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## Regulation of Blood and Lymphatic Vessels by Immune Cells in Tumors and Metastasis

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### Abstract

Research over the last decades has provided strong evidence for the pivotal role of the tumor-associated blood and lymphatic vasculature in supporting immunoevasion and in subverting T cell-mediated immunosurveillance. Conversely, tumor blood and lymphatic vessel growth is in part regulated by the immune system, with infiltrating innate as well as adaptive immune cells providing both immunosuppressive and various angiogenic signals. Thus, tumor angiogenesis and escape of immunosurveillance are two cancer hall-marks that are tightly linked and interregulated by cell constituents from compartments secreting both chemokines and cytokines. In this review, we discuss the implication and regulation of innate and adaptive immune cells in regulating blood and lymphatic angiogenesis in tumor progression and metastases. Moreover, we also highlight novel therapeutic approaches that target the tumor vasculature as well as the immune compartment to sustain and improve therapeutic efficacy in cancer.

### Keywords

tumor blood and lymphatic vessels; angiogenesis; innate and adaptive immune cells; metabolism; antiangiogenic therapy; immunotherapy

## INTRODUCTION

Similar to developing and growing organs, tumors require blood vessels to access oxygen and nutrients. Tumors at their initial stage (i.e., in situ carcinomas) grow avascular and encapsulated so that a basal lamina separates the tumor mass from the peritumoral tissue. In this situation, blood vessels do not enter and are not present in the lesion (1, 2). These tumors can remain in this dormant state for decades. Indeed, the discovery of dormant tumors during the autopsies of individuals who died from nononcological causes reinforces the idea that actually only a subset of these lesions progress to a vessel-dependent state of

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exponential growth (3). When this occurs, a vascular network infiltrates the lesion, a process known as angiogenic switch, and the tumor undergoes a malignant transition: Cancer cells can now cross the vessel wall and exploit the hematic route to disseminate and reach distant organs where they form metastasis. Notably, however, the angiogenic switch can occur at different stages during tumorigenesis, depending on the tumor type and the environment (4). The onset of neovascularization is a multistep process that can occur by different mechanisms and is orchestrated by a wealth of activating and inhibiting factors whose balance will dictate whether endothelial cells (ECs) are in a quiescent or activated state (4–6). Sprouting angiogenesis is the most common and best-studied mechanism by which new vascular branches arise from preexisting capillaries or postcapillary venules. In addition, tumors also use additional routes of vessel expansion such as vasculogenesis, vascular mimicry, intussusception, and vascular co-option to cope with oxygen and nutrient demands during propagation (for a review, see 1).

However, although blood vessel formation is tightly regulated during physiological conditions, tumors have lost the appropriate balances between positive and negative angiogenic controls. Once tumor angiogenesis is induced, it remains activated, leading to a continually and abnormally expanding tumor vasculature (7, 8). Tumor blood vessels are far from being normal (9–11). This is due to (a) the loss of the appropriate balances between positive and negative angiogenic factors, which lead to excessive proangiogenic signaling, i.e., the physiological response to oxygen shortage, namely hypoxia; and (b) because angiogenic pathways are often downstream of oncogene activation. They are rather aberrant and leaky and have loose endothelial junctions, a discontinuous endothelial lining, and a defective basement membrane, with blind ends and scarce pericyte coverage. These features are ultimately all signs of poor vessel maturation and functionality, with the consequence that a tumor remains constantly hypoxic, which leads to a negative feedback loop whereby proangiogenic signals never stop. Dysfunctional vessels characterized by a poor blood flow ultimately end up forming bulging and thicker vessels, where clotting events and hemostasis are landmarks. It follows that tumors with high vessel density can be also very hypoxic and vice versa, depending on their vascular functionality and metabolic demand.

From a therapeutic point of view, the initial concept to starve the tumor to death, as it was proposed more than 40 years ago by Judah Folkman, has now been revised (9, 12). Strategies leading to nonproductive angiogenesis and tumor vessel normalization represent the opposite side of the coin. The former strategy was initially described when the inhibition of Delta-like 4 in ECs, releasing Notch-1-mediated lateral inhibition, displayed excessive, dysfunctional vessel sprouting (13, 14). Although some tumors grew slowly due to inefficient blood supply as the result of this non-productive angiogenesis, the approach was soon abandoned because Delta-like 4 blockade could lead to the formation of vascular neoplasms (15), and Notch-1 haploinsufficiency was associated with the formation of vascular tumors and lethal hemorrhage in mice (16). The latter, namely tumor vessel normalization, was first hypothesized by Rakesh Jain in 2005 (10) (Figure 1). The idea was that drugs that heal the aberrant vessels of the tumor can alleviate hypoxia and increase the efficacy of conventional therapies if vessel perfusion is reestablished. In addition, a normalized tumor vessel, with a smoothly aligned endothelium, continuous basement membrane, and well-covered pericytes, enables to a lesser extent cancer cells to sneak into the circulation and

metastasize to distant organs (17–19). The concept comes with some limitations because it is difficult to predict the precise regimen (dose and time window) that will lead to vessel normalization instead of vessel pruning. Indeed, the same strategy such as blockade of vascular endothelial growth factor (VEGF), given at different doses or at the same dose in different tumors, can elicit vessel disruption and then worsen hypoxia or vessel normalization, and thus, tumor reoxygenation (20). In this respect, accessible biomarkers that predict the outcome of antiangiogenic drugs are needed (11, 21).

Another important route for cancer cell dissemination is the lymphatic circulation. Cancer cell dissemination from lymphatic tumor vessels to regional draining lymph nodes is an important indicator of tumor aggressiveness for most human malignancies (22, 23). The primary function of lymphatics is not to carry oxygen or essential nutrients. They instead absorb extravasated proteinrich fluids, lipids, macromolecules, and immunocompetent cells from the interstitial spaces within tissues. Normal and functioning lymphatic vessels thus maintain plasma volume, prevent increases in tissue pressure, and allow leukocyte trafficking, thereby also playing a key role in the proper functioning of the immune system. In a tumor, the persistent activation of lymphangiogenic signaling pathways leads to dysfunctional lymphatic vessels, resulting in an increased tumor interstitial fluid pressure. Uncontrolled tumor interstitial fluid pressure impairs the uptake of therapeutics by the tumor but also promotes mechanical forces that trigger cancer cell proliferation and invasion (17, 24).

Although cancer cells are certainly an important source of angiogenic and lymphangiogenic factors, recruited leukocytes and all tumor-associated macrophages (TAMs) and tumor-associated neutrophils (TANs) play a key role in these processes at both the primary and metastatic sites. Here, we review the latest advances on how various immune cells, including macrophages, affect tumor angiogenesis and lymphangiogenesis.

## INNATE IMMUNE CELLS AND TUMOR BLOOD ANGIOGENESIS

Tumors, in part owing to their hypoxic and acidic nature, recruit a substantial amount of different innate immune cells that can comprise up to 30% of the entire tumor population. TAMs, monocytic or granular myeloid-derived suppressor cells (M-MDSCs and G-MDSCs, respectively), and TANs are most commonly found and often associated with increased intratumoral vessel density (25–27). Indeed, besides tumor cells and cancer-associated fibroblasts, myeloid cells become a pivotal source of growth factors and chemokines to promote angiogenesis (2, 28), as shown in multiple mouse tumor models of skin, cervical, breast, and brain cancers (25, 29–34). Because of their high plasticity, these cells can either convey proinflammatory or anti-inflammatory features, but in the tumor setting, they are commonly found to support immunosuppression and angiogenesis (26, 35, 36) (Figure 1).

While TAMs are generally protumoral [M2/T helper (Th)2-like], macrophage polarization toward a proinflammatory, antitumoral (M1/Th1-like) phenotype induced tumor blood vessel normalization in several preclinical tumor models or human tumors. This triggered an adaptive immune response against the tumor inhibiting cancer growth and metastasis; this synergized with the effects of standard treatment such as chemotherapy (37–39). One of the

first seminal studies underscoring the functional importance of TAMs in tumor angiogenesis was conducted in the endogenous mouse mammary virus polyoma middle T-antigen (PyMT) tumor model and then confirmed in other tumor model systems (35, 40, 41). VEGF-producing TAMs were sufficient to facilitate the angiogenic switch and the progression to malignancy. This is because inactivation of TAMs by blocking the CSF1/CSF1R pathway, broadly depleting TAMs by clodronate liposomes, or genetically deleting VEGF in macrophages, delayed the angiogenic switch, whereas genetic restoration of the macrophage population rescued the angiogenic phenotype (35, 40, 41).

TAMs and TANs also express various proteases, including the matrix metalloproteinase 9 (MMP9). MMP9 was shown to release and thus increase the bioavailability of extracellular matrix-sequestered VEGF, thus providing an alternative mechanism of VEGF-induced angiogenesis by innate immune cells in pancreatic, cervical, and brain tumor models (33, 34, 42). Blocking MMP9 by genetic or pharmacological depletion inhibited the angiogenic switch in all three tumor models. Tie2-expressing macrophages (TEMs) belong to a subgroup of TAMs that is often closely aligned to tumor vessels through EC expression of the Tie2 receptor ligand angiopoietin-2 (Ang2) (43). The number of TEMs correlates with vascular density in several tumor models and certain human tumors (44). Furthermore, selective ablation of TEMs by antibody-mediated neutralization of the Tie2 ligand Ang2 or by virtue of Tie2 promoter-driven thymidine kinase expression in mammary, pancreatic neuroendocrine, and brain tumor mouse model systems underscores their significant contribution in tumor angiogenesis (45, 46). Notably, early studies had revealed that hypoxia-induced Ang2 in concert with VEGF strongly induced the angiogenic switch in co-opted tumor vessels of glioblastomas (47).

Besides TAMs, neutrophils, or granule-containing cells, which are the most abundant white blood cells and the first cells to be recruited to injuries, produce factors that regulate angiogenesis. Neutrophils are normally the first to fight invading pathogens by several means including the generation of neutrophil extracellular traps (NETs), which are webs of fibers composed of chromatin and serine proteases that trap and kill extracellular microbes (48, 49). TANs, like TAMs, can exist as Th1 (N1) or Th2 (N2) polarized cells based on their anti- or protumor activity (29, 50). In tumors and metastases, neutrophils secrete proangiogenic factors and proteases similar to those of macrophages, most predominantly VEGF, fibroblast growth factor (FGF), and MMPs (28, 51, 52). Neutrophils contain granules with different compounds and factors. They can also harbor VEGF-enriched granules that are released upon tumor necrosis factor (TNF) stimulation *in vitro*, suggesting an alternative and fast route of VEGF availability to promote blood vessel growth (53). As described above, neutrophils could secrete MMP9 to liberate extracellular matrix-sequestered VEGF in dysplastic pancreatic islets of Rip1Tag2 mice that was sufficient to induce the angiogenic switch (42, 54), whereas pharmacological neutrophil depletion impaired the angiogenic switch in these pancreatic islet lesions (54). In addition, granulocyte colony-stimulating factor (G-CSF)-stimulated neutrophils upregulate the expression of Bv8 (prokineticin 2), which stimulates EC survival, migration, and proliferation but also functions as a chemoattractant for neutrophils, providing a positive feedback loop for neutrophil recruitment and activation (36).

Immature Gr1<sup>+</sup> immune cells in mice with either a mononuclear or granular morphology also convey immunosuppressive functions in tumors and were therefore named M-MDSCs and G-MDSCs, respectively (26). This is because MDSCs have predominantly been studied for their ability to suppress human CD3<sup>+</sup> and mouse CD4<sup>+</sup> or CD8<sup>+</sup> T cells (55). Most studies relating to their proangiogenic activities during tumor progression have not differentiated between neutrophils and MDSCs but solely depicted them as Gr1<sup>+</sup>CD11b<sup>+</sup> cells. What these studies have revealed so far is that tumor-associated Gr1<sup>+</sup>CD11b<sup>+</sup> cells display angiogenic properties and promote blood vessel growth in various tumor models partly by producing VEGF and MMP9 (56–58). In addition, they produce additional chemokines and cytokines such as CXCL1, CXCL8, interleukin (IL)-1b, and IL-6 that promote tumor neovascularization (51).

Taken together, all these heterogeneous innate immune cell constituents produce various but overlapping angiogenic mediators that control many aspects of vessel formation. The most prominent factors commonly found in these cells are growth factors and cytokines [e.g., basic fibroblast growth factor (bFGF)], tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) and - $\beta$  (TNF- $\beta$ ), platelet-derived growth factor, placental growth factor (PlGF), neuropilin 1 (Nrp1), CXCL chemokines (CXCL8, 12), semaphorins, and various proteases, including MMP2, MMP7, MMP9, and MMP14, as well as cysteine cathepsin proteases (33, 39, 42, 59–66).

Because distinct myeloid cells redundantly express these angiogenic factors, it is conceivable that innate immune cells may compensate for the loss of other myeloid subpopulations during progression and targeted therapy. In line with this concept, neutrophils can compensate for macrophages to support tumor angiogenesis in tumor-bearing CCR2 knockout mice (67). Targeting GR1<sup>+</sup> immune cells in pancreatic neuroendocrine tumors relapsing from anti-VEGF therapy did not further sensitize angiogenic inhibition because the enhanced recruitment of TAMs compensated for the lack of neutrophils and MDSCs (68).

It is important to note that not only innate immune cells but also adaptive immune cells can regulate tumor angiogenesis, although their specific implications still remain obscure. Like myeloid cells, B cells can directly promote angiogenesis by producing proangiogenic factors such as VEGF, FGF2, and MMP9 (69), or indirectly by polarizing macrophages to a Th2 immunosuppressive and proangiogenic phenotype in an immunoglobulin G (IgG)-dependent manner (70). On the other hand, T cells, dependent on the subtype, can negatively or positively control tumor angiogenesis. CD8<sup>+</sup> cytotoxic T cells and CD4 Th1 cells produce interferon- $\gamma$  (IFN- $\gamma$ ) that restrains EC proliferation and induces the production of angiostatic chemokines CXCL9, 10, and 11 in TAMs (28, 71). In contrast, regulatory T cells (Tregs) suppress IFN- $\gamma$ -expressing CD4 Th1 cells and secrete VEGF via hypoxia-induced CCL28, which both contribute to a proangiogenic tumor environment (72).

## IMMUNE CELLS AND LYMPHANGIOGENESIS

Strong evidence that TAMs are involved in tumor lymphangiogenesis is based on the observation that macrophage depletion in several tumor types abates the formation of lymphatic vessels (73, 74). TAMs can promote lymphangiogenesis by expressing VEGFC

and VEGFD that bind to VEGFR3 on lymphatic endothelial cells (LECs) (Figure 2). VEGFR3 activation leads to enhanced proliferation and survival of LECs by activation of protein kinase Akt, extracellular signal-related kinases Erk1 and Erk2, focal adhesion kinase, and NF- $\kappa$ B (75, 76). This process is stimulated by cancer cells that activate macrophage-derived lymphangiogenesis by producing IL-1 $\alpha$  in a highly specific manner (77). Studies in patients with stage 1 (thus in situ) squamous cell carcinoma showed that CD163<sup>+</sup> (alternatively activated) macrophages are recruited to the peritumoral, nonlesion skin, where they release VEGFC, which is linked to increased lymphatic density and reorganization (78). Complementing these findings, IL-8 was upregulated in squamous cell carcinoma compared to normal and adjacent nontumor skin, suggesting that this cytokine may be involved in TAM recruitment because firm adhesion of monocytes to inflamed ECs greatly depends on IL-8 (79).

This observation highlights the existence of cross talk between squamous cell carcinoma and macrophages in driving progression toward malignancy. In vitro evidence further supports the communication between cancer cells and macrophages during the lymphangiogenic process (Figure 2). Zhang et al. (80) showed that Lewis lung carcinoma cells induce alternative activation of cocultured macrophages; these in turn induced VEGFC expression in cancer cells. The induction of VEGFC transcription, production, and release by TAMs has been ascribed to TNFR1. TNF- $\alpha$ -overexpressing tumors display augmented density of both lymphatics and blood vessels. VEGFR3-blocking antibodies or the replacement of wild-type TAMs with TNFR1-deficient TAMs inhibited TNF- $\alpha$ -induced lymphangiogenesis and lymphatic metastases to lymph nodes without affecting TNF- $\alpha$ -stimulated angiogenesis. This emphasizes the importance of TNF- $\alpha$  stimulation of TAMs in the induction of VEGFC and the following activation of VEGFR3 on LECs (81). Interestingly, a study in cervical cancer patients shows that the fraction of TAMs that mostly release VEGFC (and VEGFD) also express VEGFR3 on the cell surface (thus sharing a marker with LECs). Their VEGFR3-positive monocyte progeny did not produce VEGFC unless stimulated with TNF- $\alpha$  [as in the study by Ji et al. (81)] or with the VEGFR3 ligand VEGFD (75). This suggests that VEGFR3 on monocytes and TAMs can initiate a positive loop to foster the production of its cognate ligands VEGFC and VEGFD that in turn work in a paracrine manner on LECs.

However, VEGFR3 is not always found in all tumor types in either mouse or human TAMs (82, 83). Besides VEGFC and VEGFD, TAMs also secrete VEGFA, which is more characterized for its role in angiogenesis, although this factor also plays an important function in lymphangiogenesis. First, VEGFA recruits TAMs mostly via the activation of VEGFR1 on macrophages (82, 84), but it also directly induces the proliferation and migration of LECs via VEGFR2 activation (85). VEGFA also promotes tumor and peritumoral lymphangiogenesis (86) as well as sentinel lymph node lymphangiogenesis in a model of chemically induced skin carcinogenesis (87). In addition to their release of lymphangiogenic growth factors, TAMs regulate lymphangiogenesis indirectly by the production of enzymes, such as MMPs, plasmin, and urokinase plasminogen activator, that contribute to matrix remodeling and growth factor activation (88). Similar to what has been previously described for TEMs in the process of tumor blood vessel formation (46, 89), perilymphatic macrophages might support the emerging lymphatics so that only a small



fraction of TAMs that reside in close proximity to the vessels is relevant for lymphangiogenesis (M. Mazzone, unpublished data). Once in the perilymphatic space, TAMs sustain lymphangiogenesis but also lymphatic metastasis by fostering cancer cell intravasation (90, 91). A study in breast cancer patients has revealed that TEMs are associated with lymphatic vessels in the tumor but not in the peritumoral tissue. Importantly, while TEMs within the tumor express lymphatic markers such as LYVE-1, podoplanin (PDPN), VEGFR3, and PROX-1, myeloid cells in the non-neoplastic tissue did not, suggesting that a phenotypic switch is impinged by the tumor microenvironment (92). Isolated TEMs were angiogenic and lymphangiogenic in vitro and expressed high levels of VEGFA, VEGFC, and VEGFD. In vitro blockade of VEGF receptors and Tie2 substantially impaired their (lymph)angiogenic potential, thus indicating the role of these pathways. A close look at the association of TEMs with lymphatic vessels revealed that elongated cells may be either very proximal or even integrated into the vessel wall. Macrophages proximal to the lymphatics not only sustain lymphangiogenesis but also promote cancer cell intravasation (92). An in vitro study showed that IL-1 $\beta$  released by perilymphatic macrophages can contribute to this step (91); however, many other factors can be involved in this process in vivo.

Although the paracrine communication of TAMs with LECs is well recognized (93–95), the physical contribution of macrophages (or myeloid cells in general) to the vessel wall during pathological lymphangiogenesis is a matter of debate (96). A previous study shows that macrophages can form lymphatic vessel-like structures that are positive for LYVE-1, PROX-1, and PDPN (97). Yet, in vitro cultured monocytes were shown to acquire endothelial markers such as CD31, VE-cadherin, and Tie2 (98); however, the in vivo relevance of this observation is uncertain (99). In the lymphangiogenesis field, however, there is still an open debate about the possibility of perilymphatic TAM integration. Analysis of the literature suggests that macrophages can also transdifferentiate in vitro into vessel-like structures, an action accompanied by downregulation of hematopoietic markers such as CD45 and CX3CR1 (100, 101). Similarly, in the Rip1Tag2 mouse model of insulinoma and in the TRAMP-C1 prostate cancer transplantation model, F4/80<sup>+</sup> LYVE-1<sup>+</sup> TAMs directly integrate into lymphatic vessels and presumably lose their macrophage features upon integration (96). Because no cell fusion events between macrophages and LECs were detected by genetic tracing experiments, the underlying idea is that TAMs can transdifferentiate into LECs. In a different context, Maruyama et al. (97) provided evidence that transplanted CD11b<sup>+</sup> macrophages infiltrate the corneal stroma and transdifferentiate into LEC clusters that join existing lymphatic vessels.

On the contrary, He et al. (102) demonstrated that genetically marked bone marrow-derived cells do not incorporate into lymphatic vessels during subcutaneous Lewis lung carcinoma, melanoma, or VEGFC-induced lymphangiogenesis. A limitation of all these studies is that either transplantations into the cornea (97) or irradiation of recipient mice (102, 103) were applied before evaluating the physical contribution of macrophages to the vessel wall, thus leading to a nonphysiological perturbation of the host and to hyperinflammatory conditions. Finally, the possibility that this process is context and tumor dependent may hold true. Overall, the proposed transdifferentiation requires further investigation and confirmation in vivo. Finally, adaptive immunity still plays a poorly characterized role in the formation of

tumor lymphatics. Although tumor lymphangiogenesis was recently shown to promote T cell infiltration and potentiate immunotherapy in melanoma (104), the reverse cross talk—or how T cells regulate lymphangiogenesis in the context of cancer—is not well understood. It has been reported that inhibition of Th1, Th2, or Th17 cytokines increases VEGFA and VEGFC expression and, thus, lymph node lymphangiogenesis in a mouse model of inflammation (105). Mechanistically, the absence of T cells induced hypoxia-inducible factor 1 alpha (HIF-1 $\alpha$ ) in macrophages, which in turn enhanced VEGFA and VEGFC levels (105). A more recent study (using a model of tail lymphatic disruption) shows that upon lymphatic injury, CD4<sup>+</sup> T cells get activated into a mixed Th1 and Th2 phenotype by dendritic cells in the regional lymph nodes and then migrate to the injury site to initiate lymphedema pharmacological inhibition of T cell release from the lymph nodes or genetic depletion of CD4<sup>+</sup> cells. This resulted in reduced lymphedema, suggesting that CD4<sup>+</sup> T cells impair lymphatic function after lymphatic injury (106). Another mechanism whereby T cells negatively regulate lymphatic function and lymphangiogenesis is through IFN- $\gamma$  secretion, leading to the suppression of lymphatic-specific genes in LECs and consequently causing marked reduction in lymph node lymphangiogenesis (107). Thus, inflammation elicits a T cell-dependent, self-limiting response that dampens T cell trafficking. Instead, the role of T cells and adaptive immunity in the regulation of tumor lymphangiogenesis and lymphatic metastatic spread is completely unstudied and will require thorough investigation in light of the latest focus of cancer biology on T cell-mediated immunotherapies.

## MACROPHAGE-INDUCED LYMPHANGIOGENESIS IN RESISTANCE TO THERAPY

Another important aspect in cancer biology is how tumors escape the deleterious effects of chemotherapeutic drugs and irradiation, thus leading to therapy resistance. Starting from the observation that healthy mice treated with paclitaxel, FOLFOX, or gemcitabine (but not cisplatin) and breast cancer patients after paclitaxel chemotherapy showed increased levels of VEGFC, Alishekevitz et al. (108) showed that chemotherapy-educated macrophages secrete cathepsin B that cleaves proheparanase into its active form. In turn, heparinase induced VEGFC expression by TAMs and thus endorsed tumor lymphangiogenesis and lymphatic metastasis. The induction of the cathepsin B-mediated cascade is ascribed to a population of VEGFR3<sup>+</sup> TAMs in tumors (108). This is in favor of a positive feedback loop, where VEGFC enhanced its own signaling through TAMs. Importantly, based on these results, chemotherapy would presumably introduce the risk of fostering metastasis, whereas the combination of paclitaxel and VEGFC/VEGFR3 blockade would both directly inhibit lymphangiogenesis and block the prometastatic activity of macrophages. This was indeed the case in tumor-bearing mice treated with chemotherapy (108). Similarly, radiotherapy stimulated cancer cells to produce higher levels of CSF1, resulting in the enhanced infiltration of (lymph)angiogenic myeloid cells into the tumor site (109). More data are required to corroborate these findings in patients. Nevertheless, these studies highlight how TAM recruitment following a wound repair situation as it occurs after chemotherapy or radiation therapy can lead to treatment failure and/or resistance. Thus, TAM-targeting agents should be tested in combination with a specific type of chemotherapeutic drugs or irradiation regimens.



## METASTASIS-ASSOCIATED MACROPHAGES IN ANGIOGENESIS

Although angiogenesis at the primary tumor site has been studied for more than 50 years, much less attention has been paid to the molecular mechanisms and cellular players controlling the angiogenic switch in metastasis. A considerable amount of work has proven that TAMs take part in each step of cancer growth and tumor angiogenesis (110, 111), but much less is known about the distinct role of metastasis-associated macrophages (MAMs). Several studies demonstrated that myeloid cells, and in particular MAMs, are important for the preparation of the metastatic niche via the release of matrix proteins at the metastatic sites. For this reason, these cells are also entrained by the primary tumor into the premetastatic niche before the lodging of cancer cells (112–115). In breast cancer, the release of CCL2 (also known as monocyte chemoattractant protein 1, MCP1) by cancer cells and stromal cells in the lung is important to recruit macrophages to the parenchymal tissue (116). Upon CCL2/CCR2 interaction, CCL3 is released by the same macrophages. In an autocrine manner, CCL3 binding to CCR1 and its activation enhance interaction of MAMs with metastasizing cancer cells, at least in part, through the engagement of integrin  $\alpha 4$  (117). As a result, prolonged MAM retention enhances extravasation of cancer cells and therefore metastasis via macrophage-borne VEGFA that loosens the endothelial junctions and allows cancer cell extravasation (116). These MAMs are enriched for the expression of VEGFR1. Activation of this receptor in MAMs has been shown to be important for metastatic growth but not for cancer cell extravasation (116, 118). Indeed, VEGFR1 blockade was able to decrease the total metastatic burden and the average size of the lesion despite an unchanged number of metastatic nodules (119). One possible explanation for these observations is that MMP9 is downstream of VEGFR1 activation. MMP9 activity released by MAMs (and to a minor extent by cancer cells or other stromal cells) sustained the angiogenic growth in metastatic lesions (118). Together with MMP9, the coexpression of CSF1 (also known as macrophage colony-stimulating factor, M-CSF), another VEGFR1 downstream effector, is required for metastatic growth (119), likely because CSF1 is a key cytokine for macrophage function, survival, proliferation, and differentiation (120). Interestingly, we have shown that under physiological conditions, CSF2 (also known as granulocyte-macrophage colony-stimulating factor, GM-CSF) keeps the levels of caveolin-1 elevated in interstitial macrophages of the lungs; thus, it follows that caveolin-1 downregulates VEGFR1 exposure on the membrane of these macrophages, likely through the formation of caveolae (119). Together, macrophage-associated caveolin-1 is critical for restraining metastasis. It represents an intrinsic antimetastatic surveillance mechanism in the pulmonary microenvironment, whereby its upregulation prevents excessive exposure of VEGFR1 at the cell surface and thereby limits downstream MMP9 and CSF1 expression, angiogenesis, and finally metastatic growth (118). Because the lung has the physiology to encounter dangerous signals from the air, it is not surprising that blocking the metastasis by this axis in macrophages was seen in the lung but not in the liver (118). Further effort will be required to understand how the prometastatic axis represented by the CCL2/CCR2–CCL3/CCR1 axis in MAMs is specific for breast cancer and lung metastasis or whether this pathway is also observed in other tumors and/or metastatic sites.

Elegant work by Mazziere et al. (46) has shown that Ang2 antibodies not only inhibited primary tumor growth and metastatic dissemination of a breast cancer mouse model but also directly suppressed the progression from a micro- to a macrometastatic stage independently of primary tumor growth. This was, at least in part, ascribed to the fact that at both the primary tumor and metastatic sites, Tie2 expression by TEMs is instrumental to sustain their association with sprouting vessels in response to Ang2 expression by blood endothelial cells (BECs). Mirroring these findings and using a different mouse model, Srivastava et al. (121) have proven that Ang2 reduced the growth of preseeded metastases by decreasing vessel density and increasing pericyte coverage at the metastatic sites in an adjuvant setting (after primary tumor resection).

Interestingly, a combination of Ang2 and low-dose chemotherapy completely regressed metastasis in a model that is refractory to maximal doses of paclitaxel. Mechanistically, Ang2 signaling in ECs is able to elicit an inflammatory response via endothelial production of CCL2 that recruits CCR2<sup>+</sup>Tie2 MAMs and indirectly via endothelial expression of adhesive molecules such as ICAM1, which is instrumental for inflammatory cell intravasation (121). Given the observation that anti-Ang2 antibodies also sensitized anti-VEGF treatment in metastatic lesions that were generally resistant to anti-VEGF alone, it is relevant to note that in this context, blocking Ang2 shielded the effects of Bv8 on ECs (121) [Bv8 having been released by CD11b<sup>+</sup>Gr1<sup>+</sup> myeloid cells and conferring resistance to VEGF-targeted therapies (122, 123)]. Further investigation is needed to understand which inflammatory pathways are specifically important for the angiogenic switch in micrometastasis, whether these are the same as for the metastatic growth of existing lesions, and whether these targets can be translated into human cancers.

Although we have started to gain a better understanding of the implication of macrophages in angiogenesis at metastatic sites, we still know very little about the contribution of all other immune cells (and especially cells of adaptive immunity) in this process. Studies in mice using sarcoma, melanoma, and pancreatic cancer models have all pointed to CD4<sup>+</sup> T cells and/or CD8<sup>+</sup> cytotoxic T cells as the main executors of metastatic dormancy (124–126). However, to which extent this depends on blood vessel expansion and angiogenesis remains to be elucidated.

## **INFLAMMATION, HYPOXIA, AND METABOLISM IN THE CONTEXT OF TUMOR ANGIOGENESIS**

When inflammatory cells infiltrate into tumors, they encounter different oxygen tensions fluctuating from 60 mmHg (i.e., 8% oxygen) to almost anoxic conditions (almost no oxygen), depending on the tumor type. Yet, the oxygen tension of most tumors varies from anoxia to 7.5 mmHg (i.e., 1% oxygen), a condition known as hypoxia (127). In addition, immune cells also face an acidic tumor environment due to the increased anaerobic glycolysis of tumor cells. Thus, low oxygen tension and different metabolic fingerprints of specific cancer cell types will greatly affect metabolite availability and cause metabolic restrictions (128–130). Oxygen and metabolite availability can thus define what is coined a metabolic niche. It follows that TAMs (and other immune cells) display specific alterations

in metabolic gene expression because they are forced to adapt their metabolism in relation to the metabolic niche they encounter (38, 128–132). The questions of how these different niches are causatively linked to a phenotypic shift of inflammatory cells and how this impinges on tumor progression have gained much attention in the last few years. As the focus of this review is on how cancer inflammation controls angiogenesis, we describe how differing metabolite and oxygen availabilities can affect the angiogenic properties of TAMs and other immune cells within the tumor.

### Control of Inflammation by Hypoxia in the Process of Tumor Angiogenesis

Several mechanisms can drive myeloid cell accumulation into the hypoxic niche of the tumor. For example, HIF-1 $\alpha$  stabilization under low oxygen tension promotes the transcriptional induction of CXCL12 (also known as SDF1) (133) and its receptor CXCR4 (134) in ECs (133) and myeloid cells (33), respectively, thus allowing recruitment and retention of bone marrow–derived angiogenic cells (33). In a similar way, the ligand for Tie2, Ang2, is produced by angiogenic tumor vessels and is a chemoattractant for TEMs. Hypoxia upregulates Tie2 expression on TEMs and Ang2 binding to Tie2 to downregulate their antitumor functions and promote their proangiogenic functions (46, 135). Depleting TEMs or silencing Tie2 in macrophages greatly abates the angiogenic sprouting of several tumors (46, 99). Hypoxic cancer cells and stromal cells also upregulate semaphorin 3A (Sema3A) that engages neuropilin 1 (Nrp1)<sup>+</sup> TAMs into the hypoxic niche via a VEGFR1/PlexinA1/PlexinA4 signaling platform. Once in the hypoxic niche, hypoxia-driven Nrp1 downregulation in TAMs will retain them in loco via a PlexinA1/PlexinA4 pathway, therefore countering external migratory signals (136). Here, TAMs become angiogenic and immunosuppressive (82, 126, 137). Nrp1 genetic knockout or knockin in macrophages of an Nrp1 form that does not signal through Sema3A strongly reduces angiogenesis, promotes cytotoxic T cell responses, and reduces tumor growth and metastasis in mouse pancreatic, lung, and breast cancer models (138). Similar findings were confirmed by Miyachi et al. (139) in gliomas, where both genetic knockout of Nrp1 in microglia and macrophages and systemic pharmacological inhibition of Nrp1 via a compound named EG00229 had a strong antitumoral effect via the reshaping of the inflammatory response. Human mast cells have also been shown to express Nrp1 (as well as Nrp2, VEGFR1, VEGFR2, Tie1, and Tie2) and to release a large array of angiogenic and lymphangiogenic molecules such as VEGFA, VEGFC, and VEGFD (140). Immunologically activated human basophils selectively produce VEGFA but not VEGFC and VEGFD. However, they also produce Ang1 that activates Tie2 on human mast cells or on BECs and LECs, promoting a mast cell–mediated cascade that leads to indirect or direct tumor angiogenesis and lymphatic angiogenesis (140–142).

Although hypoxia-induced release of growth factors, such as VEGF, and of chemokines, such as CXCL12 and CCL2, is important for the recruitment of specific monocyte subsets (127, 143, 144), the effect of hypoxia on the macrophage phenotype is less understood. This may be because of the complex interaction between cell autonomous signaling pathways induced by hypoxia in macrophages and their response to hypoxia-induced stimuli coming from neighboring cancer and stromal cells (127, 144). It is well established that HIF-1 $\alpha$  has an important role in the phenotypic response to hypoxia. HIF-1 $\alpha$  positively controls diverse

inflammatory responses by promoting glycolysis and energy production in myeloid cells at the inflammatory hypoxic site (145). As a consequence, macrophages display reduced activation and motility when HIF-1 $\alpha$  is genetically deleted (145). However, whether metabolic changes in HIF-1 $\alpha$  knockout macrophages impinge on cancer progression and tumor angiogenesis is unknown. Instead, it has been shown that TAMs react to hypoxia by increased expression of HIF-1 $\alpha$ -mediated proangiogenic genes such as VEGFA (40, 132, 146). VEGFA release by TAMs is responsible for tumor blood vessel dysfunction and abnormalities and the consequent increase in tumor hypoxia. This has been shown by the genetic deletion of VEGF in myeloid cells, which results in tumor vessel normalization and restores tumor perfusion, thus resulting in a better response to chemotherapy (40). Besides its induction of VEGF, HIF-1 $\alpha$  in TAMs mediates the suppression of adaptive immunity, as the loss of HIF-1 $\alpha$  in myeloid cells directly abrogates hypoxia-induced suppression of T cell activation in an inducible nitric oxide synthase (iNOS)-dependent manner (146).

Although hypoxic TAMs upregulate both HIF-1 $\alpha$  and HIF-2 $\alpha$  (146, 147), it seems clear that HIF-1 $\alpha$  has an impact only on the expression of angiogenic (and metabolic) genes (40, 132, 146), whereas hypoxia-induced HIF-2 $\alpha$  has a different function (148). Myeloid cell-specific loss of HIF-2 $\alpha$  in murine hepatocellular and colitis-associated colon carcinoma models reduces TAM infiltration due to impaired expression of CXCR4 and CSF1R. This alteration is linked to a drop in cancer cell proliferation and tumor progression, while blood vessel density is unchanged (148). Additional data and the analysis of different tumor models are required to support a general idea that HIF-2 $\alpha$  is more relevant for the recruitment of TAMs and their effect on cancer cells, whereas HIF-1 $\alpha$  is more related to angiogenic and immune functions of TAMs. Based on a noncancer study, we reported that in conditions of muscle or cardiac ischemia, the downregulation of the HIF-prolyl hydroxylase PHD2 by genetic deletion of one allele or by activation of Tie2 signaling in macrophages increases NF- $\kappa$ B activity that leads to the transcription of CXCL12 and platelet-derived growth factor B (149, 150). These are at least partly responsible for the recruitment of mural cells around blood vessels and thus for the arteriogenic process (149, 150). However, we could not observe a difference in tumor vessel coverage when analyzing myeloid cell-specific *Phd2*-haplodeficient mice (17), supporting the notion that different macrophage subsets in different tissues can rely on alternative pathways in support of their angiogenic activity.

HIF-1 and HIF-2 are not only stabilized by hypoxia but also by paracrine stimuli and signaling pathways that involve kinase, phosphatases, and other interacting molecules (151–153). It has been shown that mainly PI3K $\gamma$  and partly PI3K $\delta$  elicit AKT activity in TAMs, which leads to HIF-1 $\alpha$  and HIF-2 $\alpha$  accumulation and the production of several angiogenic factors. Loss of PI3K $\gamma$  in TAMs was sufficient to induce, also in hypoxia, HIF-1 $\alpha$  and HIF-2 $\alpha$  degradation via the 26S proteasome pathway, which resulted in reduced growth and distant dissemination of subcutaneous Lewis lung carcinoma tumors (151). The attention to these results is also due to the fact that researchers have defined PI3K $\gamma$  as a macrophage-specific mediator of resistance to therapies such as immunotherapy and antiangiogenic therapy (68, 154, 155). However, the role of HIF proteins and hypoxic TAMs in this resistance process remains to be further characterized.

In addition, because it is not yet well understood how the HIF-dependent hypoxic response regulates tumor angiogenesis through the phenotypic alterations of T cells, this will likely be an important topic in the near future. A recent publication provides evidence that HIF-1 but not HIF-2 is essential for the cytotoxic activity of CD8<sup>+</sup> cells and for their ability to cross the endothelial barrier, a limiting step for their recruitment into the tumor (156). However, although HIF-1 in T cells appears to mediate cytotoxicity (157, 158), hypoxic tumor niches prevent cytotoxic T cell activation via the influence of other hypoxic cells and the paracrine interaction of ligands and receptors that are controlled by hypoxia (159, 160). It is more surprising that tumors in T cell-specific HIF-1 $\alpha$  knockout mice display less tortuous and more perfused vessels (with increased tumor vessel normalization); this is based on reduced expression of the HIF-1 $\alpha$  target VEGFA (156). It is not clear whether or not the effect on blood vessels in T cell-specific HIF-1 $\alpha$  or VEGFA knockout mice is epiphenomenal and thus whether the increased tumor growth in these mice is rather due to angiogenesis-independent mechanisms. Indeed, other reports show that reduction of vessel normalization, not the induction, increases T cell infiltration (161, 162). More intriguingly, activation of CD4<sup>+</sup> T cells by immuncheckpoint inhibitors correlates with vessel normalization, and adoptive Th1 transfer in xenopatientes resolves primary tumor hypoxia via vessel normalization (161). The intertwined cross talk between hypoxia, T cells, and blood vessels and how it affects disease outcome certainly warrants future in-depth analysis.

### **Control of Inflammation by Metabolism in the Process of Tumor Angiogenesis**

Despite the growing interest in immunometabolism, how metabolic pathways and metabolite availability affect immune cell phenotype in a cancer context remains poorly explored. Even less is known about how metabolic pathways in immune cells affect the formation of a tumor vascular network (128, 130, 163). Interestingly, angiogenic and metabolic genes exhibited the highest-expression differences between hypoxic and normoxic TAMs (164). We found that REDD1 (regulated in development and DNA damage response 1), a physiologic inhibitor of the mechanistic target of rapamycin (mTOR), is synergically induced in TAMs by hypoxia and soluble stimuli from the tumor. REDD1 upregulation in hypoxic TAMs will excessively inhibit the mTOR pathway to a lower extent than it does in healthy tissues (131). As a consequence, mTOR release upon genetic knockout of REDD1 leads to enhanced glucose uptake. In this way, REDD1 knockout TAMs are able to subtract glucose from newly forming blood vessels and compete with tumor-associated ECs for glucose (131). Reduction of glucose availability and decreased glycolysis in ECs foster endothelial quiescence and the stabilization of blood vessels (165). Conversely, in tumors where REDD1 is excessively induced in hypoxic TAMs (which turn off the mTOR pathway), competition between ECs and macrophages is in favor of blood vessels; the glycolytic endothelium is thus hyperactive and less sessile (165). This is the first proof that in a tumor, both the surge of angiogenic factors and metabolic cross talk play in favor of an abnormal and dysfunctional vascular network. REDD1 knockout in TAMs reinstalls mTOR activity and thus reestablishes glucose up-take by macrophages and glucose competition with ECs. This results in more normalized, mature tumor blood vessels that prevent hypoxia and cancer cell intravasation (131).

Not only can macrophages affect angiogenesis, but a mutual control exists whereby ECs in turn sustain the M2-like, proangiogenic phenotype of macrophages. They install a positive feedback loop in cancer that keeps blood vessels hyperbranched, leaky, dysfunctional, tortuous, and poorly covered. This mutual regulation of EC and macrophage behavior at least partly relies on a metabolic pathway. At the vascular niche of the tumor, in particular in glioblastoma, EC-specific production of IL-6 and a more diffuse release of CSF1 promote an M2-like, alternative activation of TAMs (166). This alternative activation depends on the downstream activation by both IL-6 and CSF1 of the peroxisome proliferator-activated receptor gamma (PPAR- $\gamma$ ), a key transcriptional factor involved in the control of lipid uptake and glucose metabolism. In TAMs, PPAR- $\gamma$  binds the promoter of HIF-2 $\alpha$ , inducing its transcriptional (hypoxia-independent) accumulation. HIF-2 $\alpha$  is then associated with alternative macrophage activation (153). EC-specific knockout of IL-6 was sufficient to reduce microvascular proliferation in the tumor, to promote extensive necrosis, to decrease arginase-1 expression by TAMs (a marker of M2 activation), and to increase the survival of glioblastoma-bearing mice. This observation may indeed provide a link between obesity, macrophage polarization, tumor angiogenesis, and cancer because polyunsaturated fatty acids bind and activate PPAR- $\gamma$ , and long-chain polyunsaturated fatty acid status has recently been related to the pathogenesis of obesity (167). If true, could PPAR- $\gamma$  antagonists be used in immunotherapy to block M2-like macrophage polarization and thus abnormal angiogenesis and cancer malignancy? Phenolic acids such as oleuropein found in olives inhibit adipogenesis and adipocyte lipolysis. This means that mice fed a high-fat diet will display less circulating fatty acid and reduced body weight gain when treated with oleuropein (168). In B16 melanoma-bearing mice subjected to a high-fat diet, cancer and metastasis are more pronounced than in mice on a lean diet, but oleuropein strongly abrogates the effect conferred by the high-fat diet on tumor progression. Interestingly, this was associated with a reduction in M2 macrophage polarization as well as a consequent decrease in VEGFA, VEGFC, and VEGFD expression, altogether leading to the inhibition of tumor angiogenesis and lymphangiogenesis (168). Although more observational than mechanistic, this study underlines the strong effect of systemic metabolism on tumor inflammation, angiogenesis, and lymphangiogenesis.

An important metabolic pathway in macrophages implies the degradation of the amino acid arginine. Although arginase-1 is more abundantly expressed in M2-like, protumoral macrophages and degrades arginine into ornithine and urea, iNOS or Nos2 is enriched in M1-like, antitumoral macrophages and utilizes arginine and NADPH to generate citrullin and nitric oxide. Arginine depletion by macrophage-borne arginase-1 has been linked to the inhibition of antitumor T cell responses (127, 169). Similarly, nitric oxide release by hypoxic TAMs is also immune suppressive (147). Although the effect of TAM-associated arginase-1 on tumor blood vessels is unexplored, a recent report has shown that in several pancreatic cancer patients and in several mouse tumor models, local radiotherapy of the tumor induces iNOS in TAMs (because of a direct effect of  $\gamma$ -irradiation on macrophages). Instead, it was more unexpected that iNOS was responsible for tumor blood vessel normalization, EC activation, and recruitment of host T cells as well as increased delivery of adoptively transferred antitumor and cytotoxic T cells, all of which were accompanied by a reduction of Th2 (protumoral) and an increase of Th1 (antitumoral) cytokines (170). These



results demonstrate the positive antitumor effects of macrophage-borne nitric oxide on blood vessels and the indirect effects on T cell activation and recruitment (170) that are in sharp contrast to the immunosuppressive function of nitric oxide release by hypoxic TAMs (likely in avascular areas of the tumor) (147). Altogether, tumor context, association with therapy, and localization of immune cells and their interaction within different compartments can give rise to opposite effects (171).

Another amino acid involved in defining the phenotypic features of TAMs and their impact on tumor blood vessels is glutamine. Its role has always been considered proinflammatory, as the amino acid has been widely recognized as an important metabolic fuel for immune cells as well as a required foundation for lymphocyte and macrophage functions (172). However, in certain circumstances, glutamine supplementation is clearly anti-inflammatory (173, 174). This is likely due to the effect that glutamine availability has on the induction of glutamine synthetase, the enzyme responsible for glutamine production starting from glutamate. We found that an anti-inflammatory stimulus such as IL-4 as well as glutamine deprivation induce glutamine synthetase in mouse and human macrophages (38). Deletion of glutamine synthetase results in glutamate reroute to the GABA shunt with accumulation of succinate at the tricarboxylic acid (TCA) cycle. Succinate directly and indirectly (through HIF-1) sustains a rewiring of TAMs into M1-like macrophages. This leads to increased T cell recruitment and activation but also tumor blood vessel normalization, with increased perfusion, reduced permeability, and prevention of cancer cell dissemination (38). In this case, tumor vessel normalization (despite a reduction in vessel number) likely fosters a feedback loop of enhanced host T cell response, as described above (161, 162, 170).

Overall, it is apparent that the hypoxia/oxygen-sensing and metabolic machinery are contestants in the same game. Finally, given the large armamentarium of metabolic drugs currently tested in clinical settings (175), the repurposing of these molecules as a strategy to reshape inflammation and thus the angiogenic (and immune) landscape within the tumor is a novel frontier of cancer therapy.

## MYELOID CELL-INDUCED ANGIOGENESIS IN RESISTANCE TO THERAPY

A variety of standard and targeted therapies, including those that reduce blood vessel density, generate intratumoral hypoxia. Therapeutically induced low oxygen tension instigates the same course of action as naturally arising hypoxia to mobilize innate immune cells from the bone marrow and retain them at the tumor site (33, 46). Thus, intratumoral innate immune cells not only sustain angiogenesis but also possess the capacity to protect tumors from the deleterious effects of anti-VEGFR therapy by stimulating VEGF-independent pathways, as shown in several preclinical tumor studies. In addition to VEGF, TAMs and neutrophils express other proangiogenic factors like FGF1, FGF2, MMP9, and Ang2, and enhance those in response to VEGF inhibition (73, 176, 177) (Figure 1). Specifically, Gr1<sup>+</sup> immune cells, including TANs and MDSCs, have been found to be enriched in several tumor types relapsing from antiangiogenic therapy where they convey a proangiogenic relapse from VEGF blockade by secreting increased levels of angiogenic mediators including Bv8 (68, 178). As shown recently, the enhanced attraction of neutrophils seems to be in part orchestrated by CXCL5-producing CX3CR1<sup>+</sup> Ly6C<sup>lo</sup>

monocytes that are recruited to the tumor site due to the upregulation of CX3CL1 on tumor ECs in response to antiangiogenic therapy; this triggers transmigration of these monocytes across the endothelium (179).

In addition, accumulating TEMs in pancreatic tumors of the Rip1Tag2 mice undergoing VEGFR2 blockade contributed to a proangiogenic relapse that could be suppressed with a dual ANG2/VEGFR2 inhibitor targeting both TEMs and VEGFR2 (176). CXCR4<sup>+</sup> TEMs were also found to participate in the revascularization of MMTV-PyMT mammary tumors that had undergone treatment with the vascular-disrupting agent combretastatin A4 phosphate (CA4P). CA4P is known to induce substantial intratumoral hypoxia due to tumor vessel obstruction and, thus, enhanced HIF-induced CXCL12 expression and subsequent infiltration of CXCR4<sup>+</sup> TEMs (180). Combination of CA4P and a CXCR4 inhibitor blocked TEM accumulation and enhanced CA4P-induced tumor necrosis concomitant with reduced tumor growth, and consequently, sustained response. These results demonstrate that therapeutically induced hypoxia can induce expression and secretion of Ang2 and CXCL12, which in turn mediate Tie2-mediated VEGF-independent angiogenic activity of TEMs in tumors.

It is important to emphasize, however, that the different innate cell populations in tumors appear to express quite a similar profile of multiple angiogenic factors; thus, it is conceivable that they can compensate for each other in regulating angiogenesis. Interestingly, in the pancreatic Rip1Tag2 tumor model, depleting specific myeloid subpopulations resulted in increases in non-targeted myeloid cells, creating an oscillating pattern of resistance (68). Although most tumor model systems will respond to antiangiogenic therapy by slowing down tumor growth, pancreatic neuroendocrine tumors in Rip1Tag2 mice first respond very well to VEGF signaling blockade exhibiting reduced density of vessels, which are overall normalized and show growth stasis. Subsequently, they relapse and reinstate growth within 2–8 weeks, depending on the specific drug regimen (177, 181, 182). By comparing innate immune cells in responding and relapsing pancreatic neuroendocrine tumors, it became apparent that macrophages, monocytes, and neutrophils all exerted angiostatic and immunostimulating features in responding tumors when associated with the upregulation of CXCL14 and other angiostatic chemokines (Figure 1). This led to an influx of cytotoxic CD8 cells, whereas in relapsing tumors, myeloid cells converted back into an immune-suppressive and angiogenic phenotype, and CD8 influx ceased. Importantly, myeloid cells activated their PI3K $\gamma$  pathway, which disabled their repolarization and consequently promoted the proangiogenic tumor relapse (68). In support of these results, myeloid PI3K $\gamma$  signaling was shown to induce a transcriptional program that promoted immunosuppression by inhibiting NF- $\kappa$ B and activating C/EBP $\beta$  (155). Thus, pharmacological inhibition of myeloid PI3K $\gamma$  improved and sustained the tumor response to antiangiogenic therapy by converting all innate immune cells to an angiostatic and immune-stimulatory state associated with enhanced cytotoxic T cell infiltration and activity (68). These results further support the emerging proposition that angiogenesis and inflammation are functionally interregulated and that immune cells play a pivotal role in regulating both processes.

In line with these observations, tumors can also endorse the adaptive immune system to escape from antiangiogenic therapy. Recent studies have shown that relapsing tumors upregulated the negative immune checkpoint regulator, programmed cell death-ligand 1 (PD-L1), in tumor and stromal cell constituents (183, 184). As a result of PD-L1 binding PD-1 on the surface of activated T cells, T cell anergy or exhaustion was produced, which thereby triggered immunosuppression. Combining immunotherapy using anti-PD-L1 with antiangiogenic therapy (either anti-VEGF or anti-VEGF/Ang2) had reciprocally beneficial effects in that immunotherapy targeted evasion from antiangiogenic therapy, whereas vascular normalization elicited by antiangiogenic treatment could increase lymphocyte infiltration and activation (183, 184). Surprisingly, in successfully treated tumors, antiangiogenic immunotherapy induced high endothelial venule (HEV)-like structures that are normally found in secondary lymphoid organs and specialized to facilitate lymphocyte trafficking (185). Indeed, intratumoral HEVs substantially enhanced cytotoxic T cell infiltration and activity, and thereby furthered tumor cell destruction, leading to overall improved outcome (183). These preclinical studies support the notion that immune checkpoint inhibitors can sensitize and prolong efficacy of the VEGF signaling blockade and, conversely, antiangiogenic therapy can improve immunotherapy by supporting vascular changes such as vessel normalization and HEV formation in tumors.

Further support for this concept stems from a recent study by Tian and colleagues (161, 186) who demonstrated that intratumoral T lymphocyte infiltration promoted blood vessel normalization; in addition, a normalized vasculature has the ability to enhance T cell infiltration. Mice deficient in CD4 and CD8 T cells involved tumors with more abnormal tumor vessels and hypoxic areas than those of CD4- and CD8-proficient mice. Checkpoint immunotherapy (anti-PD1 and/or anti-CTLA4) or adoptive Th1 transfer generating activated cytotoxic T cells in tumor model systems was sufficient to induce blood vessel normalization and reduce both hypoxia and metastases. These results were congruent with a normalization plus T cell receptor signaling pathway signature obtained by transcriptional profiling of patient tumors and was associated with good prognosis. These studies provide evidence in patient-derived tumors that blood vessel normalization and T lymphocyte infiltration provide positive feedback loops conferred by each compartment. They also underscore the notion that tumor vessels can be modified by the immune system, which enables enhanced T cell infiltration and improves immunotherapies.

## **CONCLUSION: FROM MOUSE TO HUMAN—ADVANTAGES AND LIMITATIONS**

Although the mouse tumor model systems discussed in this review have greatly helped to elucidate the various mechanistic underpinnings of myeloid-directed tumor angiogenesis and adaptive tumor resistance, it remains to be validated to which extent some of these mechanisms are also functionally implicated and significant in the human tumor setting. Notably, a number of clinical trials (NCT03024437, NCT02659384, NCT02873962, and NCT02017717) are already evaluating combinatorial approaches of VEGF/VEGFR and PD-1/PD-L1 inhibition for a number of cancer types, such as renal cell carcinoma, colorectal cancer, ovarian cancer, and recurrent glioblastoma. Thus, ongoing clinical trials already

combine antiangiogenic agents and immunotherapies such as immune checkpoint blockade or target innate immune cells as well as various approaches to enhance infiltration and activation of T cells. These trials will be instrumental to validate the concept of targeting the functional and regulatory interaction between the immune system and vascular system in cancer (187–189).

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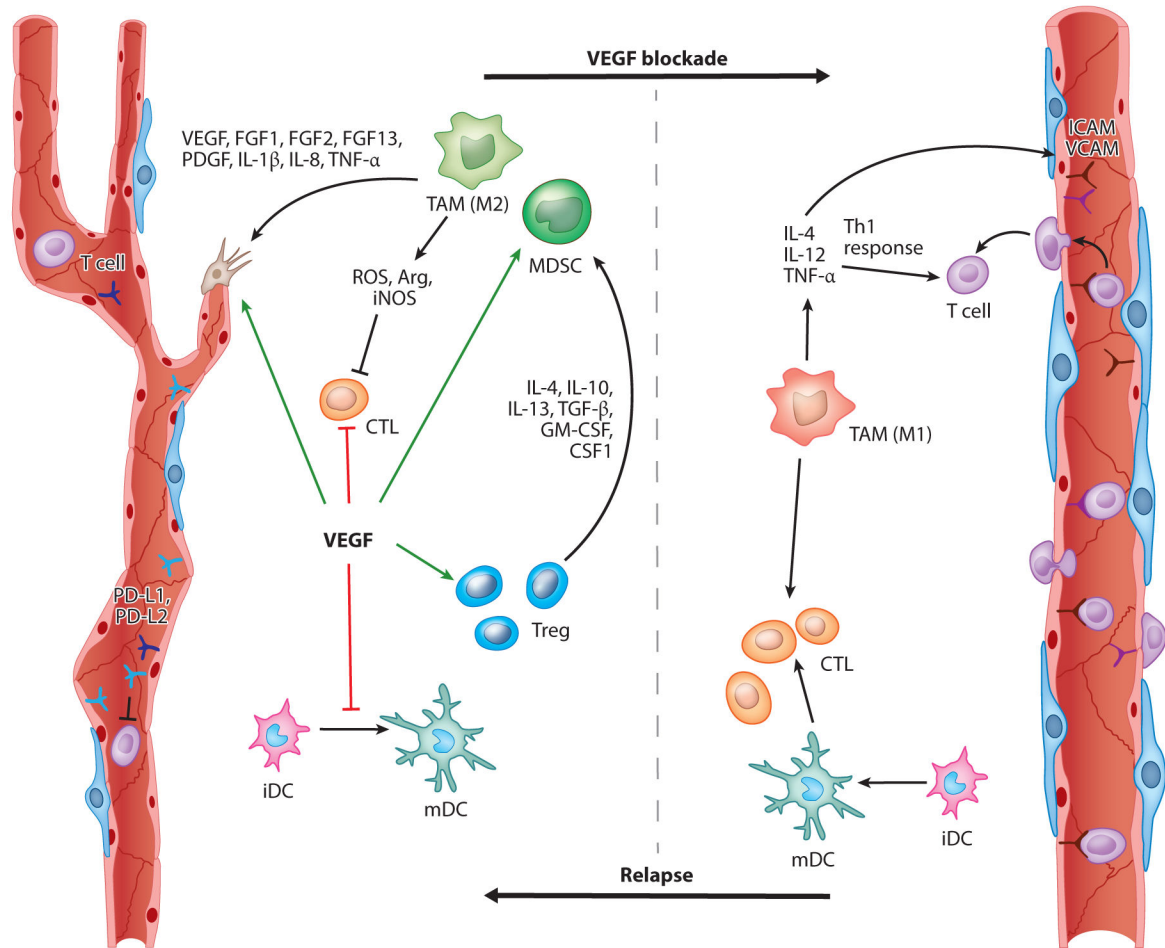
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**Figure 1.**

VEGF/VEGFR signaling controls angiogenesis and tumor immunity. VEGF facilitates several aspects of vessel formation and also promotes immunosuppression by acting on different cell types. In endothelial cells, VEGF inhibits the expression of the T cell adhesion molecules VCAM and ICAM and induces expression of the PD-1 ligands PD-L1 and PD-L2 that interact with PD-1 on T cells, resulting in reduced T cell proliferation and effector function. VEGF also directly impairs DC maturation and induces PD-L1 expression on mature DCs. It inhibits the proliferation and effector function of CTLs but induces the proliferation of Tregs. Tregs in turn recruit MDSCs and TAMs, which produce ROS, iNOS, and Arg to suppress T cell proliferation, viability, and activity. In contrast, inhibition of VEGF signaling enables enhanced T cell infiltration due to vessel normalization accompanied with an increase of ICAM and VCAM, which enhances DC maturation and thus provides more intratumoral effector T cells. VEGF/VEGFR blockade also increases the presence of Th1/M1-polarized myeloid cells (e.g., macrophages, neutrophils). Taken together, anti-VEGF therapy should promote an antitumor response by affecting the vasculature and the immune system. Continuous vessel pruning, however, induces hypoxic areas that drive the recruitment and polarization of immunosuppressive and angiogenic myeloid cells. Abbreviations: Arg, arginase; CSF1, colony-stimulating factor 1; CTL, cytotoxic T cell; DC, dendritic cell; FGF, fibroblast growth factor; G- or M-MDSC,

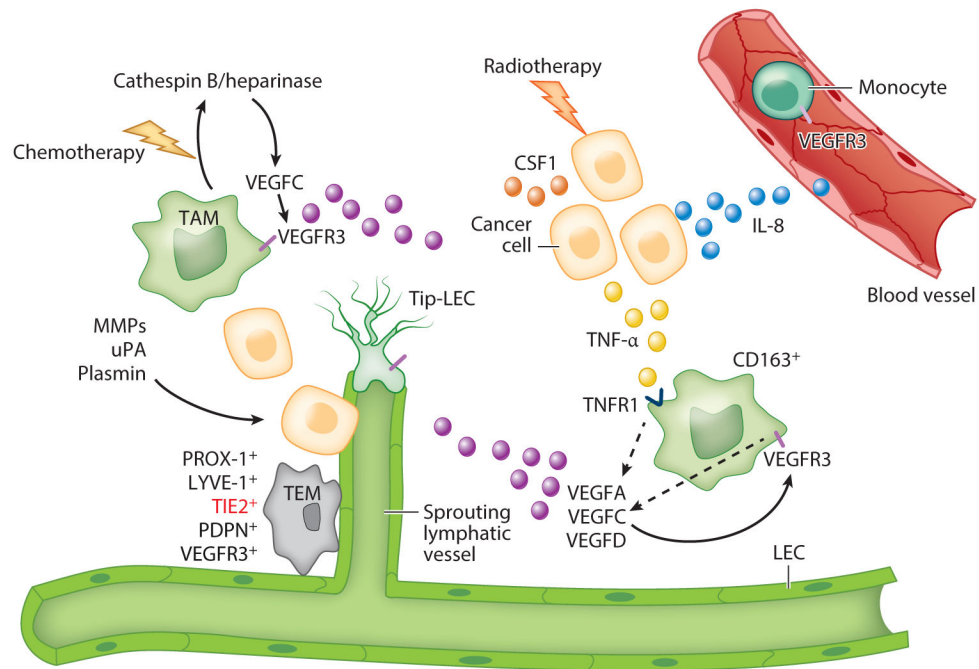
granulocytic or monocytic myeloid-derived suppressor cell; GM-CSF, granulocyte-macrophage colony-stimulating factor; ICAM, intercellular adhesion molecule; iDC, immature DC; IL, interleukin; iNOS, nitric oxide synthase; mDC, mature DC; PDGF, platelet-derived growth factor; PD-L1/2, programmed death-ligand 1/2; ROS, reactive oxygen species; TAM, tumor-associated macrophage; TGF- $\beta$ , transforming growth factor-beta; Th1, T helper 1; TNF- $\alpha$ , tumor necrosis factor- $\alpha$ ; Treg, regulatory T cell; VCAM, vascular cell adhesion molecule; VEGF, vascular endothelial growth factor; VEGFR, VEGF receptor.

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**Figure 2.**

The role of macrophages in tumor lymph angiogenesis. Adhesion of circulating VEGFR3<sup>+</sup> monocytes to tumor BECs in response to cancer cell (and stromal cell) secreted IL-8 (*blue circles*) favors monocyte extravasation. Once inside the tumor parenchyma, monocyte differentiation into TAMs, which in human tumors are mostly CD163<sup>+</sup>, elicits their production of VEGFA, VEGFC, and VEGFD (*purple circles*) upon TNF- $\alpha$ /TNFR1 signaling (*yellow circles* depict TNF- $\alpha$ ). Autocrine stimulation of VEGFR3 by its cognate ligands VEGFC and VEGFD further increases VEGFC production. VEGFA, VEGFC, and VEGFD as well as MMPs, uPA, and plasmin favor the migration of tip-LECs and the formation of new lymphatic sprouts. They also contribute to the junction disassembling of LECs and thus to the promotion of cancer cell intravasation through the lymphatics. TEMs are in close proximity to the tumor lymphatics but not in lymphatics of normal tissue. These perilymphatic macrophages (that share other lymphatic markers such as PROX-1, LYVE-1, PDPN, and VEGFR3) support new sprout growth in a paracrine manner, but it is still debated if they can integrate into the vessel wall. Chemotherapy will also act on TAMs and induce the initiation of a cathepsin B/heparinase cascade that leads to enhanced VEGFC release by TAMs and thus lymph angiogenesis and cancer cell intravasation. Mirroring this, radiotherapy induces the release of CSF1 (*orange circles*) by cancer cells that boosts the recruitment and differentiation of VEGFR3<sup>+</sup> (prolymph angiogenic) TAMs. Abbreviations: BEC, blood endothelial cell; CSF1, colony-stimulating factor 1; IL-8, interleukin 8; LEC, lymphatic endothelial cell; LYVE-1, lymphatic vessel endothelial hyaluronan receptor 1; MMP, matrix metalloproteinase; PDPN, podoplanin; PROX-1, prospero homeobox protein 1; TAM, tumor-associated macrophage; TEM, Tie2-expressing macrophage; Tip-LEC, lymphatic endothelial tip cell; TNF- $\alpha$ , tumor necrosis factor-alpha; TNFR1, tumor necrosis factor receptor 1; uPA, urokinase-type plasminogen activator; VEGFA, vascular endothelial

growth factor A; VEGFC, vascular endothelial growth factor C; VEGFD, vascular endothelial growth factor D; VEGFR3, vascular endothelial growth factor receptor 3.

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