Monocyte Subpopulations in Angiogenesis

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Abstract

Growing understanding of the role of the tumor microenvironment in angiogenesis has brought monocyte-derived cells into focus. Monocyte subpopulations are an increasingly attractive therapeutic target in many pathologic states, including cancer. Before monocyte-directed therapies can be fully harnessed for clinical use, understanding of monocyte-driven angiogenesis in tissue development and homeostasis, as well as malignancy, is required. Here, we provide an overview of the mechanisms by which monocytic subpopulations contribute to angiogenesis in tissue and tumor development, highlight gaps in our existing knowledge, and discuss opportunities to exploit these cells for clinical benefit. *Cancer Res; 74(5); 1287–93. ©2014 AACR.*

Introduction

Angiogenesis is the predominant method of blood vessel formation during adulthood. It also represents a central hallmark of cancer (1). Although early studies of angiogenesis focused on endothelial cells, the importance of other compartments of the microenvironment in vascular growth, such as cancer-associated fibroblasts and infiltrating leukocytes, is evolving. Among these, monocyte subpopulations are increasingly recognized contributors to angiogenesis. Here, we provide an overview of the mechanisms by which cells of mononuclear origin contribute to angiogenesis in tissue and tumor development, highlight gaps in our existing knowledge, and discuss opportunities to exploit these cells for clinical benefit.

Angiogenic Pathways

Although the mechanisms regulating angiogenesis are complex, VEGFA is a dominant stimulator, working through its receptor, VEGFR-2, and has been the principal target of anti-angiogenic therapy. In addition, VEGFR-3 interacts with homologs VEGFC and VEGFD and regulates branching morphogenesis and lymphangiogenesis. Importantly, VEGFR-3 augments VEGF-induced angiogenesis and sustains blood vessel growth, even in the presence of VEGFR-2 inhibitors (2). VEFGR-1 is involved in vascular patterning and is a chemotactic factor for monocytes (3, 4). Other pathways related to Delta-Notch and Angiopoeitin-2 (Ang-2)–Tie2 signaling are also of interest for tumor angiogenesis (5).

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Monocyte Subpopulations

Macrophages, first recognized by Metchnikoff, are abundant in tumors and were initially hypothesized to have antitumor properties. However, macrophages are now acknowledged to actively contribute to tumor growth and metastasis. They also play critical roles in both acute and chronic pathologic inflammation. In addition, other cells of monocyte lineage have been identified and their influence on the microenvironment is increasingly recognized.

A common myeloid progenitor cell gives rise to monocytes in the bone marrow. Monocytes enter the circulation and are capable of differentiating into macrophages, monocyte-derived dendritic cells (MDC), and tissue-specific phagocytic cells, such as Kupffer cells and osteoclasts (Fig. 1). Although the majority of this review will focus on macrophages, a brief discussion of other cells of this lineage is included.

Tie2-expressing monocytes

Tie2-expressing monocytes (TEM) are a distinct subpopulation, accounting for approximately 2% of circulating monocytes (6). TEMs play direct roles in tumor angiogenesis and are intimately associated with vasculature in the microenvironment (7). Ang-2 recruits TEMs to sites of injury and inflammation, as well as tumors, where they secrete proangiogenic factors such as VEGF, MMP9, COX2, and Wnt5A (Fig. 1; ref. 8). The selective depletion of TEMs impairs tumor growth and vessel formation (9). TEMs do not express CCR2, suggesting that their recruitment is regulated by different mechanisms than the CCL2-dependent monocytes (3).

MDCs

MDCs differ in origin from plasmacytoid dendritic cells and function as potent migratory antigen-presenting cells (10). MDCs emerge immature from the bone marrow and after encountering foreign antigen, undergo maturation, migrate to lymphoid tissue, and stimulate T-cell proliferation. These cells are able to prime naïve T cells, unlike macrophages and B cells, which can only present antigens to activated T cells (11). In addition, they are capable of cross-priming antigen-specific
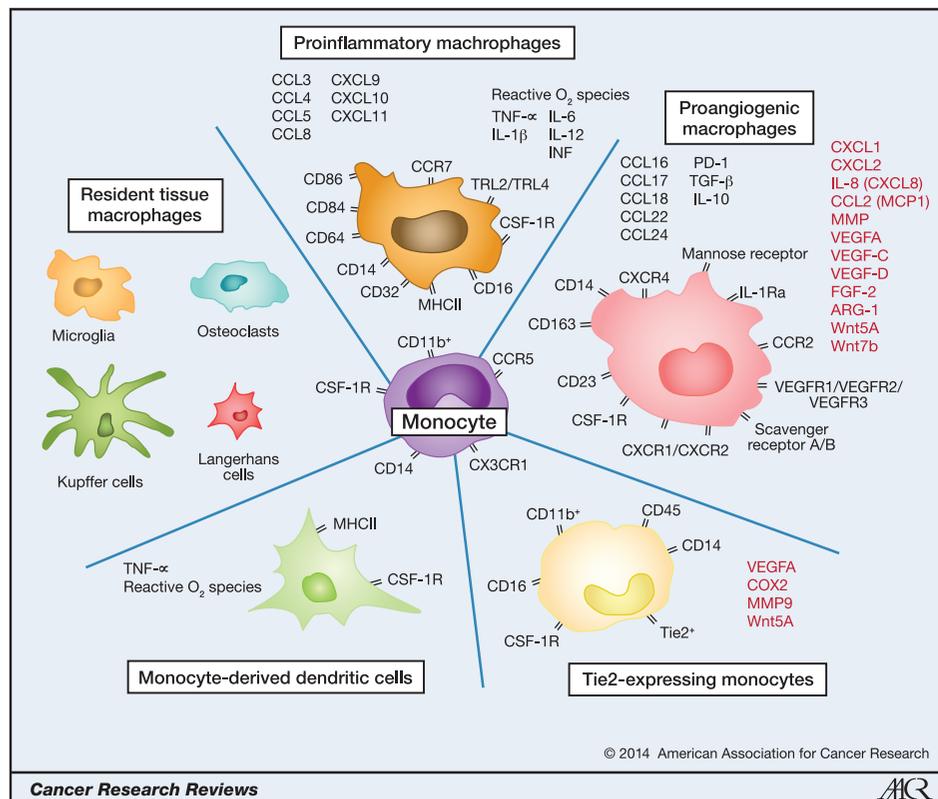
Figure 1. Phenotypic diversity of monocyte subpopulations. Cells are shown with characteristic surface receptors, along with chemokines, cytokines, and other characteristic factors when appropriate. Known proangiogenic factors are highlighted in red.

CD8$^+$ T cells. They can also exert an antimicrobial effect through the secretion of TNF-α and reactive oxygen species (Fig. 1; ref. 12). Recent studies have shown that these cells can differentiate from macrophages, as well as monocytes (13).

Resident tissue macrophages

A variety of tissue-specific cells of monocyte lineage exist, including microglia, osteoclasts, Kupffer cells, Langerhans cells, and alveolar macrophages. These cells acquire unique phenotypes in response to signals in the microenvironment and become highly specialized. For example, microglia are intimately involved in the regulation of neuronal patterning, whereas osteoclasts regulate bone remodeling (4). Peritoneal macrophages, now known to include two distinct subsets, represent another specialized population and play roles in bacterial phagocytosis (14). Although it was originally thought that these tissue-specific macrophages differentiated from hematopoietic bone marrow progenitors, lineage studies suggest derivation from embryonic yolk sac progenitors. These findings have challenged previous conceptions about resident macrophage populations, which were thought to be seeded in tissues throughout the body exclusively by bone marrow progenitors (4). Interestingly, resident tissue macrophages can proliferate in response to macrophage colony-stimulating factor (M-CSF), especially in the setting of inflammation (15).

Macrophages

Macrophages have an extremely complex transcriptome, reflective of their diverse and often tissue-specific functions

(16). Efforts have been made to categorize macrophages, but have failed to completely capture the complexity of their diversity. A frequently used, but now controversial system functionally categorizes macrophages into classically activated M1 or alternatively activated M2 phenotypes. The M1 phenotype is characterized by high levels of proinflammatory cytokines, has considerable tumoricidal activity, and is involved in the response of Th1 cells. M2 macrophages promote tissue remodeling and tumor growth and play immunoregulatory roles in coordination with Th2 cells. M1 and M2 phenotypes likely represent extremes in a continuum of macrophage activation, and have been reversed, highlighting their plasticity in response to environmental signals (17, 18).

Phenotypic M1 macrophages, henceforth referred to as proinflammatory macrophages, are induced via STAT (STAT1) signaling pathways through the response to INF-γ and activation of Toll-like receptors (TLR; ref. 19). IRF5, NF-κB, and AP-1 are upregulated and are essential for the induction of their characteristic proinflammatory cytokines and chemokines (Fig. 1; refs. 20, 21). These cytokines contribute to the proinflammatory properties of this phenotype and include TNF-α, interleukin (IL)-1β, IL-6, IL-12, type I INF, as well as reactive oxygen species (22). Arginine metabolism predominates, leading to the production of inducible nitric oxide. This subset also has elevated MHC II, facilitating interaction with Th1 cells (23).

In the M2 phenotype, henceforth referred to as proangiogenic macrophages, IL-4 and IL-13 activate STAT3 and STAT6, with resultant transcription of characteristic genes (e.g., *Mrc1*,

Fizz1, and *Chi3l3*; 24). NF-κB activity is reduced and the arginase pathway predominates, leading to the production of orinthine and polyamines (25). Phenotypic chemo- and cytokines, along with membrane receptors, are seen in Fig. 1.

Macrophages also secrete factors (e.g., VEGFA, VEGFC, and VEGFD, TNF-α, IL-8, and FGF-2) that directly contribute to angiogenesis in normal tissue development and tumor growth. (26). Macrophages differentially express VEGFR-1, VEGFR-2, and VEGFR-3, facilitating angiogenesis through interaction with their respective isoform ligands (26, 27). Subsets of macrophages express Tie2, an important regulator of angiogenesis. Through the secretion of proteinases, macrophages liberate growth factors, degrade the basement membrane, promote vessel sprouting, and facilitate endothelial cell migration.

Monocyte Subpopulations in Physiologic and Homeostatic Angiogenesis

Macrophages can be found in both murine extraembryonic and embryonic tissues by postcoital day 8 and have proangiogenic gene expression signatures, including Tie2 expression (28). In the retina, resident macrophages play critical regulatory roles in angiogenesis and programmed vascular regression through the canonical Wnt pathway, producing Wnt7b and signaling vascular remodeling (29). Macrophages are also important in the development of the hindbrain, where they chaperone the fusion of tip and stalk cells during vessel formation (28). During murine embryo implantation, depletion of uterine MDCs inhibits decidualization, resulting in termination of pregnancy as a result of defective angiogenesis in the absence of MDC-derived VEGF and TGF-β1 (30).

The significance of macrophages in development was demonstrated in the *Csf1op/op* knockout mice, in which most macrophage populations are absent. This model lacks the specialized bone-resorbing macrophages, osteoclasts, resulting in impaired hematopoiesis and compromised bone integrity. Decreased vascular anastomoses are seen in the hindbrain, as macrophages are not present to oversee vessel maturation. Remodeling deficiencies are present in the brain, mammary gland, kidney, and pancreas, pointing to the role of macrophages in branching morphogenesis and stromal patterning (13).

Macrophages and monocytes play a vital role in wound healing. CXCL12, also known as stromal cell–derived factor 1 (SDF-1), is released by vascular injury, and modulates the differentiation of circulating monocytes through CXCR4. CXCL12 is also a potent monocyte chemotactic factor, allowing the CXCL12–CXCR4 signaling axis to play roles in monocyte-driven angiogenesis through both chemoattraction and differentiation (31). Tissue injury quickly recruits circulating monocytes, which differentiate into proinflammatory macrophages in response to signals in the microenvironment, especially CCR2 (32). This initial inflammatory response initiates antimicrobial defenses through the production of radical oxygen species and the activation of Th1 cells. Subsequently, macrophages undergo a shift toward the developmental and proangiogenic phenotype, with the expression of Wnt ligands. Wnt5A stimulates endothelial cell proliferation and migration,

and upregulates Tie2 in macrophages and endothelial cells. TEMs are capable of polarization into a more proangiogenic phenotype in response to Ang-2, also released by endothelial cells, and oversee the fusion of migrating tip cells in angiogenic sprouting.

Macrophages are actively involved in recovery and reperfusion following ischemic injury and are recruited to the ischemic microenvironment by CCL2 released from endothelial cells. In rabbit models of hind limb ischemia, CCL2 infusion into the proximal end of the ligated femoral artery resulted in increased collateral artery formation as well as monocyte and macrophage infiltration (33). Injection of polarized macrophages improved reperfusion following induced ischemia in the mouse hind limb (34). In contrast, hind limb ischemia in macrophage-deficient *Csf1op/op* mice resulted in a 40% reduction in reperfusion recovery and decreased the formation of collateral vasculature (35). Similar patterns are seen in models of myocardial infarction.

Monocyte Subpopulations in Aberrant Angiogenesis

Subversion of macrophage-driven angiogenesis is implicated in many pathologic states, including proliferative retinopathy and reperfusion injury. Unlike the programmed angiogenesis occurring in development, signals in the pathologic microenvironment can trigger aberrant vessel formation through macrophage-dependent pathways. Disruption of Fzd4 and Fzd5 proteins, critical to the canonical Wnt pathway and macrophage participation in retinal development, is linked to familial exudative vitreoretinopathy and its characteristic abnormal vasculature (36). Macrophages are involved in the development of choroidal neovascularization the vision-threatening complication in retinal degenerative and immune disorders, including macular degeneration. Their depletion *in vivo* with clodronate produced smaller and less severe choroidal neovascularization lesions with decreased vascularity (37). Macrophages also play a role in atherosclerosis, where they are actively recruited into the vascular intima and subintima, take up oxidated low-density lipoprotein to become foam cells, and contribute to the plaques characteristic of peripheral and coronary artery disease (38). Although recruitment to the site of plaque formation is known, correlation between the density of macrophage recruitment and disease severity is not well established.

The factors that make macrophages essential for adaptive recovery to ischemia in the heart and limb can prove detrimental in other tissues. In murine and rat models of ischemic stroke, macrophages expand neuronal damage, mediated through IL-1β and TNF-α (39, 40). The benefits of therapeutic hypothermia following cerebral hypoxia or ischemia may occur through decreased free radical production and reduced infiltration of inflammatory cells, especially macrophages (41).

Monocyte Subpopulations in Tumor Angiogenesis

The ultimate subversion of macrophage-driven angiogenesis is seen in cancer. Tumor-associated macrophages (TAM) are abundant in established tumors and their presence is

associated with increased tumor invasion, migration, and poor clinical prognosis in 80% of solid tumors (42). TAMs differentiate from circulating monocytes actively recruited to the tumor microenvironment and are frequently biased to the benefit of tumor cells. Tumor and stromal cells produce numerous monocyte chemoattractants including CSF1, CCL2 (MCP-1), CCL3, CCL5, and placental growth factor (19). The interaction between tumor cells and macrophages has been studied in solid tumors of the abdominal cavity, which is rich in resident macrophages. In ovarian cancer models, stimulating peritoneal macrophages with thioglycolate significantly increased peritoneal metastases. This effect is abrogated by macrophage inhibition with intraperitoneal acetyl salicylic acid (43). $Csf1^{op/op}$ knockout breast cancer models show significant inhibition of tumor progression with nearly absent metastasis, whereas overexpression of Csf1 stimulated tumor growth and progression (42). In addition, hypoxia is a powerful monocyte and macrophage attractant and is widely present in established tumors. VEGF acts as both a potent monocyte chemoattractant and stimulator of angiogenesis (3).

Importantly, TAMs are responsible for the angiogenic switch governing the transformation to malignancy (4). Multiple studies have confirmed the correlation between TAM number and increased capillary density (4). Transcriptional profiling of TAMs reveals features similar to the proangiogenic phenotype, with high levels of IL-10, TGF-β, ARG-1, and the mannose receptor in conjunction with low levels of the proinflammatory markers IL-12, TNF-α, and IL-6 (3, 44). Macrophages respond to hypoxia by upregulating hypoxia-inducible factor-1α (HIF-1α) and HIF-2α, which facilitate the transcription of genes involved in angiogenesis (3). TAMs also upregulate Tie2, augmenting their proangiogenic polarization. Tie2-expressing macrophages are capable of vascular mimicry through the expression of endothelial cell markers and formation of capillary-like structures in response to VEGF, possibly paving the way for vessel maturation with replacement by true endothelial cells (45). Under the control of VEGF, monocytes can be educated to serve as transient building blocks for blood-vessel expansion (21).

Macrophage-secreted VEGF, largely stimulated through NF-κB, promotes vascular permeability, facilitating the extravasation of metastatic tumor cells. Furthermore, perivascular macrophages have been directly observed to facilitate tumor cell intravasation and thereby, hematogenous metastasis (46). TAMs may help tumor cells evade effective immune detection via the expression of immunosuppressive factors such as IL-10, TNF-β, PD-1 (programmed death-1), and biasing the immune response toward Th2 cells while suppressing CD8+ T-cell function (3, 47). STAT3, a dominant factor in polarization to the proangiogenic phenotype, suppresses dendritic cell maturation, impairing the priming of naïve T cells. Knockout of STAT3 in hematopoietic stem cells inhibits tumor growth, decreases metastasis, and enriches dendritic cell, natural killer (NK) cell, T cell, and neutrophil activity (19).

TAMs also modulate response to anticancer therapies. Although macrophages can increase tumor radiation sensitivity via the production of reactive oxygen species, they can also yield unfavorable effects (48). Following radiotherapy, CSF1 is upregulated, leading to the recruitment of CSF1R-expressing macrophages and enhanced tumor regrowth through the angiogenic avenues previously discussed. In a prostate cancer model, treatment with a CSF1R inhibitor improved response to radiation (7). TEMs are also implicated in postradiotherapy tumor regrowth following recruitment by CXL12 in response to hypoxia. After radiation, TEMs are found in close proximity to remaining tumor vasculature, suggestive of their role in vascular regrowth (7). Treatment with platinum increases the number of proangiogenic macrophages present in tumor (49). TEMs and macrophages are specifically implicated in the development of resistance to anti-VEGF therapy through their ability to activate proangiogenic pathways (5). Vascular regrowth following therapy-induced vascular injury is also directly linked to TEMs and macrophages (50).

Therapies Targeting Monocyte Subpopulations

Monocyte-driven angiogenesis is an attractive therapeutic target in cancer and other pathologic conditions, and methods to deplete or reprogram cells of this lineage are currently the subject of intense investigation. Bisphosphonates, clinically approved for the treatment of osteoporosis and bony metastases, are one modality currently being explored. Multiple studies have demonstrated the reduced risk of breast and colon cancers in patients receiving bisphosphonates (51). Furthermore, in patients with breast cancer, bisphosphonates reduce bone metastasis, and in those with prostate or renal cell carcinoma and preexisting bone metastasis, results in a trend toward increased survival (52). The clinical efficacy of bisphosphonates has been attributed to the inhibition of osteoclast activity, which contributes to the growth of solid tumors through the liberation of bone marrow–derived growth factors such as TGF-β and insulin-like growth factor (IGF). Importantly, they also directly induce apoptosis in TAMs, thus decreasing their infiltration and, as a result, decrease the proangiogenic factors that aid tumor growth and spread. In various mouse models of cancer, bisphosphonate treatment significantly reduced angiogenesis (53).

Trabectedin, a DNA-binding agent, has activity against cells of monocytic lineage. Selective blood monocyte cytotoxicity has been confirmed *in vitro*, with rapid decreases in CD45+ CD11+ CD115+ circulating monocytes after administration. TAM infiltration was reduced, resulting in significantly reduced tumor growth and metastasis, as well as decreased angiogenesis (54). Trabectedin mediates these effects through the induction of the extrinsic apoptosis pathway and demonstrates selectivity for cells of monocytic lineage, as other populations such as neutrophils and lymphocytes are unaffected. Leukocytes differ in their expression of signaling and decoy TRAIL receptors, where monocytes and TAMs express functional TRAIL receptors. In contrast, neutrophils and lymphocytes have decoy TRAIL receptors, imparting protection from trabectedin (54).

Because CSF1 is a potent cytokine in the recruitment of macrophages and monocytes and is unregulated in the tumor microenvironment, selective blockade of this pathway has been explored with the CSFR1 inhibitor, GW2580. In prostate

cancer models, CSFR1 inhibition reduced tumor regrowth following irradiation and reduced the infiltration of monocyte lineage cells (55). The small-molecule CSF1R kinase inhibitor, PLX3397, reduced TAM recruitment in parallel with decreased vascular density in breast cancer models and reduced tumor growth in prostate cancer models. In addition, treatment with PLX3397 improved chemosensitivity through enhanced CD8^{+} T-cell response (47). This compound is now in phase I/II clinical trials in a variety of cancer types, including glioblastoma, acute myeloid leukemia, and advanced solid tumors.

The CCR2 inhibitor, PF-04136309, depletes TAMs, reduces metastasis, and enhances chemosensitivity in pancreatic cancer models (56). Anti-STAT3 agents offer additional possibilities for macrophage modulation (56). Antibodies directed at Ang2 on TEMs resulted in the regression of tumor vasculature and decreased tumor progression in murine models of pancreatic and breast cancer (5).

Reprogramming of macrophages has been explored through the PD-1 pathway. PD-1, an inhibitory factor secreted by macrophages in the tumor microenvironment, is known to reduce CD8^{+} CTL activity and induce immune tolerance. PD-L1 blockade, in combination with whole tumor antigen vaccination, increased immune activity and facilitated tumor rejection through the stimulation of CD8^{+} T cells in ovarian cancer (57). Reprogramming macrophages to a tumoricidal phenotype has been accomplished through the administration of an agonistic CD40 antibody for "priming," followed by a "triggering" signal mediated through TLRs. This method of macrophage activation has produced tumor regression *in vivo* (58).

In addition, mechanisms to exploit the phagocytic capability of macrophages are promising. Tumor cells are known to express "don't eat me" signals, largely CD47 mediated. Tumor cell receptor CD47 interacts with signal regulatory protein α (SIRPα) on the surface of TAMs, inducing a powerful antiphagocytosis signal. CD47 inhibition produces frank tumoricidal responses from macrophages and anti-CD47 antibody therapies are currently in clinical development (58).

Finally, a rapidly emerging field of study uses monocyte- and macrophage-derived exosomes to achieve targeted drug delivery to the tumor microenvironment. Exosomes are endogenous nanovesicles capable of delivering biologic information between cells, and are readily expressed by macrophages. Recently, macrophages have been broken down into exosome-mimetic nanovesicles, which maintain the inherent targeting ability of the cell of origin through the preservation of plasma membrane proteins. These nanovesicles have been successfully loaded with various chemotherapeutic agents and track to the tumor microenvironment *in vivo*, resulting in decreased tumor growth without the adverse effects associated with the administration of free drug. (59)

Perspectives

Biologic understanding of monocyte subpopulations is quickly expanding. Transcriptional profiling has shed light onto the diverse origins of resident tissue macrophages and broadened a previously linear derivation from bone marrow progenitors. The plasticity of this lineage is highlighted by their capability to differentiate into many different cell types in response to signals from the microenvironment. Even as macrophages have long been recognized to play a role in angiogenesis, the contribution of cells such as TEMs and dendritic cells has only recently come into focus. Given their intimate involvement in vascular formation, targeting cells of mononuclear origin is a currently underdeveloped clinical tool with application in many pathologic states, especially cancer.

Despite the advances made, many areas remain unexplored and unexplained. Evidence suggesting different origins of recruited and resident macrophages provides the opportunity to target recruited macrophages, associated with pathologic angiogenesis and tumors, while leaving homeostatic resident macrophages intact. To exploit these differences, however, complete proteome and transcriptional profiling of macrophage subsets is needed. The strict phenotypic categorization of macrophages has been disproven, as macrophages express a variable range of phenotypic markers, even within the same tumor microenvironment. Polarization is reversible, but mechanisms allowing for polarization and reversibility are incompletely understood. Furthermore, there is a differential expression of receptors like TRAIL in TAMs, which modulates the response to some therapies (54). The signals in the microenvironment responsible for this differential receptor expression are not known, but could potentially be exploited for sensitization to receptor-dependent chemotherapeutic agents. Comprehensive understanding of these processes will provide new opportunities to harness the plasticity of this lineage for therapeutic gain.

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