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Macrophage Diversity Enhances Tumor Progression and Metastasis

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Abstract

There is persuasive clinical and experimental evidence that macrophages promote cancer initiation and malignant progression. During tumor initiation they create an inflammatory environment that is mutagenic and which promotes growth. As tumors progress to malignancy, macrophages stimulate angiogenesis, enhance tumor cell migration, invasion, and suppress anti-tumor immunity. At metastatic sites macrophages prepare the target tissue for arrival of tumor cells and then a different subpopulation of macrophages promotes tumor cell extravasation, survival, and subsequent growth. Specialized subpopulations of macrophages may represent important new therapeutic targets.

Introduction

Tumors have a complex cellular ecology that establishes the malignant potential of the tumor. In these ecosystems innate immune cells are highly represented and among the most abundant of these are macrophages. Although the original hypotheses proposed that macrophages are involved in antitumor immunity, there is substantial clinical and experimental evidence that in the majority of cases these tumor-associated macrophages (TAM) enhance tumor progression to malignancy. The tumor promoting functions of macrophages at the primary site include supporting tumor-associated angiogenesis, promotion of tumor cell invasion, migration and intravasation as well as suppression of antitumor immune responses (Condeelis and Pollard, 2006; Pollard, 2004). Macrophages also potentiate the seeding and establishment of metastatic cells and play a role in tumor

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initiation when inflammation is a causal factor. This review focuses on the diverse roles and functions of macrophages in the primary tumor and at metastatic sites. Accumulating evidence suggests that tumor initiation, progression, and metastasis are affected by dynamic changes in the phenotypes of macrophages and that defined subpopulations of macrophages are responsible for these tumor-promoting activities.

Macrophage Phenotypes

Macrophages are differentiated cells of the mononuclear phagocytic lineage (Pollard, 2009)and references therein) that are characterized by specific phenotypic characteristics and by the expression of particular markers, none of which are entirely restricted to the lineage (Gordon and Taylor, 2005). In mice, macrophages are phagocytic and express CD11b, F4/80, colony stimulating factor-1 receptor (CSF-1R; CD115) and do not express Gr1 (more specifically Ly6G that is detected by anti-Gr1 antibodies). In humans, phagocytosis, CD68, CD163, CD16, CD312 and CD115 are the major markers of the lineage. When combined these characteristics discriminate macrophages from other members of the myeloid lineage such as the polymorphonuclear neutrophils and eosinophils (Joyce and Pollard, 2009).

Macrophage origins, lineage, and regulation by growth factors have been recently reviewed and attempts have been made to define macrophage subsets (Pollard, 2009) and references therein). The most successful classifications have been applied to subtypes participating in particular immunological responses. These include the “activated” macrophage involved in the responses of type I helper T cells (Th1) to pathogens. This population is activated by interferon gamma and engagement of Toll-like receptors (TLRs) and is characterized by elevated expression of major histocompatibility complex (MHC) class II, expression of interleukin (IL-12) and tumor necrosis factor α (TNF α) generation of reactive oxygen species and nitric oxide (NO) and the ability to kill pathogens and cells. In contrast, the “alternatively-activated” macrophages that differentiate in response to IL-4 and IL-13, are involved in Th2-type responses including humoral immunity and wound healing (Gordon, 2003). Another population is the antigen-presenting, migratory dendritic cells that are a branch of the mononuclear phagocytic lineage. There are also other macrophage populations involved in tissue development and homeostasis that are largely regulated by CSF-1 and that do not fall easily into these immunological categories (Pollard, 2009). This argues that there are many populations of macrophages ranging from trophic macrophages involved in developmental processes (often with specialized functions such as the bone remodeling osteoclast), those active in tissue repair, and the immunological subsets described above (Pollard, 2009). Mantovani and collaborators have suggested that macrophages in tumors are biased away from the activated (M1) to the alternatively activated type named “M2” (Mantovani and Sica, 2010). Recent gene profiling experiments on TAMs support this shift to an immunoregulatory type (Biswas et al., 2006; Ojalvo et al., 2009; Pucci et al., 2009). However, in contrast to this binary MI/M2 definition, TAMs are comprised of several distinct populations that often share features of both types, but with greater overall similarity to macrophages involved in developmental processes (Ojalvo et al., 2009; Ojalvo et al., 2010). The functions of these subpopulations in promoting malignancy will be discussed below.

Macrophages and Cancer

Clinical studies make a strong case that macrophages promote tumorigenesis. In one meta-analysis it has been reported that over 80% of studies show a correlation between macrophage density and poor patient prognosis (Bingle et al., 2002) and recent studies have further supported this conclusion. For example, there is a strong association between poor survival and increased macrophage density in thyroid, lung, and hepatocellular cancers (Chen et al., 2005; Ryder et al., 2008; Zhu et al., 2008). However, as before there are some exceptions with high macrophage densities correlating with increased survival in pancreatic cancer (Kim et al., 2008). An unbiased transcriptome analysis of follicular lymphoma shows that a macrophage transcriptional signature is a predictor of a poor prognosis, as is increased macrophage density (Farinha et al., 2005). Analysis of the transcriptome of TAMs derived from studies in mouse models of breast cancer has also provided evidence that an enrichment in macrophage transcripts is predictive of poor prognosis and reduced survival in human breast cancer (Ojalvo et al., 2009; Zabuawala et al., 2010).

Macrophage differentiation, growth and chemotaxis are regulated by several growth factors including CSF-1, granulocyte-macrophage (GM)-CSF, IL-3, and chemokines such as CCL-2 (Pollard, 2009). Over-expression of CSF-1, the major lineage regulator for macrophages (Pollard, 2009), is, associated with poor prognosis in breast, ovarian, endometrial, prostate, hepatocellular and colorectal cancer among others (Groblewska et al., 2007; Lin et al., 2002; Mantovani and Sica, 2010; Mroczko et al., 2007; Ojalvo et al., 2010; Sapi and Kacinski, 1999; Smith et al., 1995; Zhu et al., 2008). CCL-2 is also over-expressed in a wide-range of cancers (Mantovani and Sica, 2010) and is associated with poor prognosis in breast, colorectal and thyroid cancers (Bailey et al., 2007; Saji et al., 2001; Tanaka et al., 2009; Yoshidome et al., 2009) whereas its absence is associated with increased survival in cervical cancer patients (Zijlmans et al., 2006). In melanoma there appears to be an inverse relationship between CSF-1 and CCL2 expression although in this case, TAM density still correlates with invasiveness and poor prognosis (Varney et al., 2005). A CSF-1 response transcriptional signature has been found in ductal carcinoma in situ (DCIS) of the breast (Sharma et al., 2009) and in a subset of breast cancers where it correlates with higher tumor grade (Beck et al., 2009). In leiomyosarcomas the CSF-1 expression signature has been reported as the only independent prognosticator in multivariate analysis (Espinosa et al., 2009). These association studies are highly suggestive of the involvement of these growth factors and chemokines in tumor macrophage biology. However, a note of caution should be added for this interpretation, given that in some cases tumor cells express the receptors for these growth factors and chemokines and this expression can result in an increased malignant phenotype (Mantovani et al., 2008; Patsialou et al., 2009; Scholl et al., 1994; Smith et al., 1995).

Experimental evidence for the tumor promoting activation of macrophages has been derived from several different types of experiments. Genetic ablation of the macrophage growth factor CSF-1 in the polyoma middle T (PyMT) oncoprotein mouse model of breast cancer greatly reduces macrophage density in tumors, slows the rate of tumor progression to malignancy and severely inhibits metastasis (Lin et al., 2001). In these studies macrophages are the only cells expressing the CSF-1 receptor (CSF-1R). Over-expression of CSF-1 in

wild type tumors results in earlier macrophage recruitment and an accelerated rate of tumor progression and increased metastasis. Genetic ablation of CSF-1 also affects tumor development and reduces malignancy in a genetic model of colon cancer (Oguma et al., 2008) and in an osteosarcoma xenotransplant model (Kubota et al., 2009). Furthermore genetic ablation in myeloid cells of the *Est-2* transcription factor, a direct effecter of the CSF-1 pathway, results in an inhibition of metastasis in both PyMT and orthotopic transplant breast cancer models (Zabuawala et al., 2010).

Similar results have been obtained with therapeutic approaches. Treatment with antisense or antibodies that inhibit CSF-1 or its receptor reduces macrophage recruitment or function in mice bearing xenotransplants of human tumor cells, thereby inhibiting tumor growth and metastasis (Abraham et al., 2010; Aharinejad et al., 2009). Further, specific depletion of macrophages using clodronate-encapsulated liposomes reduces tumor growth in melanoma, ovarian, Lewis lung, teratocarcinoma, rhabdomyosarcoma, and prostate graft models (Gazzaniga et al., 2007; Halin et al., 2009; Kimura et al., 2007; Robinson-Smith et al., 2007; Zeisberger et al., 2006).

It should be recognized however, that although the experimental and clinical data largely support the hypothesis that macrophages promote malignancy there are exceptions. For example, in the bone marrow macrophages play a gatekeeper role by phagocytosing cells that do not express the anti-death receptor CD47. Leukemic cells up-regulate this receptor to escape destruction (Jaiswal et al., 2009). Older data also show that liver macrophages (known as Kupffer cells) engulf and kill circulating tumor cells, such that the depletion of Kupffer cells in rats enhances metastasis (Heuff et al., 1993). Further, in peritoneal xenograft experiments in which cancer cells ‘metastasize’ to the liver, depletion of Kupffer cells worsens the prognosis. In addition these tumors exhibit greater differentiation and less malignancy but grow faster in the absence of macrophages, the mice dying of an increased tumor load (Oosterling et al., 2005). Thus although these experiments provide further support for a role for macrophages in malignant progression, ironically the outcome of their depletion was earlier death. These data contrast with the observations that Kupffer cells provide essential mitogens to hepatocellular carcinoma. The mitogen synthesis is due to NFκB signaling in these cells and ablation of their NFκB signaling results in a reduction in tumor burden (Karin and Greten, 2005).

Although the dynamics within tumors is undoubtedly complex, with macrophages playing both positive and negative roles, the data from animal models as well as from the clinical correlates indicates that in the vast majority of cases macrophages promote tumor progression and metastasis.

MACROPHAGES IN CANCER INITIATION AND PROMOTION

There is a growing appreciation that inflammation is the root cause of many cancers. Mantovani and colleagues have called this the 7th hallmark of cancer and reviewed its characteristics as well as the epidemiological and infectious disease literature that supports this hypothesis (Mantovani and Sica, 2010). A causal basis for the role of inflammation in cancer initiation has direct experimental support. In humans chronic obstructive pulmonary

disease is associated with persistent colonization with the bacterium *Haemophilus influenzae* and leads to increased lung cancer risk. In a mouse model of lung cancer bronchial exposure with *H.influenzae* lysate results in inflammation in the lung and an increase in tumorigenesis (Moghaddam et al., 2009). Myeloid-specific ablation of intergrin αV also results in an ulcerative colitis that induces colonic tumors (Lacy-Hulbert et al., 2007). Ablation in myeloid cells of *Stat3*, a transcription factor whose function suppresses inflammatory responses because it is a major target of immunosuppressive cytokine IL-10 (Yu et al., 2007), causes inflammation in the colon. This is associated with abundant expression of TNFα and IL-6 by macrophages, and results in a chronic colitis and invasive colonic adenocarcinomas (Deng et al., 2010). Similarly global ablation of IL-10 also results in chronic colitis and intestinal tumors (Yu et al., 2007). Mice that have a genetic ablation of GM-CSF (*Csf3)* and interferon γ (*Ifnγ*) whose loss would compromise acquired immune responses to pathogens, are found to develop a wide range of cancers (Enzler et al., 2003). The causality of the inflammation in carcinogenesis in these studies comes from experiments in which suppression of the bacterial flora by antibiotic treatment reduces the inflammation and inhibits tumorigenesis (Berg et al., 1996; Deng et al., 2010; Enzler et al., 2003). These data argue that the immune system is normally in balance but once the negative controls of immune responses are compromised, a persistent inflammatory response to normally commensal organisms results. This inflammation in turn creates a tumor promoting microenvironment.

The inflammatory state in myeloid cells is controlled by the transcriptional factors NFκB and Stat3 that work in opposition to one another (Karin and Greten, 2005; Yu et al., 2007). NFκB is a central transducer of signals that cause inflammation downstream of TLR activation. Its activity results in expression of inflammatory cytokines such as IL-12 and TNFα as well as inducible nitric oxide synthase (iNOS) (Karin and Greten, 2005). In the inflammatory responses associated with cancer initiation, NFκB signaling is essential for the inflammatory phenotype (Karin and Greten, 2005). Inhibition of this activity through ablation of IκB kinase α (IKKα) in myeloid cells in mouse models of intestinal cancer reduces inflammation and inhibits tumor progression (Greten et al., 2004).

The type of inflammation associated with increased cancer risk due to chronic infection or persistent irritation is often called “smoldering inflammation” (Mantovani and Sica, 2010). This nomenclature is used because the inflammation is low grade without overt clinical consequences. Activated macrophages are central to this type of immune response and work in concert with other immune cells (Balkwill et al., 2005). It has been hypothesized that these immune cells produce a mutagenic environment (Pang et al., 2007) by generating both reactive nitrogen and oxygen species. NO in particular reacts with peroxidates to give nitrosoperoxycarbonate and this reaction is a major driver of the chemistry of inflammation. This highly reactive compound and other products cause mutations in the adjacent epithelial cells (Meira et al., 2008; Pang et al., 2007). In addition, there is evidence that the inflammatory microenvironment also promotes genetic instability within the developing tumor epithelial cells (Colotta et al., 2009). In either case, the mutations are fixed after replication of the epithelial cells, a process that is stimulated by growth factors synthesized by the infiltrating or resident immune cells that include macrophages. These growth-promoting effects on tumors are caused by the production of IL-6 in hepatocellular

carcinoma (HCC) (Lin and Karin, 2007; Naugler et al., 2007) and TNFα (Karin et al., 2006) and IL-6 in colitis associated cancers (Grivennikov et al., 2009). Interestingly, IL-6 synthesis in Kupffer cells in response to inflammation-induced liver damage is gender dependent with males who have increased risk of HCC having elevated levels. IL-6 is also required for the increased risk of HCC in female mouse models (Naugler et al., 2007).

Classical models of skin carcinogenesis show that oncogenic mutations caused by low but not initiating doses of carcinogens (such as dimethybenzanthracene) need to be "fixed" by application of a tumor promoter. The promoter application causes an acute inflammatory response that is dominated by macrophages. TNFα action through NFkB is a causal agent in this promotion through mechanisms that act directly on epithelial cells and on the inflammatory cells in the surrounding stroma, particularly the macrophages (Balkwill, 2009). Similar mechanisms operate in colon cancer (Luo et al., 2004). Together these data strongly support causal roles for inflammation in cancer initiation and promotion. Although not definitive, given that macrophages have not been uniquely targeted in any system, the data suggest that macrophages are key cells in cancer induced by inflammation.

Macrophage functions in the Primary Tumor

The macrophage phenotype associated with cancer initiation and promotion is comparable to the "activated" one (Gordon, 2003). However, once initiated and the tumors progress towards malignancy, the macrophage phenotype changes from the "inflammatory" type to one that resembles macrophages that promote tissue formation during development (Figure 1) (Pollard, 2004, 2009). In established tumors, NFκB signaling is inhibited by the constitutive expression of p50 homodimers that negatively regulate NFκB and the macrophages display the M2/trophic phenotype with reduced iNOS and TNFα expression (Saccani et al., 2006). Indeed, blocking NFkB function by inhibition of IKKα in cultured macrophages reduces the inflammatory gene expression signature and pushes cells to the trophic/M2 type (Porta et al., 2009). This transition from stimulated to inhibited NFκB function between the initiation and the established tumor stages are poorly understood but appears central to macrophage function in the tumor microenvironment. This alternatively activated/trophic type of macrophage is also found in cancers that arise in the apparent absence of obvious inflammation, such as breast cancer, in which macrophages are recruited to benign tumors in large numbers just as the tumors transition to malignancy (Figure 1).

Macrophage recruitment to tumors has been well-documented in the PyMT mouse model of breast cancer (Lin et al., 2003; Pollard, 2009) where a sizeable populations of macrophages are recruited at the adenoma/mammary intraepithelial stage once the tumors have progressed to early malignancy (Lin et al., 2003; Lin et al., 2006; Lin et al., 2001; Wyckoff et al., 2007). Similar patterns also occur in human endometrial and breast cancers (Lewis and Pollard, 2006; Smith et al., 1995). These macrophages are recruited in the presence of CSF-1, which promotes a trophic phenotype, and IL-4 and IL-10, which makes them immunomodulatory (DeNardo et al., 2009; Hamilton, 2008; Lin et al., 2001). Thus it is a misnomer to consider the leukocytic infiltrate in established tumors to be "inflammatory" as there are few of the hallmarks of inflammation, such as edema, swelling and fever.

Macrophages have the most complex transcriptome known (Suzuki et al., 2009). Because of their potential diversity of gene products, blood origin, and motile nature they are ideally suited to perform specific tasks in a timely and spatially appropriate manner. Thus macrophages, despite sharing many features in common, distinct tasks appear to require subtypes of macrophages. This is the case in the tumor microenvironment where macrophages are put into service to support the tumor. These pro-tumoral functions of macrophage subpopulations are discussed below and are indicated in Figure 2.

Tumor cell invasion, migration, and intravasation—Using a combination of intravital imaging and an in vivo assay for invasive tumor cells in the PyMT mouse model and in breast cancer cell xenografts, macrophages have been shown to be required for tumor cell migration and invasion (Condeelis and Pollard, 2006). They are the key that unlocks the gate to allow tumor cells to escape. Mechanistically, tumor cells synthesize CSF-1 that stimulates macrophages to move and produce epidermal growth factor (EGF) that in turn activates migration in the tumor cells (Wyckoff et al., 2004) (Figure 1). The macrophages and tumor cells move in lock-step, and inhibition of either the EGF or CSF-1 signaling pathways results in inhibition of migration and chemotaxis of both cell types. This is despite the fact that the CSF-1 receptor and the EGF receptor (ErbB1) are restricted to macrophages and tumor cells, respectively (Wyckoff et al., 2004; Wyckoff et al., 2007). A number of experimental systems provide evidence that macrophages and tumor cells are sufficient for this EGF-CSF-1 paracrine interaction: macrophage-induced migration can be re-capitulated with these two cell types in an in vitro collagen overlay assay (Condeelis and Pollard, 2006), in mammary epithelial organoid culture system (DeNardo et al., 2009) or in co-culture (Green et al., 2009). However, in this latter case EGF is not involved whereas CSF-1 is essential. In human breast cancer EGF expression is restricted to macrophages whereas CSF-1 is in the tumor cells (Leek et al., 2000; Scholl et al., 1994). In PyMT tumor cells CSF-1 is regulated by steroid hormone receptor co-activator (SRC)-1 and in SRC-1's absence, although tumor growth is not affected, macrophage recruitment is impaired and tumor cell intravasation and metastasis are inhibited (Wang et al., 2009). Macrophage polarization to the invasion-promoting phenotype is in turn regulated by Il-4 synthesized by CD4+ T cells or tumor cells. In the absence of IL-4 macrophages are unable to promote invasion and migration of tumor cells and metastasis is dramatically reduced in the PyMT model (DeNardo et al., 2009; Gocheva et al., 2010).

The co-migration of macrophages and tumor cells can be initiated by other growth factors such as heregulin and CXCL12 (stromal derived factor-1;SDF-1) dependent on the breast cancer model. However once initiated, the migration of both cell types still requires paracrine CSF-1-EGF signaling (Hernandez et al., 2009). Given that heregulin and CXCL12 can be synthesized by tumor cells, fibroblasts, or pericytes, this data suggests that a specialized microenvironment is formed that can initiate tumor cell-macrophage invasion (Figure 1). This is consistent with intravital imaging of mammary tumors that shows tumor cell invasion is not uniform but occurs sporadically in particular locations and the observations that tumor cell movement in vivo occurs adjacent to macrophages in the PyMT mammary tumor model (Wyckoff et al., 2007) and in xenotransplants on the chick allantoic membrane (Green et al., 2009). Pertinently, CSF-1 expression in human tumors is highest at

the invasive edge, a site abundantly populated by macrophages (Lin et al., 2001; Scholl et al., 1994; Smith et al., 1995; Zhu et al., 2008).

Other molecules that may be involved in the macrophage stimulation of invasiveness in vivo have also been suggested by tissue culture experiments. For example Wnt5a acting through the non-canonical pathway (Pukrop et al., 2006) in organoids and TNFα via NFκB in co-culture (Hagemann et al., 2005) can promote tumor cell invasion. Further, macrophages have been shown to compensate for the loss of motility in tumor cells following a knockdown of osteopontin (SPP1) (Cheng et al., 2007).

The extracellular matrix plays a major role in modifying tumor cell invasiveness. Macrophage synthesize SPARC/osteonectin (secreted protein, acidic rich in cysteine), which is important for deposition of collagen IV, enhanced tumor cell invasion, and adhesion to other ECM components (such as fibronectin). SPARC/osteonectin has been shown to be required for spontaneous metastasis from the primary tumor (Sangaletti et al., 2008). Fibrillar Collagen 1 also enhances the invasion process as tumor cells and macrophages move ~10 times faster on these structures than through the stroma itself. This has the unfortunate consequence of recruiting cells towards blood vessels given that these collagenous fibrils also anchor these structures (Condeelis and Segall, 2003). At least during development of the mammary gland, macrophages have been shown to promote collagen fibrillogenesis (Ingman et al., 2006). Intravital imaging has shown that intravasation occurs through clusters of macrophages located on the abluminal side of the vessels (Wyckoff et al., 2007). Thus macrophages on vessels give come-hither signals that result in tumor cell migration down collagen fibrils toward vessels where the tumor cells escape into the vasculature aided by macrophages. This localized movement near to vessels has been confirmed by intravital imaging of xenografted tumors (Gligorijevic et al., 2009). Reduction in the number of tumor associated macrophages using genetic means (Wyckoff et al., 2007), or inhibition of EGF (DeNardo et al., 2009; Wyckoff et al., 2007) or CSF-1 (Wyckoff et al., 2007) signaling in wild type mice bearing mammary tumors reduces the numbers of circulating tumor cells. This data suggests that the paracrine loop between the two cell types is required for egress into the circulation in vivo (Wyckoff et al., 2007). Importantly, analysis of clinical material indicates that a structure named the “tumor microenvironment of metastasis (TMEM)” defined by co-localization of macrophages, tumor cells, and endothelial cells is a prognostic marker for poor survival in breast cancer (Robinson et al., 2009). These data may also tie together the clinical observations described above as CSF-1, EGF, CXCR4 are prognostic markers in many cancers.

The macrophages that stimulate tumor cell invasion in vivo have been isolated and their transcriptome interrogated on DNA microarrays. Unsupervised clustering shows this population uniquely separated from a general TAM or a reference population of splenic macrophages. Comparisons with other datasets show that the “invasive macrophages” are most similar to those found during embryogenesis. They are enriched in developmental pathways, in particular the Wnt signaling pathway (Ojalvo et al., 2010). Given that macrophage-produced Wnts promote vascular remodeling in developmental contexts (Lobov et al., 2005) this array data led to the hypothesis that these invasive macrophages link angiogenesis and tumor invasion.

The studies cited above have focused upon invasion and intravasation of single tumor cells typically found in breast cancers. However there are other types of invasion, including the collective invasion of sheets of cells such as that found in colon cancer. In a mouse model of this disease caused by a mutation in the APC gene and hemizygosity of Smad4, a unique population of immature myeloid cells (which express CD34, CD45, CD11b, and CCR1, but not F4/80) surrounds the invasive front. Depletion of CCL9, the ligand for CCR1, blocks the accumulation of these cells with a consequent inhibition of tumor cell invasion (Kitamura et al., 2007). These myeloid cells display an unusual phenotype and, as yet, no complete lineage relationship has been established.

Tumor cell migration also requires proteolytic destruction of the matrix to allow the escape of tumor cells from the confines of the basement membrane. Subsequently, proteolysis is required for tumor cells to migrate through the dense stroma. Macrophages are potent producers of many proteases, including cathepsins, matrix metalloproteases, and serine proteases (Egeblad and Werb, 2002). In many tumors proteases play a role in tumor progression and metastasis (Egeblad and Werb, 2002; Gocheva et al., 2006; Joyce and Pollard, 2009). Depletion of cathesin B (Gocheva et al., 2010; Vasiljeva et al., 2006) and S (Gocheva et al., 2010) from macrophages results in reduced tumor cell invasion and inhibition of metastasis in the PyMT model. Urokinase/Plasminogen activator (uPA) is mostly produced by macrophages and in the PyMT model its loss also inhibits metastasis (Almholt et al., 2005). In the colon model of collective cell migration described above, the immature myeloid cells produce both MMP9 and MMP2 that are required for the tumor cell invasion (Kitamura et al., 2007).

Angiogenesis—In most tumors there is a dramatic enhancement of vascular density from the benign to malignant transition, a process referred to as the angiogenic switch (Hanahan et al., 1996). The formation of a complete vasculature is a complex process with many cell types, often with overlapping functions influencing its outcome in tumors. Cells of the mononuclear phagocytic lineage cells, and macrophages in particular, are major contributors to this process (Zumsteg and Christofori, 2009). Studies in which macrophages are reduced in mammary tumors using the null mutation in the *Csf1* gene show that these cells are required for the angiogenic switch. This effect is reversed by the re-expression of CSF-1 in the mammary epithelium (Lin et al., 2006). Over-expression of CSF-1 in wild type mice results in the premature accumulation of macrophages into hyperplastic lesions and a dramatic early angiogenic switch that in turn accelerates the transition to malignancy. These data strongly argue for the role of the angiogenic switch in regulating the malignant transition and for macrophages to be important players in this regulation (Lin and Pollard, 2007). These studies also show that macrophages play a significant role in vascular remodeling as tumors progress to late carcinoma stages (Lin et al., 2006). A similar macrophage depletion strategy also reduces angiogenesis in an osteosarcoma model (Kubota et al., 2009). Further, most TAM depletion strategies using liposome-encapsulated clodronate described above, inhibit angiogenesis in transplanted tumor models (Gazzaniga et al., 2007; Halin et al., 2009; Kimura et al., 2007; Zeisberger et al., 2006). This effect seems to be the most likely cause of reduced tumor growth following macrophage depletion seen in these transplants models, as their growth is very dependent upon rapid angiogenesis.

A subpopulation of CD11b-positive myeloid cells characterized by expression of Tie2, a marker of mature endothelial cells, has been described in tumors. These myeloid cells appear to be derived from Tie2-expressing monocytes (TEM) that are found in human cancer patients and in mice (Murdoch et al., 2008). Co-injection of tumor cells with these cells enhances angiogenesis. In contrast ablation of these cells impairs angiogenesis in several mouse models of cancer (De Palma et al., 2005). Transcriptional profiling of Tie2-positive and negative monocytes show that they are distinct classes although highly related (Pucci et al., 2009). We have also identified two subpopulations of macrophages in the PyMT model that express differing levels of Tie2 (Data not shown). They are probably equivalent to the two populations described by (Pucci et al., 2009) who defined their populations by a reporter gene assay rather than the more sensitive analysis of cell surface markers by fluorescence-activated cell sorting. Indeed, *Tie2* mRNA is expressed in the Tie2-negative population albeit at a 2-fold lower level than the expression in the Tie2-positive population (Pucci et al., 2009).

Transcriptional profiling on high-density oligonucleotide arrays of these TAMs shows that they are highly enriched in transcripts that encode angiogenic molecules (Ojalvo et al., 2009). Reinforcing this result, in the PyMT mammary cancer model, gene ablation of the *Ets2* transcription factor in the myeloid lineage inhibits angiogenesis. Transcriptional profiling of the *Ets2*-deficient TAMs shows that ETS2 controls the expression of transcripts encoding proteins that regulate angiogenesis (Zabuawala et al., 2010). In both cases comparisons of TAM transcriptomes with available clinical databases shows that these transcriptional signatures are predictive of survival (Ojalvo et al., 2009; Zabuawala et al., 2010). These data make a strong case that this population of TAMs plays important roles in tumor progression through their effects on angiogenesis. Moreover, the pro-angiogenic role of TAMs in mouse models is consistent with clinical observations in breast cancer that correlate macrophage density with microvessel density and poor prognosis (Leek and Harris, 2002).

Hypoxia is a major driver of angiogenesis. Macrophages accumulate in hypoxic areas of the tumor and are particularly associated with necrotic tissue (Murdoch et al., 2008). HIF1α whose expression is constitutive in macrophages, modulates the recruitment of macrophages to hypoxic regions of the tumor. This recruitment is through chemokines especially CCL-2 and endothelins (Grimshaw et al., 2002; Murdoch et al., 2008). At the hypoxic site, HIF1α regulates the transcription of a large panel of genes associated with angiogenesis including *VEGF* (Lewis and Hughes, 2007; Murdoch et al., 2008). These genes then mediate the revascularization of the necrotic zones (Murdoch et al., 2008). This process can be modeled in *in vivo* angiogenesis models such as in T47D tumor cell spheroids (Murdoch et al., 2008) or in transplant tumor models (Zumsteg and Christofori, 2009).

Macrophages produce VEGF in both human and mouse mammary tumors (Leek and Harris, 2002; Lin et al., 2006). VEGF over-expression in macrophage depleted mice increases vascularization and also accelerates the transition to malignancy (Lin et al., 2007). This rescue is also associated with the recruitment of macrophages even in the absence of CSF-1 and these cells and their angiogenesis regulating gene products may be partially responsible for the angiogenic response. Targeted ablation of the *Vegfa* gene in myeloid cells results in

the inhibition of the angiogenic switch (Stockmann et al., 2008). However, despite the failure of the angiogenic switch, the tumors that grow out are more aggressive and are characterized by a less dense but more coherent vasculature. This aggressive growth suggests strong selection in response to hypoxic stress for tumor cells that are able to use glycolysis as a source for energy (Stockmann et al., 2008).

Macrophages can produce VEGF but in other cases they also make it bioavailable through the production of MMP9, which releases VEGF from extracellular depots. Targeting of macrophages with bisphosphonate in a model of cervical carcinogenesis inhibits of angiogenesis because macrophages are the major producers of MMP9 in this model and are recruited by CCL2 (Giraudo et al., 2004). Lack of CCL2 signaling reduces macrophage infiltration but has only a modest effect on tumor progression because of a compensatory recruitment of MMP9-producing neutrophils (Pahler et al., 2008). However, in other reports CCL2 recruitment of macrophages is required for angiogenesis (Fujimoto et al., 2009; Gazzaniga et al., 2007). In a glioblastoma mouse model, stromal-synthesized SDF-1 (CXCL12) recruits a myeloid cell population that expresses MMP9 and releases matrix-bound VEGF. This VEGF not only stimulates angiogenesis but also tumor cell invasion (Du et al., 2008).

Immunoregulation—Macrophages are central to many immune responses and are clearly immunoregulatory cells within the tumor. In some cases this can result in rejection as both macrophages and dendritic cells are able to present antigens to cytotoxic T cells and macrophages are adept at tumor killing. Pioneering work from Fidler and colleagues indicated that activated macrophages can kill tumor cells and eliminate metastases (Fidler and Schroit, 1988). Similarly inhibition of tumor growth in xenograft models have recently been obtained by activating macrophages by either over-expressing GM-CSF (Eubank et al., 2009) or treating tumors with CpG together with anti-IL10 (Guiducci et al., 2005). These latter treatments activate Toll-like receptors and block immunosupression, respectively. However, in the vast majority of tumors there does not appear to be substantial immunological limitation of tumor growth. This suggests that the tumor microenvironment suppresses any immune response and alters the phenotype to one that promotes the tumor (Swann et al., 2008). The exact role of macrophages or their cousins in this process has not been fully delineated. Phenotyping of the transcriptome of TAMs has suggested that they represent an immunological regulatory type. This is characterized by downregulation of transcripts involved with immunological activation such as IL-12, IL-18 and the TLR signaling pathway and upregulation of transcripts found in alternatively activated macrophages such as arginase (Biswas et al., 2006; Ojalvo et al., 2009). Importantly in the PyMT model this polarization of macrophages is caused by IL-4 synthesized by CD4-positive T cells (DeNardo et al., 2009). Furthermore, macrophages in tumors develop in high concentrations of CSF-1 that support their differentiation to trophic macrophages and away from immunologically-activated ones, which are controlled by GM-CSF (Hamilton, 2008; Mantovani and Sica, 2010).

Macrophages can inhibit cytotoxic T-cell responses through several mechanisms. For example, macrophages produce IL-10 that in turn induces monocytes to express the costimulatory molecule programmed death ligand (PD)-L1 and suppresses cytotoxic T cell

responses (Kuang et al., 2009). Macrophages in human ovarian cancers produce CCL22, a chemokine that regulates the influx of regulatory T cells (T_{reg}) that suppress cytotoxic T cell responses. The abundance of these T_{regs} in ovarian cancer predicts poor survival (Curiel et al., 2004). In mammary tumor xenografts a newly recruited macrophage population suppressed immune responses through synthesis of PGE2 and TGF-β (Torroella-Kouri et al., 2009).

Myeloid-derived suppressor cells (MDSCs) are another immunosuppressive immune cell population related to macrophages that has come to the forefront in recent years. MDSCs are a mixed population of myeloid cells that accumulate in pathological conditions including cancer. Morphologically, these populations consist of monocytes and granulocytes and immature myeloid cells and are identified by their capacity to suppress cytotoxic T cell responses (Gabrilovich and Nagaraj, 2009). In mice, MDSCs express both the myeloid cell marker CD11b and the granulocyte marker Gr1. The presence of these markers satisfy the classical definition of neutrophils and therefore MDSCs are not mononuclear phagocytes (Gabrilovich and Nagaraj, 2009). This cell surface marker analysis has also been reinforced by gene expression analysis that shows that MDSCs are markedly different from TAMs (Pucci et al., 2009). However, a key question remains whether these cells can differentiate solely into mature granulocytes or whether they can become macrophages in vivo.

Macrophages at the Metastatic Site

Most studies have focused upon events occurring in the primary tumor often with metastasis as an end point. However, metastasis not only requires the release of cells from the primary site but also their transit through the circulation or lymphatics to arrive at a distant site where the cells need to extravasate, survive, and prosper. This process of metastasis is very inefficient. In humans there are many thousands of circulating cells released by tumors every day but only a few successfully make metastases. In fact the most likely fate for these cells is death, with extravasation and establishment of micro-metastases being major rate-limiting events (Joyce and Pollard, 2009). Although it has long been known that macrophages populate metastatic lesions (Joyce and Pollard, 2009) only recently has their role in metastasis been appreciated.

Cancer is a systemic disease and primary tumors secrete factors that influence metastatic outcome at distant sites. For example the tumor-derived extracellular matrix protein, versican, stimulates metastasis in the Lewis lung carcinoma model through TLR2 signaling in myeloid cells (Kim et al., 2009). Aggressive tumors systemically influence less aggressive indolent ones to grow faster and to stimulate the growth of micro-metastases through the acquisition of bone marrow derived cells. In these studies osteopontin expressed from the aggressive tumor has been shown to be necessary but not sufficient for the mobilization of bone marrow cells (McAllister et al., 2008). In neither study were the phenotype of the bone marrow cells that influence the metastatic site fully characterized, however it is likely that macrophages are major payers in these responses.

Primary tumors have also been shown to cause the accumulation myeloid derived cells at distant sites and this process enhances metastatic efficiency (Kaplan et al., 2005). These primed sites are termed the pre-metastatic niche. Their location can be altered, for example

from lung to bone, by serum conditioned by the primary tumor giving organ specificity to the metastases (Kaplan et al., 2005). Among the tumor-produced factors required for the pre-metastatic niche are the myeloid chemoattractants S100A8 and A9 whose synthesis is induced by the primary tumor. S100 proteins induce the synthesis of amyloid protein A that signals through TLR4 in myeloid and endothelial cells (Hiratsuka et al., 2006; Hiratsuka et al., 2008). In addition, lysyl oxidase crosslinks the collagen at the pre-metastatic site and is essential for the myeloid recruitment (Erler et al., 2009). These myeloid cells secrete MMP9 that releases matrix-bound VEGF whose function is in turn required for the increase in metastatic efficiency (Hiratsuka et al., 2002). Although the myeloid cells that accumulate in these niches have not been fully characterized, they are CD11b, Mac1 and VEGFR1-positive, characteristics of mononuclear phagocytic cells (Hiratsuka et al., 2006; Kaplan et al., 2005). Inhibition of VEGFR1 signaling by antibody inhibition or receptor mutation also inhibits the formation of the pre-metastatic niche (Hiratsuka et al., 2002; Kaplan et al., 2005). However, there is a contradictory report challenging these findings, albeit in a different model of metastasis (Dawson et al., 2009). It has been proposed that these niches provide sites for tumor cells to adhere and prosper (Psaila and Lyden, 2009). Alternatively, these niches might simply prepare the tissue for successful colonization by being a reservoir of monocytes that can be rapidly mobilized to differentiate into macrophages in response to incoming tumor cells, a process that enhances metastatic seeding and growth (Qian et al., 2009).

Metastatic cells can colonize and grow in particular tissues even in the absence of a primary tumor(Joyce and Pollard, 2009). Recent ex-vivo imaging studies of lungs indicate that macrophages are recruited to these extravasating tumor cells and ablation of these macrophages dramatically reduce the extravasation efficiency and the subsequent tumor cell survival such that metastatic cell seeding efficiency is markedly reduced (Qian et al., 2009). Furthermore, ablation of these recruited macrophages limits subsequent metastatic growth, even after metastatic lesions had been established. These effects were accompanied by physical interactions between macrophages and metastatic tumor cells suggesting short-range transmission of growth and survival signals (Qian et al., 2009). Phenotyping revealed that the metastasis-associated macrophages differ from the CD11c-positive lung interstitial resident macrophages. Instead they are by CSF-1 and characterized by cell surface expression of CD11b, F4/80, VEGFR1, and CXCR2 and the absence of Gr1 and CD11c (Qian et al., 2009). Thus this pro-metastatic macrophage is another population with a distinct phenotype (Figure 2) that is found not only in these experimental models but also in metastases derived from autochthonous (spontaneous and native) models (Qian et al., 2009).

In summary mononuclear phagocytes appear to not only set up preferred sites for metastatic cell seeding but also enhance tumor cell extravasation, establishment, and subsequent growth of metastatic lesions (Figure 3).

Perspectives

Malignant cells can be reverted to a quiescent differentiated state by incorporation into an embryonic microenvironment (Joyce and Pollard, 2009). This indicates the microenvironment is dominant over malignancy. Thus for tumors to progress and become

malignant they must manipulate their microenvironment to one that is at least permissive if not promoting. This is probably due to selection of oncogenic mutations that lead to secretion of molecules that alter the cellular composition and function of the microenvironment. Among these changes is the recruitment of bone marrow derived cells of which, macrophages are particularly abundant. Macrophages in primary and secondary tumors confer several properties that enhance progression and metastasis. Each function is effected by a particular macrophage subpopulation (Figure 2). Elsewhere we have reviewed data that macrophages play important trophic roles during development and have argued that these roles are recapitulated in tumors (Pollard, 2009). This conjecture is supported by transcriptome analysis on high-density arrays that show the TAMs are most similar to macrophages involved in developmental processes (Ojalvo et al., 2009). These trophic macrophages are also characterized as immunomodulatory as would be expected for cells involved in normal processes. However, in contrast to developing systems tumors have lost their “off” switches because of oncogenic mutations, thus losing control of positional identity. Consequently tumors continue to inappropriately call for trophic support from macrophages. This leads to macrophage enhancement of malignancy at every step of the way.

Undoubtedly, as we dig deeper many other macrophage subpopulations will be revealed. For example, macrophages in prostate cancer cause the tumor cells to become resistant to the inhibitory effects of therapeutic androgen receptor antagonists and instead these drugs become agonists for growth. This resistance is mediated by a transcriptional cofactor TAB2 that acts as a sensor of inflammation in the form of IL-1β and whose phosphorylation causes release of gene transcription that is normally repressed by the anti-androgen. Similar mechanisms operate with anti-estrogens in MCF-7 breast cancer cells (Zhu et al., 2006). Macrophages might therefore have a further pro-tumor function in the progression of sex steroid hormone-dependent cancers to steroid independence.

Many experimental challenges remain. For example, caution needs to be exercised in the interpretation of experiments involving tumor transplantation, often into immunocompetent animals. These experiments are confounded by anti-graft reactions even in syngeneic contexts as well as the skewed immune reactions in immuno-compromised mice. They cannot represent the subtlety of immune cell interactions that occurs during the progression of autochthonous tumors. Thus for macrophage biology, immune competent animals need to be used and although valuable results can be obtained from transplant experiments, the conclusions need to be validated in models of spontaneously arising tumors. Another challenge is to confirm that the macrophage subtypes and functions found in rodents are present in human tumors. It is likely that comparable macrophage populations exist given that the individual TAM expression signatures derived from mice are represented in human tumor datasets and can even be predictive (Ojalvo et al., 2009; Zabuawala et al., 2010). Yet, this validation of the rodent data in humans is essential before anti-macrophage therapeutics are designed.

The evidence that macrophages provide tropic support to tumors and the genetic experiments that show if you remove this support that malignancy is suppressed, strongly argue that these cells or their unique signaling pathways are therapeutic targets. Unlike

tumor cells, the genomes of macrophages are stable suggesting that they may not as readily become drug resistant. Significant progress has been made in identifying the molecular basis for both macrophage phenotypes and their actions in promoting specific aspects of tumor behavior. Some important signaling pathways have been defined as described above, including those in response to VEGFa, TNFα, EGF and CSF-1. In addition, some transcriptional regulators (NFκB, Stat3) that bias macrophage phenotypes from pro- to anti-tumoral and molecules that recruit these cells to tumors such as CCL-2 have been identified. Pan-macrophage inhibitors such as drugs that inhibit CSF-1 signaling are in early clinical trials. However, in the future there may also be targeted therapies that uniquely strike macrophages in the tumor microenvironment and macrophage therapeutics that enhance the activities of conventional treatments.

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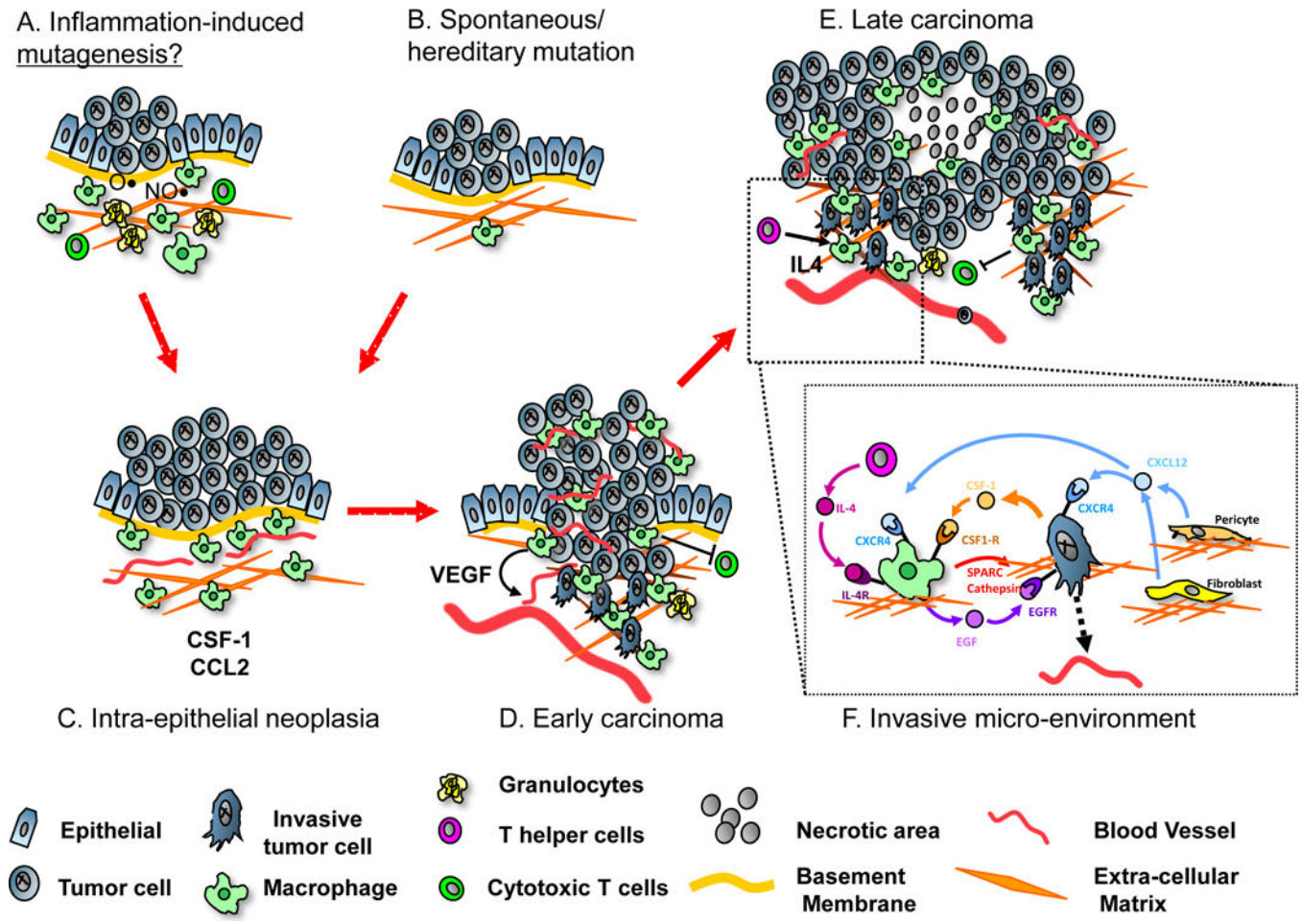

Figure 1. Macrophages Promote Tumor Initiation, Progression, and Malignancy

A) Chronic smoldering inflammation in response to pathogens or chronic irritants creates a mutagenic and growth-promoting environment in the subepithelial stroma. This environment potentiates the acquisition of oncogenic mutations in the overlying epithelial cells. Central to the inflammatory process are activated macrophages, which are the major producers of reactive oxygen and nitrogen species as well as a wide range of growth factors. B) Spontaneous or hereditary mutations cause tumor initiation and progression in cancers not associated with inflammation. C) The hyperplastic lesions progress to an intraepithelial neoplasia. This process results in the recruitment of monocytes by chemoattractants from the blood, such as colony stimulating factor-1 (CSF-1) and the chemokine CCL-2. These monocytes differentiate into macrophages in the tumor. These macrophages, unlike those in the initiating inflammatory environment, are not classically activated but instead resemble trophic, immunomodulatory macrophages found during development. D) The transition from an intraepithelial neoplasia/adenoma to an early carcinoma is promoted by macrophages in part through their stimulation of the angiogenic switch. Macrophages deliver vascular endothelial growth factor (VEGF) and other angiogenic molecules in a temporal and spatial fashion to avascular areas resulting in angiogenesis. In addition, macrophages produce growth factors and proteases that facilitate the escape of tumor cells from their constraining basement membranes. Furthermore, macrophages suppress cytotoxic

T cell responses to the invading tumor cells. E) After progression to malignancy, and as tumors because late carcinomas, macrophages are continuously recruited through similar mechanisms as before. In the tumor they differentiate into different subpopulations that have functions in: (1) angiogenesis, (2) tumor cell invasion and intravasation, and (3) immunosuppression. The dotted box designates an invasive microenvironment as defined in mouse models of breast cancer. In this model tumor cell motility and invasion is sparked by the production of growth factors/chemokines, such as CXCL12 that binds to its receptor (CXCR4) expressed on both macrophages and tumor cells. Once motility is initiated it is driven by an obligate epidermal growth factor (EGF)-CSF-1 paracrine loop with macrophages and tumor cells moving in lock step. Invasion also requires matrix formation and destruction through cathepsins and SPARC. Macrophages promote vasculogeneisis through angiogenic factors such as VEGF. Tumor cells egress through macrophage clusters on the blood vessels thus the macrophages increase both the invasion/intravasation of tumor cells and the number of vascular targets. This allows increased numbers of tumor cells to enter the circulation and thereby enhance tumor metastasis.

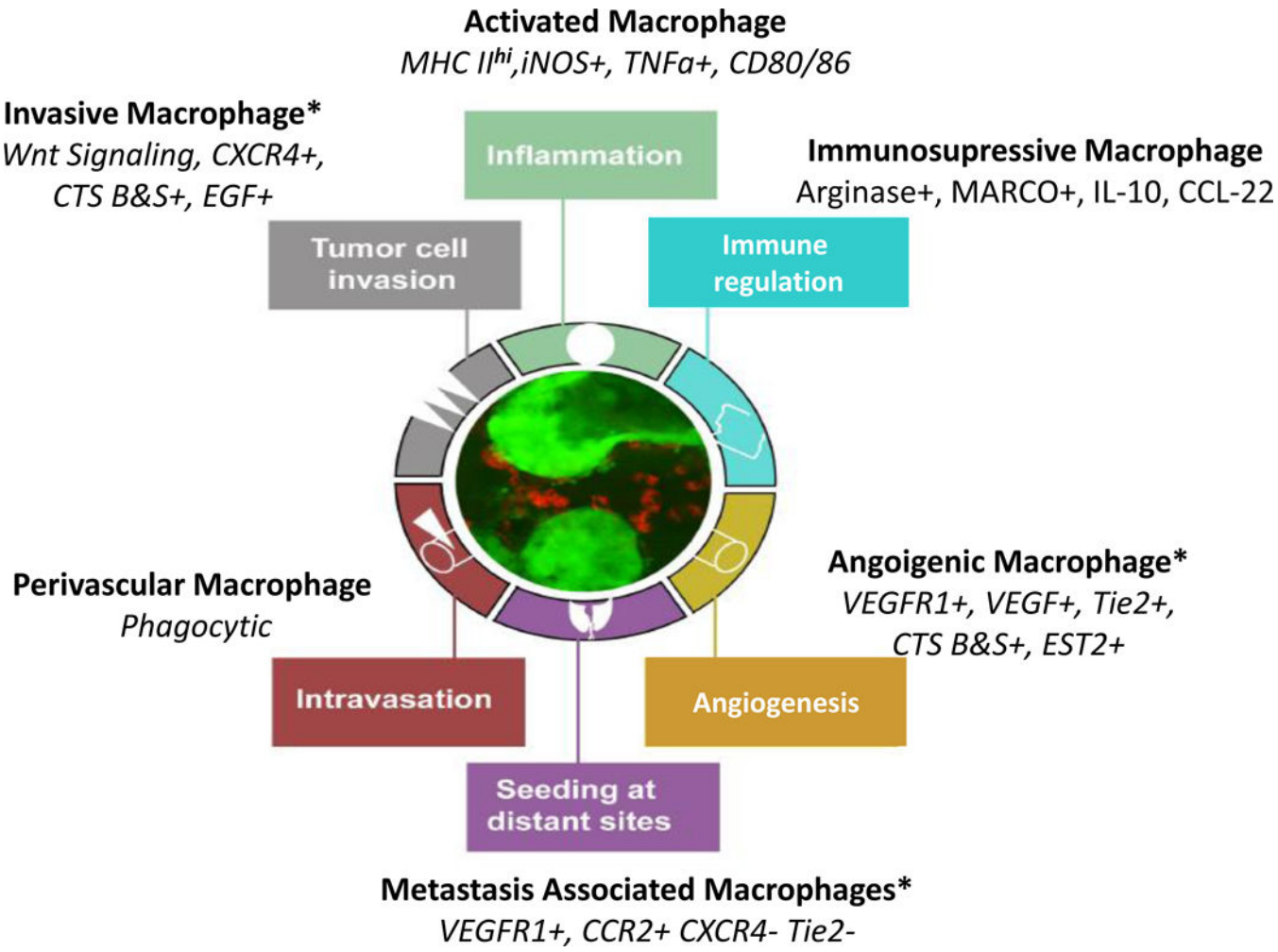

Figure 2. Macrophage Phenotypes and Tumorigenesis

Shown are six macrophage functions that provide extrinsic support to a tumor. Each of these extrinsic activities can be ascribed to a unique macrophage subpopulation. All of these macrophage subtypes are defined by the expression of the canonical markers Cd11b, F4/80 CSF-1R, and the absence of Gr1 but they are educated by microenvironmental cues to adopt a particular phenotype and perform the tasks as shown. The population listed as "perivascular" are probably the same as the invasive macrophage population as they have similar activities but they are localized to the abluminal surface of vessels often in cluster. * Populations whose transcriptomes have been analyzed

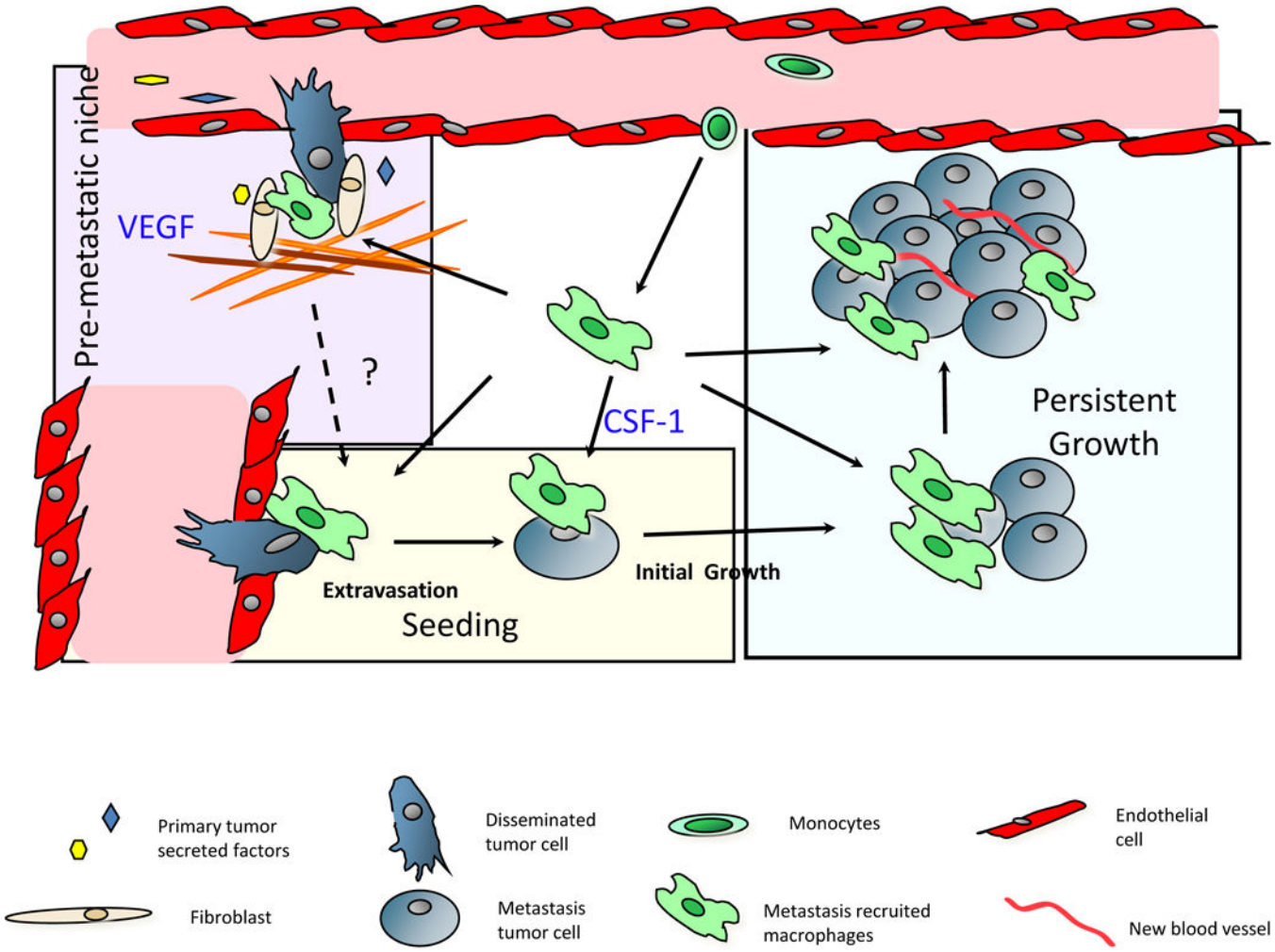

Figure 3. Macrophages Promote Seeding and Growth of Metastatic Cells

Myeloid cells, most likely macrophages, are recruited to the pre-metastatic niche in response to secreted products from the primary tumor. The metastatic target organs contain fibroblasts and elaborate extracellular matrix consisting of fibronectin and collagen. These niches direct and enhance tumor cell seeding in sites distant from the primary tumor. Once the tumor cells arrive at the metastatic site and begin to extravasate they recruit macrophages that are differentiated from blood borne monocytes. These macrophages enhance the ability of tumors cells to extravasate and promote their subsequent survival and growth. They continue to accumulate in metastatic lesions where they stimulate the growth and survival of the metastatic cells. Several growth factors and signaling pathways are important for these macrophage functions including vascular endothelial growth factor (VEGF) in the pre-metastatic site and colony stimulating factor-1 (CSF-1) for growth of the tumor cells.