mechanisms (Van Ginderachter *et al.,* 2006b, Movahedi *et al.,* 2008). Distinguishing these different myeloid cell types in tumors is not always trivial, as they are highly related, often express similar markers and are in some cases able to perform similar functions. Even within the TAM compartment, the existence of different TAM populations with different functions according to their presence in different regions of the tumor – areas of invasion, the stroma, perivascular areas, avascular and perinecrotic areas – has been predicted. In recent years, real-life images of different tumor microenvironments were produced and cancer cell/stromal cell dynamics were visualized in real-time (Wyckoff *et al.,* 2007, Egeblad *et al.,* 2008, Kedrin *et al.,* 2008). In mouse mammary tumors, macrophages are present in large numbers at the margins of the tumor and then decreasingly deeper in the tumors,

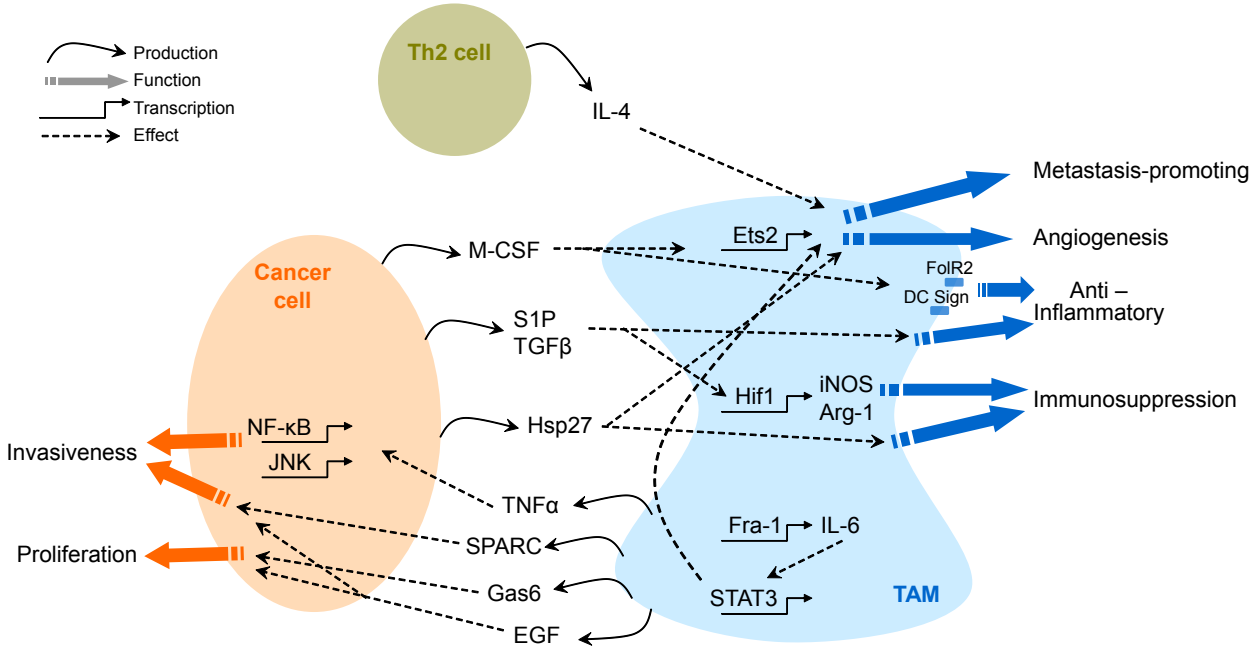

Fig. 1. Bidirectional interaction between tumor-associated macrophages from breast carcinomas and cells in their environment, including cancer cells and T cells. *Distinct mediators secreted by breast cancer cells and tumor-infiltrating lymphocytes instruct a tumor-promoting phenotype on the resident macrophages, including metastasis-promoting, angiogenic, anti-inflammatory and immune suppressive functions. Several signalling pathways and transcription factors are implicated in this phenomenon. Vice versa, tumor associated macrophages (TAM) secrete a number of factors with a direct impact on cancer cell proliferation and invasiveness.*

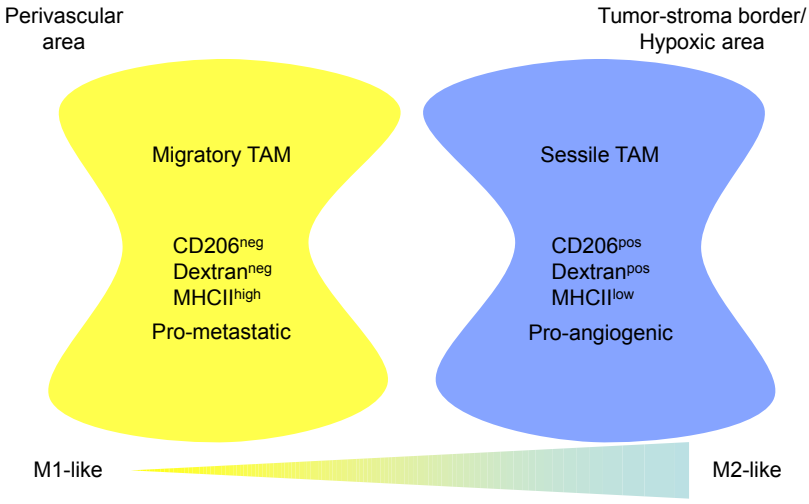

Fig. 2. Tumor-associated macrophage (TAM) heterogeneity in breast carcinoma tumors. *Scheme summarizing observations by several independent research groups, hinting to the existence of distinct TAM subsets, which are localized in different tumor compartments, exhibit a different molecular profile and exert specialized functions.*

where many were found in association with blood vessels either as single cells or in clusters (Wyckoff *et al.,* 2007). The presence of TAM near blood vessels is of great importance for the course of the disease, as these TAM attract cancer cells and move with them in a directed fashion, resulting in the extravasation of cancer cells only in the neighbourhood of such macrophages. As a matter of fact, the density of such tripartite interactions between invasive cancer cells, macrophages and endothelial cells (also called the tumor microenvironment of metastasis or TMEM) is able to predict the presence of distant metastases in breast cancer patients (Robinson *et al.,* 2009). Mechanistically, the coordinated movement of cancer cells and perivascular macrophages can be explained by a paracrine positive feedback loop, whereby M-CSF produced by cancer cells and EGF produced by perivascular TAM are instrumental for cell migration and invasion (Wyckoff *et al.,* 2007, Goswami *et al.,* 2005). Triggering of this loop can occur under the influence of growth factors such as heregulin-β1 and CXCL12 (Hernandez *et al.,* 2009). Movement of cancer cells in the neighbourhood of blood vessels has also been observed in a study using cancer cells that express the photoswitchable protein Dendra2 (Kedrin *et al.,* 2008). Regions in different tumor microenvironments of the same orthotopically grown tumor were photoswitched and cancer cell motility was followed using intravital microscopy through a mammary imaging window. While there was little migration in avascular regions, photoswitched cells were more mobile in a vascular microenvironment containing perivascular macrophages. In the latter regions, cancer cells infiltrated larger areas and lined up along blood vessels, illustrating the existence of at least two distinct microenvironments within the same tumor. Along the same line, Egeblad *et al.,* (2008) showed migratory behaviour of M-CSFR^{+} myeloid cells at the tumor-stroma borders, but not within the tumor mass, using spinning disk confocal microscopy on mouse mammary tumors. These authors also identified other markers with the ability to discriminate between migratory and sessile cells. Non-migratory cells present within the tumor mass were mostly CD68^{+} CD206^{neg} and did not ingest intravenously injected dextran (dextran^{neg}). Cells at the tumor-stroma border could be distinguished as migratory CD68^{+}MMR/CD206^{neg} dextran^{neg} myeloid cells and sessile CD68^{+} CD206^{+} dextran^{+} M2-type TAM, altogether clearly illustrating the existence of distinct macrophage types in breast tumors. In agreement with these data, a recent study employed CD206 and MHC II expression to discriminate between two TAM subpopulations in orthotopically grown mouse breast tumors (Movahedi *et al.,* 2010). Interestingly, MHC II^{high} TAM are excluded from hypoxic avascular areas (hence more perivascular), are CD206^{neg} and in general more M1-oriented, while hypoxic MHC II^{low} TAM express higher levels of CD206 and other M2-associated markers. At the functional level, the MHC II^{low} CD206^{+} hypoxic TAM were more proangiogenic. Though clinical data on TAM heterogeneity in human breast tumors are almost non-existing, one study hints to this possibility. In breast carcinoma patients, expression of thymidine phosphorylase (TP) in TAM provided an independent prognostic value, with macrophage TP^{+} tumors having a significantly worse prognosis (Toi *et al.,* 1999). Even patients having an extensive accumulation of CD68^{+} TAM could be categorized in two subgroups with strikingly different diagnoses: a good prognostic macrophage TP^{neg} group and a poor prognostic macrophage TP^{pos} group. These data suggest the existence of both antitumor and protumor TAM types in human breast carcinomas, whereby their balance possibly influences outcome of the disease.

The existence of TAM subpopulations has obvious consequences for the interpretation of existing gene expression data sets of these cells. For example, a high-density gene expression analysis of TAM from PyMT tumors was performed on the M-CSFR^{+}F4/80^{+}Gr-1^{neg}dextran^{+} TAM population, but not on M-CSFR^{+}F4/80^{+}Gr-1^{neg}dextran^{neg} tumor-associated cells, though these cells are most likely also TAM (Ojalvo *et al.,* 2009). Hence, the conclusion that PyMT TAM express higher levels of genes related to immune suppression, development and angiogenesis is only true for the TAM subpopulation studied. The fact that conclusions for one TAM population can not be extrapolated to another one is exemplified by follow-up work from the same lab, comparing the dextran^{+} TAM to the TAM population comigrating with cancer cells in an *in vivo* migration assay (Ojalvo *et al.,* 2010). The latter are probably similar to the perivascular TAM in the imaging study by Wyckoff *et al.,* (2007), and the CD68^{+} CD206^{neg} dextran^{neg} TAM in the imaging study by Egeblad *et al.,* (2008). Surprisingly enough, these TAM populations were very different at the gene expression level. Of interest, the dextran^{+} TAM expressed elevated levels of nearly all genes belonging to a consensus gene signature for in vivo-induced M2-like macrophages, including CD206 (Hassanzadeh Ghassabeh *et al.,* 2006). In that respect, these cells resemble the MHC II^{low} CD206^{+} M2-like TAM subpopulations from orthotopically grown mammary tumors, while the MHC II^{high} CD206^{neg} M1-like TAM from these tumors might be similar to the migratory/perivascular TAM (Movahedi *et al.,* 2010). Overall, the existence of following two TAM subpopulations seems consistent throughout several independent studies by different researchers: (i) M-CSFR^{+}Gr-1^{neg}Dextran^{neg}CD206^{neg}MHC II^{high} perivascular TAM that can be co-opted by cancer cells to migrate and are generally less M2-oriented; and (ii) sessile M-CSFR^{+}Gr-1^{neg}Dextran^{+}CD206^{+}MHC II^{low} TAM found at tumor-stroma borders and/or hypoxic regions that

resemble more M2-like or "trophic" macrophages (Fig. 2).

Concluding remarks

For many years, the development of cancer therapeutics was mainly a search for ways to interfere with cancer cell-intrinsic characteristics. However, evidence in breast carcinoma patients and experimental mouse mammary tumor models make a strong case for the implication of TAM in regulating tumor progression and metastasis. Hence, significant efforts have been made to better characterize this "other half of the tumor", and data are available to proclaim TAM as potential targets for therapeutic intervention.

In order to develop novel strategies for TAM-directed anticancer therapies, several ongoing research lines will be important. These include the determination of TAM molecular signatures, using –omics approaches, which might yield novel targets for intervention. In addition, the better characterization of distinct TAM subsets, residing in different tumor regions and performing specialized functions, promises to gain better insights in fundamental TAM/cancer cell crosstalk and might provide the opportunity to specifically target the most protumoral TAM. Finally, it might well be that diverse tumor-associated host cells (such as myofibroblasts) will fall into a diversity of functional subtypes like it was found for TAM.

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