Elusive Identities and Overlapping Phenotypes of Proangiogenic Myeloid Cells in Tumors

Seth B. Coffelt,* Claire E. Lewis,* Luigi Naldini,† J. Martin Brown,‡ Napoleone Ferrara,§ and Michele De Palma†

From the Academic Unit of Pathology,* Medical School, University of Sheffield, Sheffield, United Kingdom; the Angiogenesis and Tumor Targeting Research Unit,† and HSR-TIGET, San Raffaele Scientific Institute, and Vita-Salute San Raffaele University Medical School - via Olgettina, Milan, Italy; the Department of Radiation Oncology,‡ Stanford University School of Medicine, Stanford, California; and Genentech, Inc.,§ South San Francisco, California

It is now established that bone marrow–derived myeloid cells regulate tumor angiogenesis. This was originally inferred from studies of human tumor biopsies in which a positive correlation was seen between the number of tumor-infiltrating myeloid cells, such as macrophages and neutrophils, and tumor microvessel density. However, unequivocal evidence was only provided once mouse models were used to examine the effects on tumor angiogenesis by genetically or pharmacologically targeting myeloid cells. Since then, identifying the exact myeloid cell types involved in this process has proved challenging because of myeloid cell heterogeneity and the expression of overlapping phenotypic markers in tumors. As a result, investigators often simply refer to them now as “bone marrow–derived myeloid cells.” Here we review the findings of various attempts to phenotype the myeloid cells involved and discuss the therapeutic implications of correctly identifying—and thus being able to target—this proangiogenic force in tumors. (Am J Pathol 2010, 176:1564–1576; DOI: 10.2353/ajpath.2010.090786)

There is now compelling evidence that bone marrow–derived cells (BMDCs) play an important role in regulating angiogenesis during tumor progression and recovery after antiangiogenic or cytotoxic therapy. Myeloid-lineage BMDCs shown to be ‘proangiogenic’ in mouse tumor studies include monocytes/macrophages,1–7 dendritic cell (DC) precursors,8,9 mast cells,10,11 neutrophils,12–14 and the so-called ‘myeloid-derived suppressor cells’ (MDSCs, or CD11b+Gr-1+ cells).15–17 Such cells (see Table 12,3,5,9,15,16,18–31 for a glossary) are thought to promote angiogenesis in tumors largely by expressing factors that promote the growth and expansion of new blood vessels from the pre-existing vasculature—either by the direct stimulation of endothelial cells (ECs) or the remodeling of the extracellular matrix (reviewed in 32–37).

As will be seen, various experimental approaches have been used in mice to define the role of myeloid cells in tumor angiogenesis including their genetic manipulation or pharmacological targeting, sometimes in combination with BM transplantation (BMT). However, identifying the exact myeloid cell types involved in tumor angiogenesis is proving increasingly difficult, not only because considerable heterogeneity and functional redundancy exist among tumor-infiltrating myeloid cells, but also because these cell types often express overlapping phenotypic markers (Figure 1). In this review, we discuss the advantages and limitations of using different techniques and various markers to identify myeloid cells implicated in tumor angiogenesis as well as evaluate what they tell us about the identity and function of the cells involved.

In addition to ‘classic’ myeloid cells, other BMDCs have been implicated in tumor angiogenesis. These include various progenitor or precursor cell populations, such as hematopoietic stem/progenitor cells (HS/PCs),38,39 endothelial progenitor cells (EPCs),6,38,40–44 pericyte precursor cells,2,45,46 and mesenchymal stem/stromal cells.47,48 The many unresolved issues related to

Supported by the Breast Cancer Campaign and Yorkshire Cancer Research, UK (to S.B.C. and C.E.L.), Associazione Italiana per la Ricerca sul Cancro (AIRC) and the European Union (FP6 Tumor-Host Genomics; to L.N. and M.D.P.), and NIH grant RO1 CA128873 (to J.M.B.).

Accepted for publication October 15, 2009.

Address reprint requests to Claire E. Lewis, Ph.D., Academic Unit of Pathology, Medical School, University of Sheffield, Beech Hill Road, Sheffield, S10 2RX, UK; or Michele De Palma, Ph.D., Angiogenesis and Tumor Targeting Research Unit, and HSR-TIGET, San Raffaele Scientific Institute, and Vita-Salute San Raffaele University Medical School - via Olgettina, 58, 20132 Milan, Italy. E-mail: claire.lewis@sheffield.ac.uk or depalma.michele@hsr.it.

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the phenotypic and functional identification of some of these progenitor/precursor cells—EPCs, in particular—in the context of tumor angiogenesis have stimulated a long-standing lively debate in the vascular biology field (reviewed in 33,49–53). The functional importance of EPCs in tumor angiogenesis has been discussed elsewhere (see references above) and is beyond the remit of this article.
Figure 1. Various myeloid-lineage cell types implicated to date in the regulation of tumor angiogenesis. Markers expressed (or not expressed) by each of the distinct cell types are indicated. Note that several markers are coexpressed by different myeloid cell types, raising the question of whether some of these represent distinct cell types, rather than overlapping cell subsets or differentiation states of a same cell type.

Myeloid Cells Implicated in Tumor Angiogenesis

Monocytes/Macrophages

These cells are released from the bone marrow as promonocytes, mature into monocytes in the circulation, infiltrate tumors, and differentiate further into tumor-associated macrophages (TAMs). Once resident in tumors, macrophages express a wide array of proangiogenic factors, including vascular endothelial growth factor (VEGF)-A and matrix metalloproteinase (MMP)—9. Evidence for their role in tumor angiogenesis has been established by several different experimental approaches. First, the combination of human macrophages and small avascular human tumor nodules implanted into a murine skinfold window chamber assay results in a significant increase in new blood vessels compared with tumor nodules containing no macrophages. By using MMTV-PyMT mammary tumor-prone mice carrying a colony stimulating factor-1 (CSF1) null mutation (Csfl) null mutation, Lin and co-workers demonstrated that the absence of CSF-1 markedly decreased macrophage infiltration in pre-malignant tumors, and this, in turn, resulted in inhibition of tumor angiogenesis and delayed metastasis. Furthermore, inhibition of tumor-derived TAM chemoattractants, ablation of TAMs by DNA vaccination, or pharmacological neutralization of TAM-produced proangiogenic molecules also impaired tumor angiogenesis in various tumor models. It should be noted, however, that in Ccr2-deficient, K14-HPV cervical tumor-prone mice, the genetic depletion of TAMs unleashed a compensatory neutrophil response that rescued tumor angiogenesis and progression. This interesting observation highlights that a certain degree of functional redundancy exists among tumor-infiltrating proangiogenic myeloid cells and that this may vary in a tissue- or organ-specific fashion.

TAM depletion from tumors removes each of the many aspects of macrophage involvement from tumor progression. These include, in addition to the production of VEGF-A and other proangiogenic factors, the release of cellular mediators that promote immunosuppression and enhance tumor cell survival, migration, and invasion. To specifically analyze the role of myeloid cell-derived VEGF-A in tumor angiogenesis, Stockmann and coworkers crossed mice carrying loxP-flanked Vegfa alleles to mice carrying a lysis M (LysM) promoter-driven Cre recombinase. Interestingly, the authors found that the targeted deletion of Vegfa in myeloid cells failed to inhibit angiogenesis and tumor growth or decrease the overall amount of VEGF-A expressed in tumors. Rather, it attenuated the vascular abnormalities commonly observed in tumors and improved tumor perfusion (a phenomenon previously referred to as ‘vascular normalization’). These results suggest that VEGF-A production by TAMs promotes the formation of chaotic, poorly functional tumor-associated vasculature—at least in the murine tumor models used.

As defined here, TAMs comprise distinct macrophage subpopulations. Egeland and coworkers developed and applied multicolor imaging techniques (reviewed in 63) to analyze the recruitment and behavior of macrophages and related myeloid cells in different tumor microenvironments in live mice. MMTV-PyMT mice were crossed with a transgenic mouse line expressing GFP under the control of the promoter of the Csf1r gene, which is specifically expressed in monocyte/macrophage-lineage cells. The movement of GFP+ cells within tumors was then visualized along with fluorescently labeled dextran (to label blood vessels and macrophages that engulf dextran) and fluorescently labeled monoclonal antibodies (to better identify the myeloid cell subsets involved). Based on their motility, expression of surface markers (such as mannose receptor-1, Mrc1/CD206), and ability to phagocytize dextran, the macrophages could be classified into distinct subpopulations, including low-migratory Mrc1+/dextran+ macrophages, which are found in the peritumor areas, and sessile Mrc1+/dextran+ macrophages, which are found within the tumor mass. This real-time analysis of myeloid cell location, behavior, and gene expression within intact live tumors showed that the functional heterogeneity of tumor-infiltrating myeloid cells—macrophages in particular—may be greater than anticipated by the analysis of static markers on fixed tissues. In this regard, a recent study identified a distinct macrophage subpopulation that mediates metastatic cancer cell extravasation and pulmonary seeding in mouse tumor models.

Several recent reports have shown that both mouse and human monocytes can be grouped into different functional subsets. In murine blood, these include ‘inflammatory’ monocytes, which can give rise to several subsets of macrophages (possibly including TAMs) and DCs under inflammatory conditions; as well as ‘resident’ monocytes, which appear to patrol blood vessels and promote tissue remodeling, and vascular healing, possibly by differentiating toward tissue-resident proangiogenic macrophages. One particular subset of circulating monocytes, the Tie2-expressing monocytes (TEMs), has been shown recently to play an important role in tumor angiogenesis. TEMs express the an-
whether the different monocytic/macrophage subsets found in tumors represent distinct lineages or more plastic differentiation states, and whether they are related to each other by a precursor-to-mature cell relationship, is the object of current investigations.

Polymorphonuclear Cells

Granulocytes, neutrophils in particular, and mast cells are regarded a key source of proangiogenic factors in certain experimental tumors. An early study examining the importance of MMP9 and VEGF-A in pancreatic tumors of RIP1-Tag2 mice noted that inflammatory cells are the main producers of these two proangiogenic molecules in tumors.67 Neutrophils, together with monocytes/macrophages, have since been identified as the predominant source of MMP9 and other proangiogenic molecules in RIP1-Tag2 mice.12,13 Although neutrophils are found in lower frequency than macrophages in RIP1-Tag2 insulinomas, their elimination by means of anti-Gr-1 antibodies (which, however, also bind inflammatory monocytes; see below) in this tumor model reduces the levels of MMP9 in the tumors, which in turn inhibits the association of VEGF with VEGF receptor-2 (VEGFR-2) on ECs, thus suppressing angiogenesis. Interestingly, a recent study showed that neutrophils can either express a protumoral or antitumoral phenotype according to the levels of transforming growth factor (TGF)-β present in the tumor.68 Indeed, TGF-β blockade enhanced the recruitment of neutrophils with cytotoxic properties and inhibited the growth of transplanted tumors. Of note, the specific depletion of these neutrophils by means of anti-Ly6G antibodies rescued tumor growth. Thus, like TAMs,24 neutrophils may sense the tumor microenvironment and express different activation states, which determine their protumoral versus antitumoral activity in tumors. The antitumor activities of cytotoxic neutrophils include the expression of immuno-activating cytokines and enhanced capability of killing tumor cells in vitro; it will be worth investigating whether these “cytotoxic” neutrophils also display enhanced expression of antiangiogenic cytokines (or lower expression of proangiogenic ones) as compared with their “normal” counterpart.

MDSCs, often referred to as ‘CD11b+Gr-1+ cells,’ are a heterogeneous population of myeloid cells that encompasses immature monocytes, granulocytes, DCs, and their precursors. Their multifaceted function in tumors, phenotype, and complexity has been recently reviewed.18,19,69 The defining characteristics of MDSCs are largely based on their ability to suppress innate and adaptive immunity and expression of CD11b and Gr-1, whereas expression of other phenotypic markers by MDSCs varies because of their diversity and inclusion of myeloid cells at various stages of differentiation. Recent attempts at narrowing down specific subpopulations of these cells conclude that this group of cells can be divided into monocytic (mononuclear-MDSCs) and neutrophilic (polymorphonuclear-MDSCs) and DC subpopulations, which express phenotypic markers characteristic of each respective subset.70–73 There is also some evidence indicating that these subsets may have different functions in tumors,70 but the predominant phenotype and differentiation status of these cells once they migrate...
into premalignant tissue and tumors is unclear and is likely dependent on the tumor model and tumor type. Although MDSCs are believed to promote tumor progression through immunosuppression and other mechanisms, these cells (or subsets of them, such as neutrophils) may also influence angiogenesis—an effect mediated, at least in part, by their release of proangiogenic factors. In this regard, Shojaei and coworkers found that tumor reactivity to anti-VEGF therapy correlates with the marked accumulation of CD11b+ Gr-1+ myeloid cells (possibly neutrophils) within certain mouse tumors. This finding suggests that some tumors may co-opt VEGF-independent proangiogenic programs that are executed by the tumor-infiltrating myeloid cells. Prokinectin 2, also known as Bv8 (a VEGF-like proangiogenic factor), is upregulated in myeloid cells by tumor-derived granulocyte colony-stimulating factor and appears to be a major player in driving reactivity for anti-VEGF therapy. Yet, an immunosuppressive function has not been conclusively demonstrated for the proangiogenic CD11b+ Gr-1+ cells, raising the question of whether or not they can truly be referred to as MDSCs.

A number of experimental animal models have shown mast cells to be important for tumor angiogenesis. Mast cells accumulate during the premalignant stages of tumor progression and at the periphery of invasive tumors. They have direct proangiogenic activity attributable to their production of MMPs, particularly MMP-9, and secretion of other proangiogenic molecules such as basic fibroblast growth factor, VEGF, and IL-8. In addition, mast cells indirectly stimulate angiogenesis by secreting mast cell–specific serine proteases that activate pro-MMPs and stimulate stromal fibroblasts to synthesize collagens. Tumors grown in transgenic mice deficient in mast cells exhibit delayed tumor vascularization and progression. For instance, premalignant angiogenesis is abated in a mast cell–deficient skin tumor-prone K14-HPV transgenic mouse, as well as during pancreatic islet cell carcinogenesis, and melanoma progression, and adeno-matous colon polyposis.

**BM Transplantation in the Study of Tumor Angiogenesis**

A number of studies have used myeloablative BMT to investigate the role of BM-derived myeloid cell in tumor angiogenesis. In the majority of such studies, transgenic mice ubiquitously expressing the reporter gene (e.g., GFP or LacZ) are used as BM donors so the fate of their BM-derived cells after BMT can readily be identified and tracked in recipients. Such reports consistently document the accumulation of gene-marked myeloid-lineage BMDCs in tumors, but whether these cells represent monocytes, macrophages, mast cells, neutrophils, or other cell types (including hematopoietic progenitors) has often been ignored or not assessed in detail. In some studies, such gene-marking strategies have been combined with the use of cell type–specific antibodies on tumor sections in an attempt to identify distinct BMDC subsets in tumors (Table 2). These have shown the presence of macrophages, monocytes, and granulocytes among the transplanted BM-derived cells in tumors. It should be noted, however, that ubiquitously expressed transgenes (such as those regulated by the Rosa26 locus or CMV/β-actin promoter) may preclude the correct identification of each of the different BMDC types that are recruited to the tumor microenvironment. Indeed, transgene-positive macrophages, which massively infiltrate tumors, may mask the less abundant BMDC cell types and also increase the likelihood of detecting artifactual marking of nontransgenic cell types in the tumors. Mouse lines expressing transgenes under the control of myeloid-specific transcription regulatory elements (reviewed in 77) have been used to specifically label or deplete myeloid-lineage cells in mice. However, while narrowing down the range of BMDCs that express the transgene in blood and tumors, these transgenic mice may not always provide adequate resolution of the different myeloid BMDC types recruited to the tumors. Indeed, many of the “myeloid-specific” promoters (e.g., the LysM, Csf1r, CD11c promoters) are broadly active among myeloid-lineage cells.

Regardless of the precise identity of the cell types involved, several reports have demonstrated the importance of BMDCs in tumor angiogenesis by transplanting wild-type BM cells into angiogenesis defective and/or tumor-resistant mice. In this setting, donor-derived BMDCs were able to rescue, at least in part, tumor angiogenesis and growth in the mutant mice. Different mechanisms have been proposed that may account for the ability of BMDCs to rescue the genetically hampered angiogenesis; these include the paracrine support of angiogenesis (via release of proangiogenic factors) or the direct incorporation of the BMDCs in the tumor vasculature—the latter mechanism likely representing a very rare event. Coussens and coworkers used a BMT strategy to demonstrate that the expression of MMP9 by BMDCs is crucial for tumor angiogenesis and progression in the K14-HPV skin tumor-prone mice—a finding confirmed by further BMT studies in other mouse tumor models. Du and coworkers studied the recruitment of several BMDC types, including EPCs, pericyte precursor cells, and distinct myeloid cell subsets (CD11b+ or VEGFR-1+ myeloid cells, F4/80+ macrophages, and TEMs), to glioblastomas grown intracranially in mice previously transplanted with GFP-tagged BM cells. The authors found that glioblastomas unable to express hypoxia inducible factor (HIF)-1α recruit far fewer of these BMDCs and are severely impaired in their angiogenic phenotype and growth capacity. Indeed, in agreement with previous studies, HIF-1α expressed in the highly hypoxic glioblastomas up-regulates the expression of both VEGF and stromal cell–derived factor-1 (SDF-1, or CXCL12), which in turn promote the influx and retention of the various BMDCs in the tumor microenvironment. Among the tumor-infiltrating BMDCs, TEMs were found to be an important source of...
MMP9, possibly explaining why their impaired recruitment in HIF-1α−deficient tumors or genetic elimination in wild-type tumors impair angiogenesis and tumor growth. Chan and coworkers recently showed that knockdown of prolyl hydroxylase 2 (PHD2)—a molecular oxygen sensor and negative regulator of HIF-α subunits—in human colon carcinoma xenografts increases the number of tumor-associated CD45+ and CD11b+ cells and promotes angiogenesis. However, unlike the findings of Du and coworkers, they found that BMDC recruitment to tumors is HIF-independent. Indeed, PHD2 deficiency up-regulated the expression of the proangiogenic factors, IL-8, and angiogenin, in a NF-κB−dependent but HIF-independent manner; IL-8 and angiogenin were found to be important both for the recruitment of BMDCs and the direct stimulation of angiogenesis. Regardless of precise mechanisms involved, these studies highlight the important role of tumor hypoxia for the recruitment of proangiogenic BMDCs to the tumor microenvironment.

BMT studies performed in various mouse tumor models have shown that TEMs play a crucial role in tumor angiogenesis. TEMs were originally identified in the peripheral blood and tumors of mice transplanted with BM-derived HSPCs transduced ex vivo with a lentiviral vector expressing GFP from Tie2 promoter/enhancer sequences. The generation of Tie2-GFP transgenic mice further confirmed that TEMs constitutively circulate in the mouse blood and preferentially extravasate in tumors and regenerating tissues. Importantly, TEMs have also been identified in human peripheral blood and cancer. When TEMs carrying a Tie2-driven suicide gene are selectively eliminated after BMT into tumor-bearing mice, tumor angiogenesis is inhibited and tumor growth markedly slowed; of note, the specific TEM elimination in tumor models does not affect the recruitment of other BMDCs, such as TAMs and neutrophils, to the tumors. TEMs differ from the bulk of F4/80+ TAMs by their distinguishing gene signature and are enriched for genes that regulate tissue remodeling and angiogenesis.

The BMT strategies described above show how the angiogenic program in tumors can be heavily modulated by the recruited BMDCs. However, BMT experiments usually use total body irradiation to enable the full engraftment of donor-derived cells in the recipient mice. In this regard, Ahn and Brown have shown that tissue irradiation may significantly affect the composition and proangiogenic activity of myeloid cell infiltrates in such tissues. Indeed, preirradiation of tissue before tumor cell inoculation enhances the recruitment of subsets of BM-derived CD11b+ myelomonocytic cells in the growing tumors. The enhanced recruitment of these CD11b+ cells at the site of tissue irradiation facilitates, or even rescues angiogenesis after radiation-induced EC damage. Indeed, the authors showed that newly recruited BMDCs represent the sole source of MMP9 in such irradiated tissues. In addition to the important role of BMDC-mediated MMP9 release in tumor angiogenesis, this study provides circumstantial evidence that tissue irradiation may alter the physiological fluxes of BMDCs to tumors.

Two recent reports have investigated the recruitment of BMDCs to the tumors of mice surgically joined by parabiosis. In these experiments, a GFP-transgenic mouse was surgically joined to a wild-type mouse to create a shared circulation between the two mice. The contribution of the circulating GFP-positive cells to tumor angiogenesis in the wild-type mouse was then studied without the need to myeloablate (ie, irradiate) the host hematopoietic system. In both studies, a variety of gene-marked BMDCs (mostly of the myelomonocytic lineage) were observed in the perivascular areas of both xenografted and spontaneous tumor models, with little evidence, if any, for the incorporation of bona fide EPCs in the tumor blood vessels. Although parabiotic mouse models seem to confirm BMT studies, it remains a possibility that irradiation and subsequent BMT make tumor angiogenesis more dependent on the paracrine support provided by BMDCs than in nonirradiated hosts.

### Phenotypic Markers Used to Identify Proangiogenic Myeloid Cells in Tumors

As discussed above, to identify proangiogenic myeloid cells in murine tumors, investigators have used morphological criteria, examination of reporter gene expression, and/or assessment of various myeloid-related, phenotypic markers (see Table 2). However, as shown in Table 3, the most common phenotypic markers used to date—CD45, CD11b, CD11c, F4/80, Gr-1, Tie2, CXCR4 and VEGFR-1—are expressed by more than one myeloid cell type, making it difficult to distinguish one cell from another. CD45 (leukocyte common antigen) is a transmembrane protein broadly expressed by hematopoietic-lineage cells, including myeloid and lymphoid cells. CD11b (also known as MAC-1) is generally regarded as only expressed by myeloid cells. However, several reports documented the expression of CD11b by lymphocyte and NK cell subsets, depending on their activation status.

Together with CD11b, F4/80 is a cell surface glycoprotein regularly used to identify murine TAMs. Although broadly and robustly expressed by both tissue-resident and tumor-infiltrating macrophages, F4/80 expression level has been found to diminish with some forms of macrophage activation. It is also expressed, albeit at lower levels, by circulating monocytes and mononuclear-MDSCs. Subpopulations of DCs and peripheral blood eosinophils may express this antigen in tumors. Markers/antigens expressed by neutrophils alone have proven extremely elusive. Ly6G is a surface molecule expressed almost exclusively on neutrophils and their precursors, and anti-Ly6G antibodies may thus provide a specific means to deplete these cells in mice. Another antibody used in many studies to identify or systemically deplete neutrophils in mice is anti-Gr-1, which however recognizes both Ly6C and Ly6G surface antigens. Thus,
anti–Gr-1 antibodies not only bind Ly6G+/H11001 neutrophils and their BM precursors, but also Ly6C+/H11001 inflammatory monocytes,20,21 DCs, and T cell subsets.89 On the other hand, tumor-infiltrating TEMs and their circulating precursors are reported to be Gr-1–negative.5 As mentioned earlier, Gr-1 has also been used to help identify murine MDSCs (together with CD11b), so it cannot be used to distinguish between neutrophils and MDSCs.

Many markers once regarded as EC-specific (eg, VEGFR-1, Tie2, VE-Cadherin, and Sca-1) are also expressed by subsets of myeloid-lineage cells. VEGFR-1, a receptor activated by both VEGF-A and placental growth factor, is expressed by monocytes/macrophages and their progenitors in the hematopoietic system.90–92 The expression of the angiopoietin receptor, Tie2,56 has been reported on monocytes (TEMs),5 vascular leukocytes,31 and eosinophils,93 but not neutrophils,5 mast cells,94 or MDSCs.70 Interestingly, vascular leukocytes have been shown to also express the EC-specific adhesion molecule, VE-Cadherin.9,30 It should be noted that Sca-1, which is often regarded as only expressed by HS/PCs and ECs, is now known to be more broadly expressed in the hematopoietic system, as both myeloid and lymphoid cell subsets may express this molecule. Similarly, the SDF-1 receptor CXCR4 is broadly, albeit not uniformly, expressed by murine hematopoietic cells.36 Thus, CD11b−, F4/80−, Tie2−, VEGFR-1−, and CXCR4-expressing myeloid cells, which have been reported to be proangiogenic in tumors by several studies,1,2,5,6,38 may well represent overlapping rather than distinct cell subsets.

Additional studies are now required to better understand the phenotypic and functional complexity of myelomonocytic cells found in tumors. As an example, Pucci

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Table 2. A Summary of the Phenotypic Markers Used to Date to Determine the Contribution of BMDCs to Tumor Angiogenesis in Selected Publications

<table>
<thead>
<tr>
<th>Reference</th>
<th>Mouse/tumor model used</th>
<th>Experimental strategies (selected)</th>
</tr>
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<tbody>
<tr>
<td>1</td>
<td>MT1A2 murine mammary carcinoma or radiation-induced fibrosarcoma—in wild-type mice, Mmp9+/−/− mice, or Cd11b promoter-driven diphtheria toxin receptor (DTR)/GFP mice</td>
<td>Transplantation of Tie2-LacZ or GFP-positive BM cells into pre-irradiated, tumor-bearing mice. Transplantation of wild-type BM cells into pre-irradiated, Mmp9−/− tumor-bearing mice, also before treatment with zolendronic acid to target MMP9-expressing CD11b+ cells. Transplantation of Cd11b-DTR/GFP BM cells into Mmp-9−/− tumor-bearing mice before treatment with diphtheria toxin</td>
</tr>
<tr>
<td>6</td>
<td>TS/A and N202 mammary carcinoma, B16 melanoma, and Lewis lung carcinoma (LLC) cells inoculated into nude or syngenic C57BL/6 mice</td>
<td>Transplantation of lentiviral vector-transduced BM cells expressing GFP from Tie2 prom./enh. or ubiquitous promoters (CMV, Pgk)—into nude or immuno-competent tumor-bearing mice. Transplantation of lentiviral vector-transduced BM cells expressing GFP and thymidine kinase from Tie2 prom./enh. or ubiquitous promoter (Pgk)—before ganciclovir treatment of tumor-bearing mice to ablate TEMs or myeloid cells</td>
</tr>
<tr>
<td>5</td>
<td>Transgenic Tie2-GFP and Tie2-thymidine kinase (tk) FVB mice inoculated with N202 murine mammary cells; RIP1-Tag2 spontaneous insulinoma model; nude mice bearing orthotopic U87 human glioblastoma cells</td>
<td>Transplantation of Tie2-GFP–transduced BM into RIP1-Tag2 or U87 glioma-bearing mice Transplantation of Tie2-tk BM into N202 tumor-bearing FVB mice to ablate TEMs Transplantation of Tie2-tk BM into glioma-bearing nude mice to ablate TEMs</td>
</tr>
<tr>
<td>2</td>
<td>Rag1-deficient (Rag1ko) and Rag1ko/ Mmp9−/− mice inoculated with wild-type or HIF1α-deficient murine glioblastoma orthotopically</td>
<td>Transplantation of GFP-positive Rag1ko or Rag1ko, Mmp9−/− BM into Rag1ko or Rag1ko, Mmp9−/− glioma-bearing mice</td>
</tr>
<tr>
<td>25</td>
<td>Transgenic Tie2-GFP FVB mice inoculated with N202 murine mammary cells; PyMT-MMTV mammary tumor-prone mice</td>
<td>Transplantation of Tie2-GFP BM into PyMT-MMTV tumor-bearing mice</td>
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Table continues
et al recently compared gene expression in TEMs and TAMs from murine tumors along with spleen-derived CD11b+Gr-1+ MDSCs. Although TEMs were highly related to TAMs (but not to MDSCs), several genes were found to be differentially expressed in the two macrophage subpopulations, suggesting that TEMs represent a distinct subset or differentiation state of TAMs. Among these, Arginase1 (*Arg1*) and many scavenger receptors were up-regulated, whereas Nos2 and many proinflammatory molecules were down-regulated in TEMs versus TAMs (Table 2).1,2,5,6,25 These data have helped identify a TEM surface marker profile (F4/80−Gr-1−Tie2−Mrc1−CD11clow/−CD163low/Lyve1+TLR4−Stab1+), which distinguishes them from the bulk of TAMs (F4/80+Gr-1low−Tie2−Mrc1low−CD11clow/−CD163low−Lyve1low/−TLR4low−Stab1low−). Moreover, the data have identified molecular pathways that may account for the marked proangiogenic and protumoral activity of TEMs and might be targeted to block the activity of TEMs in tumors.25 Genome-wide expression profiling of the distinct myeloid cell types identified so far in tumors may unravel, together with live imaging analysis,62 novel and/or cell-type specific markers that better address their phenotypic and functional relationships.

Table 2. Continued

<table>
<thead>
<tr>
<th>Myeloid cell(s) Identified</th>
<th>Phenotypic markers expressed by myeloid cells in tumors after bone marrow transplantation</th>
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<tbody>
<tr>
<td></td>
<td>CD45</td>
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<tr>
<td>BM-derived myelomonocytic cell*</td>
<td>ND</td>
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<tr>
<td>Tie2+ cell (BM-derived, non-endothelial cell)</td>
<td>ND</td>
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<tr>
<td>TEM</td>
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<td>Macrophage</td>
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<td>TEM</td>
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<td>Macrophage</td>
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<td>Granulocyte</td>
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<tr>
<td>Mesenchymal progenitor (non-BMDC)</td>
<td>–</td>
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<tr>
<td>Macrophage</td>
<td>+</td>
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<tr>
<td>Myeloid cell</td>
<td>+</td>
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<tr>
<td>TEM</td>
<td>+</td>
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<tr>
<td>Hemangiocyte**</td>
<td>+</td>
</tr>
<tr>
<td>BMDC***</td>
<td>+</td>
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<tr>
<td>Pericyte progenitor cell (PPC)</td>
<td>−</td>
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<tr>
<td>TEM</td>
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</table>
| TAM | + | + | + | – | – | ND | ND | ND | ND | CD11c−, CD163−, Lyve1+TLR4−Stab1+), which distinguishes them from the bulk of TAMs (F4/80+Gr-1low−Tie2−Mrc1low−CD11clow/−CD163low−Lyve1low/−TLR4low−Stab1low−). Moreover, the data have identified molecular pathways that may account for the marked proangiogenic and protumoral activity of TEMs and might be targeted to block the activity of TEMs in tumors.25 Genome-wide expression profiling of the distinct myeloid cell types identified so far in tumors may unravel, together with live imaging analysis,62 novel and/or cell-type specific markers that better address their phenotypic and functional relationships.
Relevance of BMDCs to Human Tumor Angiogenesis

As mentioned earlier, a number of studies have reported significant correlations between the number of various myeloid cell types and microvessel density/angiogenesis in human tumors (reviewed recently in 32). However, similarities between the phenotype and function of the human tumor-infiltrating myeloid cell types and the proangiogenic BMDCs described earlier in mouse models have yet to be investigated. To date, human tumor studies have focused on just individual or small combinations of hematopoietic or myeloid cell markers. For example, increased numbers of CD45^+ cells are associated with high microvessel density in some human tumor types.95,96 CD11b^+ myeloid cells have been shown to be present in human tumors,27,97 but no studies to date have correlated their number or function with human tumor angiogenesis. Moreover, the Ly6C and Ly6G antigens are specific to the mouse, so the role of Gr-1^+ myeloid cells cannot be assessed in human tumors.

TAMs are usually identified in human tumors by their expression of CD6826,27^+ rather than F4/80. Indeed, the human homolog of F4/80, the EGF-like module containing mucin-like hormone receptor (EMR)—1, is expressed by eosinophils rather than monocytes or macrophages.98 Elevated numbers of CD68^+ TAMs have been correlated with increased vascularization in human tumors.99 VEGFR-1 expression has also been reported on TAMs in tumors.100 Ruan and coworkers101 reported that aggressive human lymphoma subtypes recruit more CD68^+ VEGFR-1^+ myeloid cells than indolent lymphomas. The CD68^+ VEGFR-1^+ cells were localized around tumor blood vessels, as well as in the stromal compartment of the tumor; interestingly, these cells expressed VEGF-A, suggesting that a paracrine cross talk between myeloid cells and blood vessels occurs in aggressive lymphomas. Macrophages also express CXCR4 in human tumors and ascites fluid,102,103 whereas information regarding CXCR4 expression on other myeloid cell types in tumors is lacking.

Monocytes/macrophages expressing CD14, CD11b, and TIE2, likely corresponding to the human counterpart of murine TEMs, have also been detected in human tumor biopsies.31,27 The human TEMs represent a minor proportion of the CD45^+ tumor-infiltrating hematopoietic cells but appear to be highly proangiogenic as they promote tumor angiogenesis when cocultured with human tumor cells in immunodeficient mice. Although TIE2 expression has been reported on human peripheral blood neutrophils by some groups and not by others,26,27,104 the existence of tumor-associated TIE2^+ neutrophils has yet to be reported. Detailed colocalization studies are now warranted to see whether BMDCs expressing all or most of the markers expressed by proangiogenic BMDCs in murine tumors are present in human tumors—and whether their presence, number, or pattern of distribution positively correlates with tumor angiogenesis. The use of human/mouse chimera approaches, such as the reconstitution of the BM of immunodeficient mice with human HS/PCs, could provide an experimental tool to investigate the contribution of human BMDCs to angiogenesis in xenografted human tumors. Whereas these mouse models can be used to track the fate of transplanted human HS/PCs, they unfortunately only allow for limited human myelopoiesis and thus may fail to reliably assess the contribution of human myeloid cells to tumor angiogenesis.

Concluding Remarks

The studies discussed above show that identification of the distinct myeloid cell types responsible for driving tumor angiogenesis remains a major challenge at the present time. Not only do different myeloid cells exhibit overlapping markers, but the exact level and combination of these may alter as they differentiate and respond to local signals in their tissue microenvironment. A classic example of the latter is the heterogeneity of macrophage phenotypes seen in tumors. This may either reflect the existence of developmentally distinct subpopulations, or the influence of signals in the tumor microenvironment like hypoxia and/or various cytokines on a common myelocyte precursor (or the combination of both). Moreover, the balance of these influences may differ between tumor types and indeed between tumors of a given type.
The type and degree of myeloid cell infiltration into each tumor may vary depending on the specific BM-mobilizing and -recruiting factors released, as each tumor type mounts its own protumorigenic microenvironment. In this regard, it is generally believed that the type and concentration of cytokines and growth factors expressed in the tumor microenvironment may shape the phenotype and function of TAMs. This concept is exemplified by the macrophage polarization paradigm proposed by some authors.\textsuperscript{23,24} According to this, TAMs are driven by certain tumor- and T cell–derived cytokines to acquire a polarized “M2” phenotype, which favors tissue remodeling, angiogenesis, and the suppression of antitumor immunity. Similarly, tumor-derived factors such as granulocyte colony-stimulating factor or TGF-β may drive neutrophils to acquire protumoral and proangiogenic functions.\textsuperscript{13,17,68} This implies that neutralizing certain tumor-derived molecules may blunt the proangiogenic and protumoral activities of macrophages and neutrophils, possibly by skewing them toward and antitumoral phenotype. On the other hand, recent studies have suggested that, at least in certain murine tumors, only a specific subset of the infiltrating macrophages, the TEMs, display a profoundly “M2” skewed phenotype, whereas another subset of TAMs retains features of antigen-presenting cells and produces lower amounts of proangiogenic factors.\textsuperscript{25} These findings also suggest that distinct macrophage subpopulations (ie, the TEMs and TAMs) may arise from distinct circulating precursor cells.

Given the plethora of new studies uncovering such information about the ontogeny and diversity of myeloid cells—how and how they are influenced by the tumor microenvironment—it remains to be seen whether future studies using cell markers alone will be able to unambiguously identify the major cell type(s) involved. As discussed above, it may take new approaches like the intravital imaging of myeloid cells in live murine tumors to clarify their functions in relation to the dynamic tumor microenvironment.\textsuperscript{62} Moreover, lineage-specific cell-tracking studies are needed to investigate the developmental relationships between BMDCs that traffic to tumors. For instance, by genetic labeling and then following of monocyte precursors, it may be possible to see whether distinct monocyte/macrophage subpopulations, such as the TEMs, TAMs, and “hemangiocytes,” are derived from a single, circulating precursor cell or separate ones.

One outstanding question still needs to be addressed: is it possible to identify molecular targets that could distinguish proangiogenic protumoral myeloid cells from the antitumoral myeloid cells that regulate effective immune responses against the tumor? If future studies identify one or more cell surface proteins that are expressed by all proangiogenic myeloid cells in tumors, it is possible that they could be targeted by antibody-based or gene therapeutic methods. Such a ‘broad-brush’ approach may be important, as there is now evidence that selective deletion of just one proangiogenic myeloid cell type (eg, macrophages) from murine tumors may promote another proangiogenic one (like neutrophils) to take on the role of driving tumor angiogenesis.\textsuperscript{14,72} These strategies may also have implications for the efficacy of conventional therapies as such proangiogenic BMDCs have been implicated in the responses of murine tumors to radiation or chemotherapeutic agents.\textsuperscript{1,16,58} Alternatively, subpopulations of such proangiogenic myeloid cells like TEMs can be used to deliver gene therapy to sites of neovascularization in tumors.\textsuperscript{105}

References

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