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This is the author's manuscript

Original Citation:

Availability:

This version is available <http://hdl.handle.net/2318/1841066> since 2022-02-16T11:44:39Z

Published version:

DOI:10.1016/j.semcancer.2018.06.002

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HHS Public Access

Author manuscript

Semin Cancer Biol. Author manuscript; available in PMC 2019 October 01.

Published in final edited form as:

Semin Cancer Biol. 2018 October ; 52(Pt 2): 107–116. doi:10.1016/j.semcancer.2018.06.002.

The reciprocal function and regulation of tumor vessels and immune cells offers new therapeutic opportunities in cancer

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Abstract

Tumor angiogenesis and escape of immunosurveillance are two cancer hallmarks that are tightly linked and reciprocally regulated by paracrine signaling cues of cell constituents from both compartments. Formation and remodeling of new blood vessels in tumors is abnormal and facilitates immune evasion. In turn, immune cells in the tumor, specifically in context with an acidic and hypoxic environment, can promote neovascularization. Immunotherapy has emerged as a major therapeutic modality in cancer but is often hampered by the low influx of activated cytotoxic T-cells. On the other hand, anti-angiogenic therapy has been shown to transiently normalize the tumor vasculature and enhance infiltration of T lymphocytes, providing a rationale for a combination of these two therapeutic approaches to sustain and improve therapeutic efficacy in cancer. In this review, we discuss how the tumor vasculature facilitates an immunosuppressive phenotype and vice versa how innate and adaptive immune cells regulate angiogenesis during tumor progression. We further highlight recent results of antiangiogenic immunotherapies in experimental models and the clinic to evaluate the concept that targeting both the tumor vessels and immune cells increases the effectiveness in cancer patients.

Keywords

Angiogenesis; Immunosuppression; antiangiogenic therapy; immunotherapy immune checkpoint inhibitors; innate and adaptive immune cells; tumor hypoxia and acidosis; metabolism

Introduction

The onset of tumor neovascularization is a well-established hallmark of cancer that is initiated at a certain time during tumor progression depending on the tumor type, grade and stage. Blood vessels not only have to provide oxygen and deplete waste products to oblige

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Competing interests
RM, MM and GB declare no competing interests

the demands of a growing tumor but also form niches that enable cancer stem cell maintenance, protection and entail sites of infiltrating immune cells [1-4]. Various mechanisms for the reinstatement of blood vessel growth have been described of which angiogenesis, the sprouting of new vessels from pre-existing capillaries and postcapillary venules is the most commonly used mode [5-7]. Subsequently, tip cells anastomose with tip cells from neighboring sprouts to connect the newly formed vascular structures with the help of bridging macrophages. With the initiation of blood flow, the establishment of a basement membrane, and the recruitment of vessel-stabilizing pericytes, vascular growth is then terminated [5, 6]. Thus, under physiological conditions angiogenesis is tightly regulated by a homeostatic balance of a wealth of proangiogenic and inhibitory factors of which members of the vascular endothelial growth factor (VEGF), and angiopoietin tyrosine kinase receptor families as well as various members of the CXC and CC chemokines are the most prominent molecules to orchestrate this multistep process [1, 8]. The balance of angiogenesis-promoting and inhibiting molecules is, however, lost in tumors. Upon initiating neovascularization, tumors continue to promote angiogenic signaling for several reasons [9]. First, tumors express oncogenes and/or lose tumor suppressor genes that result in the activation of pro-angiogenic signaling pathways [10-12]. Second, expanding tumors exhibit a continually and abnormally growing tumor vasculature with a typical display of irregular-shaped, hyperdilated and poorly pericyte-covered tumor vessels that cause a leaky and sluggish blood flow [13]. These typical signs of poor vessel maturation increase interstitial pressure and generate and maintain a hypoxic and acidic environment that continues to elevate proangiogenic factors thereby further aggravating a pro-angiogenic feedback loop that never ceases.

Effects of low-oxygen tension are mediated by hypoxia-inducible factor (HIF) transcription factors that coordinate a transcriptional program to ensure metabolic and vascular adaptations under hypoxic conditions by various mechanisms [14-16]. Hypoxia upregulates various proangiogenic growth factors and chemokines that directly engage in vessel growth like VEGF, PlGF and Ang2 [17-21]. In addition, numerous experimental models have shown that both hypoxic and acidic conditions in the tumor microenvironment change the availability of metabolites and induce the secretion of molecules in tumors (e.g. CSF1, GM-CSF, G-CSF, CX3CL1, CXCL5, CXCL12, CCL17, CCL22, IL6, Sema3) that attract innate immune cells to the tumor site where they become reprogrammed to serve as an additional source of angiogenic factors [22-28]. It is important to note that innate immune cells elicit a high plasticity in order to quickly adapt and serve the homeostatic repair program. Upon injury, first neutrophils and then macrophages are recruited to the wound that are immunosupportive and angiostatic due to their responsibility to phagocytose and kill bacteria and degrade necrotic tissue. In the subsequent resolution phase, myeloid cells then convert to an angiogenic and immunosuppressive phenotype to support tissue and blood vessel restoration [29]. The ability of myeloid cells to display such opposing properties to either eliminate potential infections or hinder excessive inflammation is indeed pivotal to properly regulate tissue injury and maintain tissue homeostasis [30]. In line with the concept that tumors are wounds that never heal [31], tumors take advantage of the myeloid plasticity by reprogramming these cells to exhibit both immunosuppressive and angiogenic features to

enable tumors to escape immunosurveillance and facilitate vascular expansion. These two features have been defined as pivotal hallmarks in cancer propagation and progression [32].

1. Angiogenic factors promote immunosuppression

There is increasing evidence that tumor-supporting inflammation and angiogenesis, although being distinct and separable processes, are closely related events that are in part regulated by common chemokines [32, 33]. VEGF and Ang2 are proangiogenic factors that directly and indirectly convey immunosuppression in endothelial cells as well as innate and adaptive immune cells [34-37]. VEGF induces an immunosuppressive vasculature by on different levels (Fig.1). It downregulates the expression of the vascular adhesion molecules I-CAM1 and V-CAM1 that are necessary for T-cells to cross the endothelial layer and transit into the tumor. VEGF and Ang2 have been shown to enhance the expression of the negative checkpoint regulator programmed cell death protein 1 (PD-1)– programmed cell death 1 ligand 1 (PD-L1) in endothelial cells thus disabling the cytotoxic function of PD1+-T cells [38-41]. In addition, elevated FasL levels on tumor ECs is known to trigger apoptosis of Fas-expressing CD8+T cells [42]. Tumor-associated ECs also preferentially attract immunosuppressive Tregs by upregulating the multifunctional endothelial receptor CLEVER-1/stabilin-1 [43]. All these mechanisms consequently lead to a potent barrier that disables cytotoxic T cell infiltration into the tumor. VEGF, however, not only facilitates VEGFR-2 signaling in endothelial cells but also in VEGFR2+ immune cells. Thereby, it stimulates the expression of PD-L1 in dendritic cells and blocks the maturation and thus, functionality of dendritic cells due to their impaired ability to present tumor antigens [44]. VEGF/VEGFR2 signaling further suppresses proliferation of effector T cells but increases and promotes tumor homing of Tregs [32, 44]. This effect is dependent on neuropilin-1 (nrp1) because in an experimental tumor model, nrp1 depletion resulted in reduction of Tregs with a concomitant increase in cytotoxic T cells [45]. VEGF also upregulates several negative immune checkpoint receptors besides PD-1 (e.g. CTLA-4, Lag-3, TIM-3) on T cells contributing to T-cell anergy and exhaustion [46]. Finally, elevated levels of VEGF and/or Ang-2 as well as other tumor-secreted factors promote the recruitment and expansion of innate immune cell populations including TAMs, TEMs, neutrophils, MDSC and immature dendritic cells that secrete molecules fostering an immunosuppressive and angiogenic tumor microenvironment.

2. Immune cells promote angiogenesis

2.1. Innate immune cells promote angiogenesis—As discussed above, tumor-associated macrophages (TAM) and tumor-associated neutrophils (TAN) can either convey angiostatic and immunosupportive or proangiogenic and immunosuppressive features, but in tumors are commonly found to polarize to an immunosuppressive as well as angiogenic phenotype [47-49]. In addition, immature Gr1+ cells with either a mononuclear or granular morphology have been identified in tumors that promote immunosuppressive functions and are therefore also termed myeloid-derived suppressor cells (M-MDSC and G-MDSC respectively) [49]. Most commonly, surface marker profiling based on expression of CD11b, F4/80, CD11c, Gr1, Ly6C, and Ly6G has been used to categorize the different tumor-infiltrating myeloid cells in experimental mouse tumor models [28, 48-50]. There is a plethora of myeloid-cell secreted mediators of angiogenesis that regulate various aspects

during vessel formation including growth factors and cytokines (VEGF, FGF, TNF- α , TGF- β , PDGF-B, PIGF, Neuropilin-1, CXCL chemokines (e.g. CXCL-8,-12), semaphorins), as well as various proteases including matrix metalloproteinases (MMP-2,-7,-9, and -14) and cysteine cathepsin proteases [6, 27, 51-60]. There is also a wealth of experimental studies in various murine tumor models that have provided compelling evidence for the functional significance of myeloid cells in regulating tumor angiogenesis and subsequent tumor and metastasis progression of which some of the seminal and more recent studies will be highlighted below [24, 61, 62].

2.1.1. Tumor-associated macrophages (TAM): One of the most prominent myeloid populations that can make up to 30% of intratumoral cells are tumor-associated macrophages (TAM) and are often associated with increased vessel density in tumors [28, 49, 63]. One of the first seminal studies underscoring the functional importance of TAMs and their production of VEGF in tumor angiogenesis were conducted in the endogenous mouse mammary virus-polyoma middle T-antigen (PyMT) tumor model, with similar results forthcoming from other experimental tumor systems [24, 64, 65]. In summary, VEGF-producing macrophages facilitated the angiogenic switch and subsequent progression to malignancy because impairing TAM activity by blocking their CSF1/CSF1R signaling pathway, or macrophage-specific deletion of VEGF, or even broadly depleting TAMs by clodronate liposomes delayed the angiogenic switch while restoration of macrophages rescued the tumor's ability to promote angiogenesis [24, 64, 65]. Important to note is that VEGF loss in intratumoral TAMs caused are more aggressive tumor adaptation in multiple subcutaneous isograft models and the PyMT tumor model because it accelerated tumor growth due to reduced tumor apoptosis and intratumoral hypoxia. It was speculated that by just reducing angiogenesis to a certain level, more functional, normalized vessels with increased pericyte coverage had been generated that reduced intratumoral hypoxia and apoptosis, supported by the observation that myeloid-derived VEGF was sufficient to increase the susceptibility of these tumors to chemotherapeutic cytotoxicity; an effect that has been commonly attributed to the improved blood flow and oxygenation by intratumoral vessel normalization [11, 64]. Monocytes (TEMs) that express the angiopoietin receptor Tie 2 (TEK) represent a subset of macrophages that are often closely associated with blood vessels through endothelial cell secretion of the Tie2 ligand Angiopoietin-2 (Ang-2) [37]. Their relevance to tumor angiogenesis and subsequent tumor growth has been demonstrated by either selectively ablating TEM by virtue of Tie2 promoter-driven thymidine kinase expression or by antibody-mediated neutralization of the Tie2 ligand Ang2, in mammary, pancreatic neuroendocrine and brain tumor mouse model systems [66, 67]. Thereby, TEMs were found to not only instigate neovascularization during tumor progression but also in response to chemotherapy and radiation [68-70]. Notably, genetic deletion of Tie2 in these cells disabled their specific perivascular location and ability to promote angiogenesis [67]. As pharmacological or genetic inhibition of Tie2 in macrophages mimics the effects of Ang2 deletion in tumor endothelial cells further underscores the Ang2-Tie2 axis as a critical signaling node between TAMs and endothelial cells in promoting neovascularization [67, 71]. TAMs also express neuronal and vascular guidance molecules that regulate cell survival and migration [72, 73]. Among those, Semaphorin3A (Sema3A), like Ang2 induced by hypoxia in tumors, has also been shown to facilitate macrophage recruitment and TAM-

directed angiogenesis by mediating Nrp-1-dependent signaling of a PlexinA1/PlexinA4/VEGFR1 complex. Upon activation of this holocomplex, TAMs migrated into hypoxic regions where they secreted various immunosuppressive and proangiogenic factors. However, as soon as TAMs were positioned in the hypoxic environment, Nrp1 expression was repressed by HIF2 α -mediated activation of the NF- κ B pathway, and the migratory response of TAMs to Sema3A terminated, trapping these cells in hypoxic areas. Congruently, loss of Nrp-1 in TAMs was sufficient to disable their infiltration into hypoxic regions and thus, their hypoxia-induced immunosuppressive conversion, impaired neovascularization and thus, delayed tumor growth and metastasis in mouse models of pancreatic, lung, breast cancer [74]. Similar findings were obtained in an experimental glioma model, where genetic and pharmacological depletion of Nrp1 in infiltrating macrophages and resident microglia exhibited antitumoral effects by their reprogramming to an immunosupportive phenotype [75].

2.1.2 Tumor -associated neutrophils (TAN) and MDSC: The other major myeloid cell types implicated in angiogenesis are neutrophils, granule-containing cells, which are the most abundant white blood cells and the first cells to be recruited to injuries. Neutrophils play a key role in the front-line defense against invading pathogens where they either phagocytose micro-organisms, release soluble antimicrobial molecules from their granules or generate neutrophil extracellular traps (NETs), web-like fibers that are composed of chromatin and serine proteases and trap and kill extracellular microbes [76, 77]. In tumors and metastases, neutrophils secrete proangiogenic factors and proteases similar to those of macrophages, most predominantly VEGF, FGF and MMPs [47, 78, 79]. Interestingly, neutrophils can carry VEGF-enriched granules which are released upon TNF stimulation and enable a quick route of VEGF accessibility to promote blood vessel growth [80]. In line with this observation, neutrophils have been shown to release MMP-9 to liberate sequestered VEGF from the extracellular matrix (ECM) in dysplastic pancreatic islet lesions of Rip1Tag 2 mice. Released VEGF triggered the onset of angiogenesis in these premalignant lesions and enabled malignant conversion and progression [59, 81]. This was indeed a crucial mechanism because neutrophil depletion with antibodies against Gr1 substantially reduced the angiogenic switch in these pancreatic islet lesions [81]. Conversely, G-CSF-stimulated neutrophils upregulated the expression of the angiogenic factor Bv8 (prokineticin-2) in several tumor models which stimulated endothelial cell survival, migration and proliferation and attracted more neutrophils thus, providing a positive feedback loop for neutrophil recruitment and activation [82].

Tumors also recruit myeloid-derived immunosuppressive cells (MDSC), immature myeloid cells that can be divided into granulocytic (G-MDSC) and monocytic (M-MDSC) cell populations and convey not only immunosuppressive but also angiogenic properties [28, 83]. MDSC had first been studied for their ability to suppress human CD3 $^+$ and mouse CD4 $^+$ or CD8 $^+$ T cells [84]. In context to angiogenesis, MDSC-produced VEGF was sufficient to suppress the expression of the adhesion molecules ICAM1 and VCAM1 in tumor-associated endothelial cells, hence limiting T cell adhesion and extravasation [85, 86]. Several factors including G-CSF, IL-1 β and IL-6, recruit, activate and expand MDSCs in the tumor via STAT3 activation stimulating their pro-angiogenic properties while blocking their

differentiation into neutrophils or macrophages respectively [87, 88]. It is therefore not surprising that their pro-angiogenic expression profile and functions largely overlap with those of mature macrophages and neutrophils [79, 89].

2.2. Adaptive immune cells regulate angiogenesis—The implications of lymphocyte in tumor angiogenesis is poorly explored but recent results suggest that they have the ability to directly and indirectly regulate blood vessel growth and modulation. B-cells can directly promote angiogenesis by producing proangiogenic factors such as VEGF, FGF2, and MMP-9 [90], or indirectly by polarizing macrophages to an immunosuppressive and proangiogenic phenotype in an IgG -dependent manner [91]. T-cells on the other hand, appear to negatively or positively regulate tumor angiogenesis dependent on the T-cell type. CD8⁺ T-cells and CD4⁺ T-helper 1 cells produce IFN γ that restrains endothelial cell proliferation and induces the production of the angiostatic chemokines CXCL 9,10 and 11 in TAMs [47, 92]. In contrast, Treg cells suppress INF γ - expressing CD4⁺ Th1 cells and secrete VEGF via hypoxia-induced CCL28, which both contribute to a proangiogenic tumor environment [93]. Congruent with these results, intratumoral CD4⁺ and CD8⁺ T-cell depletion displayed an increase in dysfunctional tumor vessels and subsequent hypoxia which was reverted by CD8 influx and activity through checkpoint immunotherapy (anti-PD1 and/or anti-CTLA4) or adoptive TH1 transfer in models of murine tumors and patient-derived tumors inducing tumor vessel normalization and reduced both hypoxia and metastases [94].

3. Metabolic pathways in immune cells regulate angiogenesis

The tumor induces a major disturbance in tissue homeostasis and cellular metabolism by endorsing a hypoxic and acidic microenvironment that strongly affects metabolic availability not only for tumor cells but also other cell constituents within the tumor (Fig.2). Although it is well-established that intratumoral hypoxia induces the recruitment of immunosuppressive and angiogenic myeloid cells to the tumor site, it is less understood how subsequent changes in metabolite availability for immune cells affect their ability to support blood vessel growth. Exposure to low pH and hypoxia impacts immune cells in different ways dependent on the immune cell subtype and subsequently leads to escape of immunosurveillance, angiogenesis and cancer progression [95, 96]. T and NK cells appear to lose their antitumor function and become anergic and apoptotic while regulatory T cells are engaged to block cytotoxic T-cell activity, and myeloid cells become immunosuppressive which all together sustains tumor growth. Under normoxia, glycolytic pyruvate enters the tricarboxylic acid (TCA) cycle for oxidative phosphorylation (OXPHOS), whereas lactate conversion from pyruvate is enhanced in anaerobic conditions. In cancer cells, pyruvate to lactate conversion occurs already in the presence of oxygen due to metabolic alterations producing metabolic acidosis with an intratumoral pH of 6.0–6.5 [97]. Under low oxygen conditions, tumor cells increase their glycolytic flux, resulting in a significant secretion of lactate which induces an acidic environment [95, 96]. There is increasing evidence that tumor-secreted lactate affects the behavior of intratumoral TAMs because it induces the expression of VEGF and Arginase-1 involved in suppressing T-cell responses; these are hallmarks of TAM polarization to an immunosuppressive and angiogenic phenotype [98]. Endothelial cells undergo metabolic changes when they become activated to form new vessels. Despite their exposure to high

oxygen levels, endothelial cells, like tumor cells, divert the majority of pyruvate to lactate using aerobic glycolysis [99, 100]. Similarly, T_{eff} cells predominantly use glycolysis for sufficient ATP synthesis. Consequently, the metabolic similarities between immune cells, endothelial cells and tumor cells potentiate a competition for several substrates (Fig.2).

A metabolic competition between TAMs and endothelial cells was elegantly demonstrated in a recent study. Hypoxic TAMs were shown to express very high levels of REDD1, a negative regulator of mTOR, which blocked glycolysis in TAMs and thus made more glucose available for endothelial cells to form new blood vessels. Remarkably, genetic deletion of REDD1 in TAMs increased their glycolytic rate to a level that it competed with neighboring endothelial cells for glucose and suppressed their angiogenic activity. Indeed, competition over glucose induced vessel normalization and improved oxygenation in tumors resulting in growth reduction [101]. In support of a reciprocal regulation between TAMs and endothelial cells, tumor endothelial cells can sustain the angiogenic/immunosuppressive phenotype in TAMs by generating a positive metabolic feedback loop in cancer that keeps tumor vessels in an angiogenic mode. In an experimental model of glioblastoma (GBM), EC-secreted IL6 and a more diffuse release of CSF1 promoted the conversion to angiogenic/immunosuppressive TAMs in a HIF-2 α -dependent manner [102]. Both, IL6 and CSF1 signaling in TAMs activated peroxisome proliferator-activated receptor gamma (PPAR- γ), a key transcriptional factor involved in the control of lipid uptake and glucose metabolism, via HIF-2 α induction [103]. Endothelial-specific deletion of the *IL6* gene was sufficient to reduce microvascular proliferation in the tumor, decrease the expression of the immunosuppressive marker arginase-1 in TAMs and increase the survival of GBM-bearing mice [102]. Besides changes in glucose metabolism, amino acid metabolites can also be dysregulated in tumors and affect immune cell behavior. Increased IDO activity in tumors has been shown to deplete tryptophan and accumulate kynurenine and its derivatives, thereby inhibiting the activation and proliferation of immune cells and rendering the tumor microenvironment immunosuppressive and angiogenic. A variety of tumor cells also lack the enzyme argininosuccinate synthetase-1, the enzyme responsible for L-arginine synthesis, which makes tumor cells completely dependent on TAM-supplied L-arginine [104, 105]. The activity of two the L-arginine consuming enzymes nitric oxide synthase (NOS) and Arginase is strongly upregulated in several tumor types [106-109]. These enzymes generate reactive nitrogen species which are shown to impair T-cell mediated immune responses [110].

Another important metabolic pathway in macrophages entails the amino acid arginine. While the arginine-degrading enzyme arginase-1 is more abundantly expressed in angiogenic/immunosuppressive TAMs and degrades arginine into ornithine and urea, inducible nitric oxide synthetized (iNOS, or Nos2) is enriched in antitumoral TAMs and generates citrullin and nitric oxide from arginine and NADPH. Arginine depletion by macrophage-expressing arginase-1 has been linked to the inhibition of anti-tumor T cell responses [111, 112]. Similarly, nitric oxide release by hypoxic TAMs is immunosuppressive [113]. Important to note however is that local γ -irradiation of patient and murine pancreatic tumors induced iNOS in TAMs which in turn normalized tumor blood vessels, recruited host T cells as well as adoptively transferred cytotoxic T cells [114]. These results underscore the direct and indirect positive antitumor effects of macrophage-secreted nitric oxide on blood

vessels and T-cell influx and activation [114] which is in contrast to the immunosuppressive function of nitric oxide released by hypoxic TAMs in avascular areas of the tumor [113]. All together, these results reiterate the impact of metabolic changes in regulating the pleiotrophic functions of immune cells in angiogenesis and immunosuppression [115].

4. Immune cells facilitate resistance to antiangiogenic therapy

Compelling data from multiple laboratories have provided a convincing rationale for the development of VEGF and VEGF receptor inhibitors to block intratumoral vascular growth and subsequent tumor propagation. Subsequently, bevacizumab (Avastin, Genentech/Roche), a monoclonal antibody against VEGF, as well as sorafenib (Nexavar, Bayer) and sunitinib (Sutent, Pfizer), both kinase inhibitors that target VEGF receptor (VEGFR) were the first FDA-approved angiogenic inhibitors followed by other angiogenic tyrosine kinases, aflibercept, another VEGF-Trap antibody (Regeneron) and, more recently Ramucirumab, an antibody against VEGFR2 (ImClone/Lilly) [116]. Although encouraging effects of these inhibitors were observed in a subset of cancer patients, antiangiogenic therapy has revealed very limited and transient beneficial effects by improving progression-free-survival and quality-of-life but not overall survival, and only in a fraction of cancer patients [11, 117, 118]. Interestingly, however, experimental studies in which the treatment was beneficial, discovered that albeit tumor vessels were pruned, they also exhibited enhanced pericyte coverage concomitant with a more functional morphology and subsequent improvement in blood flow and oxygenation which was coined by Rakesh Jain as “vessel normalization” [119]. Importantly, such vascular changes were also observed by functional MRI analysis in cancer patients undergoing anti-VEGFR therapy. Normalization of tumor vessels during VEGF blockade has shown to improve drug and oxygen delivery thereby reducing intratumoral hypoxia which in turn contributed to a more immunosupporting tumor environment associated with increased CD8 cytotoxic T cell influx [120] (Fig.1). Anti-VEGF- induced vascular normalization is however transient and continuous treatment will result in further pruning and restoration of hypoxia leading to various adaptation mechanisms to overcome vascular growth restrictions (Fig.1). These imply at least two major relapse categories, in which tumors either reinstate growth by revascularization or alter their growth behavior without obligate neovascularization [121]. Adaptation to vascular growth restrictions also involve the recruitment of myeloid cells instigated by therapy-induced hypoxia which like during tumor progression, induce similar factors that mobilize innate immune cells from the bone marrow and attract them to the tumor site [27, 67]. Here, myeloid cells sustain vascular growth by stimulating VEGF-independent pathways. In response to angiogenic inhibition, TAMs, TEMs and neutrophils enhance the expression of various other angiogenic molecules besides VEGF like Ang2, FGF-1,2, chemokines, and MMP9 [122-124]. Specifically, neutrophils and MDSC (often only referred to as Gr1+/Ly6G + immune cells) were recruited in several tumor model systems relapsing from antiangiogenic therapy and thereby endorsed resistance to VEGF blockade by secreting increased levels of proangiogenic molecules including Bv8 which exerts direct proangiogenic effects on endothelial cells [89, 125]. One of the underpinning mechanisms that lead to enhanced neutrophil infiltration by VEGF inhibition was facilitated by T_H17A T-cell-induced IL17 which in turn enhanced intratumoral levels of the neutrophil attractant CSF-3 (GM-CSF) [126]. A more recent study described the implication of CXCL5-secreting

Ly6C^{lo} monocytes in the recruitment of neutrophils during VEGF inhibition [127]. Interestingly, CX3CR1+ Ly6C^{lo} monocytes also entered the tumor when VEGF signaling was blocked due to upregulation of CX3CL1 on tumor endothelial cells [127]. Besides neutrophils, macrophages also possess the capacity to protect tumors from the deleterious effects of antiangiogenic therapy. Upregulation of the hypoxia-regulated factor Ang2 activated the Ang2-Tie2 axis and enabled enhanced infiltration of TIE2-expressing macrophages (TEMs) in pancreatic and breast tumor models undergoing VEGFR2 inhibition. Conversely, dual ANG2/VEGFR2 blockade targeted both Tie2+TEMs and VEGFR2 on endothelial cells, and thus, suppressed revascularization and progression in tumors [122]. Importantly, dual Ang2-VEGFR2 blockade prolonged vessel normalization and CD8 influx in comparison to monotherapies thereby enhancing survival in different mouse tumor models of brain, pancreas and breast [41]. TEMs also protected spontaneous MMTV-PyMT mammary tumors treated with the vascular-disrupting agent combrestatin A4 phosphate (CA4P) that induced substantial intratumoral hypoxia due to tumor vessel destruction which in turn enhanced CXCL12 expression and subsequent infiltration of CXCR4+ TEMs. Conversely, combined CA4P treatment and CXCR4 inhibition restrained TEM influx, enhanced CA4P-induced tumor necrosis, and thus sustained response and further reduced tumor burden [128]. It is important to reiterate that the different intratumoral myeloid cells express a redundant profile of multiple angiogenic factors that enables them to compensate for each other if necessary. Indeed, depletion of TAMs lead to an increase of neutrophils and targeting TANs cause an enhanced influx of TAMs, an oscillating pattern between these immune cell populations that disabled the efficacy of antiangiogenic therapy in the pancreatic Rip1Tag2 tumor model [89]. While most tumor model systems will respond to antiangiogenic therapy by slowing down tumor growth, PNETs in Rip1Tag2 mice first responded very well to VEGF signaling blockade accompanied by tumor growth inhibition and vessel normalization, but then exhibited an pro-angiogenic relapse within weeks dependent on the specific drug regimen [124, 129, 130]. During the response phase, intratumoral myeloid cells in PNET tumors became angiostatic and immunostimulating which was associated with the upregulation of CXCL14 and other angiostatic chemokines leading to an influx of cytotoxic CD8 cells. In relapsing tumors, however, myeloid cells converted back into an immunosuppressive and angiogenic phenotype and CD8 influx stopped. The underlying mechanism is based on the activation of PI3K γ signaling in myeloid cells by hypoxia-induced factors like CXCL12 and IL6 that rendered the cells insensitive to VEGF/R inhibition. PI3K γ activated myeloid cells promoted reneovascularization even in the presence of VEGF/R inhibitors and caused a proangiogenic tumor relapse [89]. In support of these results, myeloid PI3K γ signaling was shown to inhibit NF κ B while stimulating C/EBP β activation, thereby inducing a transcriptional program that promoted immunosuppression [131]. Thus, pharmacological inhibition of myeloid PI3K γ improved and sustained the tumor response to antiangiogenic therapy by converting all innate immune cells to an angiostatic and immunostimulatory state associated with enhanced cytotoxic T cell infiltration and activity [89].

5. Antiangiogenic Therapy meets Immunotherapy

Various studies including those mentioned above, have provided compelling evidence that antiangiogenic therapy is most efficacious when an immunostimulatory environment is

generated. This is in agreement with the concept of vascular normalization to reduce hypoxia and polarize innate immune cells to an immunosupportive phenotype. Emerging from these studies is the proposition that angiogenesis and inflammation are reciprocally regulated and that immune cells play a pivotal role in regulating both processes. It also underscores the tumor's vigilance to conserve an immunosuppressive and angiogenic milieu to overcome growth restrictions and escape immune surveillance. Current clinical immunotherapies, most notably those using immune checkpoint inhibitors (ICI) that reactivate exhausted cytotoxic T-cells, have shown unprecedented outcomes in patients with melanoma and non-small lung cancers which lead to the accelerated FDA-approval of ipilimumab, an inhibitory antibody against the immune checkpoint receptor CTLA4 and pembrolizumab, an antibody against the PD1 receptor [132-136]. The beneficial effects by ICIs are however only observed in 15-40% of patients and there is currently no clear explanation for the differing responses. Besides insufficient abundance of tumor antigens and redundancy of negative checkpoint regulators, lack of sufficient cytotoxic T-cell-infiltration into the tumor has been suggested as a major hurdle for several tumor types. As anti-angiogenic therapy modulates the tumor vasculature and thus can enhance T-cell infiltration as well as generate myeloid cells with immunosupporting features, combining antiangiogenic therapies with ICI or immunotherapies that modulate the immune system are attractive options to increase the percentage and endurance of efficacy in cancer patients. In support, combination of the VEGFR inhibitor axitinib and anti-CTLA4 but not monotherapies substantially prolonged survival in a melanoma mouse model by increasing effector T-cell influx and dendritic cell maturation and reducing intratumoral MDSC [137]. The rationale of antiangiogenic immunotherapy is further supported by recent studies in which tumors relapsing from antiangiogenic therapy using an anti-VEGF or dual anti-VEGF-Ang2 antibody, upregulated the negative immune checkpoint regulator PD-L1 in tumor and stromal cells [38, 41]. This lead to immunosuppression triggered by PD-L1 binding PD-1 on the surface of activated T-cells to produce T-cell anergy or exhaustion. Combining immunotherapy using anti-PD-L1 with antiangiogenic therapy (either anti-VEGF or anti-VEGF/Ang2) had reciprocal beneficial effects in that immunotherapy targeted evasion from antiangiogenic therapy, while vascular normalization elicited by antiangiogenic treatment could increase lymphocyte infiltration and activation [38, 41]. In addition, antiangiogenic immunotherapy induced a specialized form of blood vessels in treated tumors that benefited from the therapy reminiscent of high endothelial venules (HEVs). HEVs are specialized blood vessels typically found in secondary lymphoid organs where they facilitate lymphocyte trafficking [138, 139]. They also have been found to sporadically appear in tumors and metastasis of patients forming tertiary lymphoid structures where they correlated with good prognosis and patient survival [138]. Congruently, intratumoral HEVs in these tumor models substantially enhanced CTL infiltration, activity and tumor cell destruction leading to improved outcome [38]. Little is known about the mechanistic underpinnings for HEV induction in tumors but it is likely to be dependent on the LIGHT/lymphotoxin- β receptor (LT β R) signaling axis in tumor-associated endothelial cells which was induced in HEV+ tumors [38, 140, 141]. In support, vessel-directed targeting of LIGHT peptide in experimental tumors normalized blood vessels and induced HEV formation and enhanced intratumoral lymphocyte infiltration [140]. Moreover, LT β R activation could also sensitize tumors to antiangiogenic immunotherapy by enhancing T-cell influx suggesting that

treatments that induce not only vessel normalization but also intratumoral HEVs may enhance the therapeutic effects with immunotherapy [38, 141]. These preclinical studies support the notion that anti-angiogenic therapy can improve immunotherapy by supporting vascular changes such as vessel normalization and HEV formation but also demonstrated that immune checkpoint inhibitors can sensitize and prolong efficacy of VEGF signaling blockade. In line with this concept, a recent study demonstrated T-lymphocyte infiltration promoted blood vessel normalization in tumors [94, 142]. Mice depleted of CD4 and CD8 T-cells developed tumors that exhibited more abnormal tumor vessels and hypoxic areas than those of wildtype mice, while activation of cytotoxic T-cells through checkpoint immunotherapy (anti-PD1 and/or anti-CTLA4) or adoptive TH1 transfer in models of murine tumors and patient-derived tumors induced blood vessel normalization and reduced both hypoxia and metastases. Indeed, transcriptional profiling of patient tumors revealed a “blood vessel normalization” signature associated with good prognosis that also implemented the T-cell receptor signaling pathway [94, 142]. These studies provide strong evidence in human tumors that blood vessel normalization and T-lymphocyte infiltration can amplify the positive effects conferred by each individual component. What all of these studies reveal is the notion that tumor vessels need to be remodeled to enable enhanced T-cell infiltration and improve immunotherapies while immunotherapies themselves can contribute to a more functional tumor vasculature providing a strong positive feedback loop that can help to restrain and eradicate tumor growth.

Conclusions

Currently, various combinatorial treatment modalities of angiogenic inhibitors, specifically those targeting the VEGF/R and Ang2/Tie axis with immune checkpoint inhibitors and other immunotherapies are evaluated in the clinical setting [116]. These trials have been initiated based on compelling evidence from mouse tumor model systems that such a combination modulates both the tumor vasculature and the immune system to foster an immunosupportive environment and thus improves and endures efficacy in cancer patients. While these clinical trials are still ongoing, the first randomized study (IMmotion150 (NCT01984242) was just published which evaluated the clinical activity of the combination of anti-PD-L1 (atezolizumab) with or without bevacizumab, against the standard-of-care angiogenic tyrosine kinase inhibitor, sunitinib, in patients with untreated renal cell cancer (mRCC) [143]. The combination of anti-PD-L1 plus bevacizumab produced encouraging and superior efficacy versus sunitinib, specifically in the subgroup of patients with tumors expressing PD-L1. While tumor mutation and neoantigen burden was not associated with progression-free survival (PFS), angiogenesis, T-effector/IFN- γ response, and myeloid inflammatory gene expression signatures were strongly and differentially associated with PFS within and across the treatments suggestive of using those as potential biomarkers [143] [144]. Notable is also the identification of myeloid inflammation as a potential resistance mechanism to anti-PD-L1 monotherapy in mRCC which may be overcome by the addition of bevacizumab. These first clinical results are encouraging and indeed confirm some of the results obtained in the preclinical setting. Results from other clinical trials will hopefully soon be available to validate the potency of antiangiogenic immunotherapy cocktails in improving the efficacy and survival of cancer patients.

Acknowledgments

Sources of funding

This work was supported by grants from the ERC CoG ImmunoFIT Methusalem (to MM) and from the Flamish government FWO (G066515N) (to MM) and FWO (G0A0818N) (to GB) and the Belgian Association Against Cancer for the project 2014-197 (to MM) and from the National Institute of Health NIH/NCI (to GB).

Abbreviations

VEGF

vascular endothelial growth factor

TNF- α

tumor-necrosis-factor α

PDGFB

platelet-derived growth factor B

PIGF

placental growth factor

Sema3A

Semaphorin3A

TAM

Tumor-associated macrophages

TEM

Tie2-expressing macrophages

TAN

Tumor-associated neutrophils

PD-L1

programmed cell death 1 ligand 1

PD-1

programmed cell death receptor 1

PyMT

virus-polyoma middle T- antigen

ICI

immune checkpoint inhibitors

(ICB)

immune checkpoint blockade

VCAM

vascular cell adhesion molecule

ICAM

intercellular adhesion molecule

iDC

immature dendritic cell

DC

dendritic cell

CTL

cytotoxic T-cell

Treg

regulatory T-cell

ROS

reactive oxygen species

NO

nitric oxide

iNOS

inducible nitric oxide synthase

Arg1

arginase-1

IL-10, -4, -13, -12, -23, -1b, -8

interleukin-10, -4, -13, -12, -23, -1b, -8

TGF β 1transforming growth factor- β 1**GM-CSF**

granulocyte/monocyte-colony stimulating factor

CSF-1

colony stimulating factor-1

G- or M- MDSC

granulocytic or monocytic-myeloid derived suppressor cell

/Th2M2-TAM

M2-polarized macrophage

FGF-1, -2, -13

fibroblast growth factor-1, -2, -13

PDGF

platelet-derived growth factor

TNF α tumor necrosis factor- α **NK-cell**

natural killer cell

Th1

T-helper 1

FAO

fatty acid oxidation

IDO

Indolamine 2,3-diogenase

LPS

lipopolysaccharide

M_{stim}

Immune stimulatory macrophage

M_{supp}

Immune suppressive macrophage

OXPFIOS

oxidative phosphorylation

PFKFB3

6-Phosphofructo-2-Kinase/Fructose-2,6-Biphosphatase 3

T_{eff}

Effector T-cell

T_H

T-helper cell

T_n

naïve T-cell

T_m

mature T-cell

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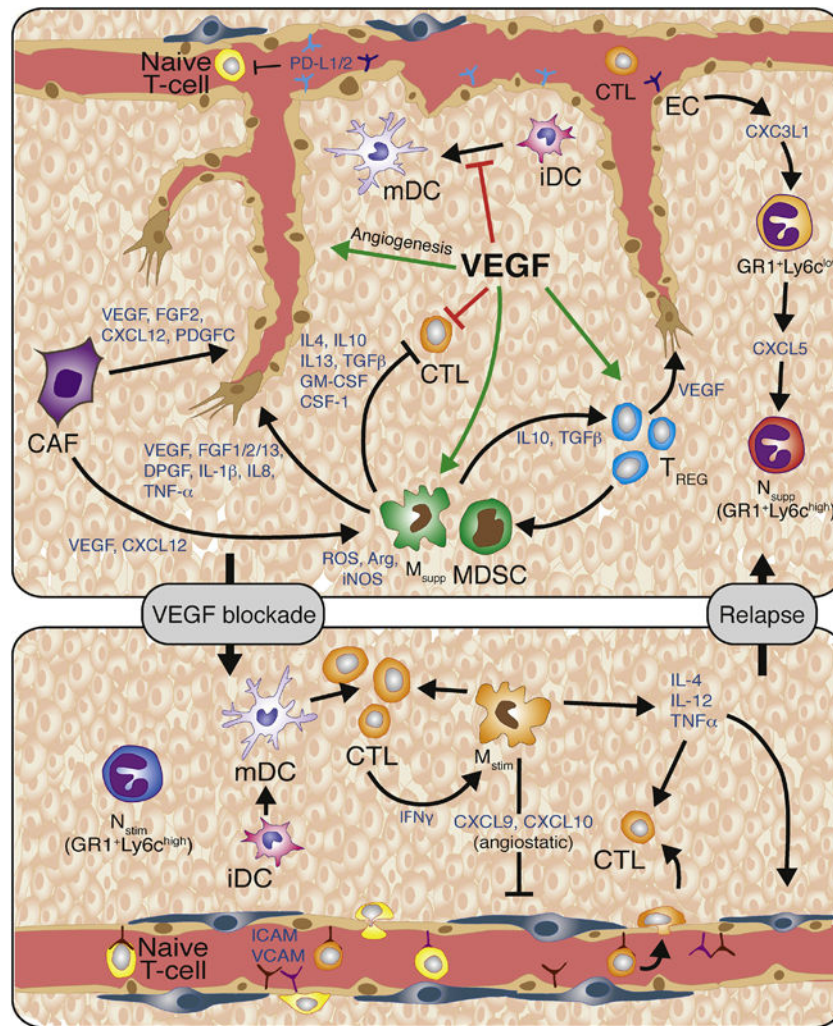


Figure 1. VEGF/VEGFR signaling promotes angiogenesis and suppresses antitumor immunity
 VEGF promotes endothelial cell proliferation, migration and survival. VEGF also promotes immunosuppression by inducing endothelial cell expression of the PD1 ligands PD-L1 and PD-L2 that interact with PD-1 on T-cells resulting in reduced T-cell proliferation and effector function. VEGF also inhibits T-cell-adhesion to the luminal surface of the vasculature and subsequent extravasation into the tumor by blocking TNF α -induced expression of VCAM and ICAM. VEGF can also directly block dendritic cell function by inhibiting dendritic cell maturation and inducing PD-L1 expression on mature dendritic cells. It inhibits the proliferation and effector function of cytotoxic T-cells, while inducing regulatory T-cell (Treg) proliferation. Tregs secrete various cytokines and growth factors, including IL-10, IL-4, IL-13, TGF β 1, GM-CSF, and CSF-1, which recruit angiogenic and immune-suppressive MDSC and TAMs. MDSC and TAMs then produce reactive oxygen species, nitric oxide, and arginase to suppress T-cell proliferation, viability, and activity. Thus, inhibition of VEGF should restore many of these phenotypes. Congruently, VEGF-blockade enables the endothelium to facilitate T-cell infiltration due to vessel normalization accompanied with elevated levels of I-CAM and V-CAM lymphocyte adhesion molecules. Furthermore, VEGF inhibition enables dendritic cell maturation and function, and thus

increases levels of intratumoral effector T-cells. Anti-VEGF therapy also increases the presence of Th1/M1-immunosupportive/angiostatic myeloid cells (e.g; macrophages, neutrophils). Subsequently, VEGF-blockade should unleash the anti-tumor immune response and lead to increased tumor cell apoptosis. Further vessel pruning, however, creates hypoxic areas that drive the recruitment and polarization of immunosuppressive and angiogenic innate immune cells. Therefore, therapeutic approaches that promote immunosurveillance, can enhance and sustain the efficacy of antiangiogenic therapy. Abbreviations: VEGF, vascular endothelial growth factor; PDL1/2, programmed death ligand 1/2; VCAM, vascular cell adhesion molecule; ICAM, intercellular adhesion molecule; iDC, immature dendritic cell; DC, dendritic cell; CTL, cytotoxic T-cell; Treg, regulatory T-cell; ROS, reactive oxygen species; NO, nitric oxide; Arg1, arginase-1; IL-10, -4, -13, -12, -23, -1b, -8, interleukin-10, -4, -13, -12, -23, -1b, -8; TGF β 1, transforming growth factor- β 1; GM-CSF, granulocyte/monocyte-colony stimulating factor; CSF-1, colony stimulating factor-1; G- or M- MDSC, granulocytic or monocytic-myeloid derived suppressor cell; M2- TAM, M2-polarized macrophage; FGF-1, -2, -13, fibroblast growth factor-1, -2, -13; PDGF, platelet-derived growth factor; TNF α , tumor necrosis factor- α NK-cell, natural killer cell; Th1, T-helper 1.

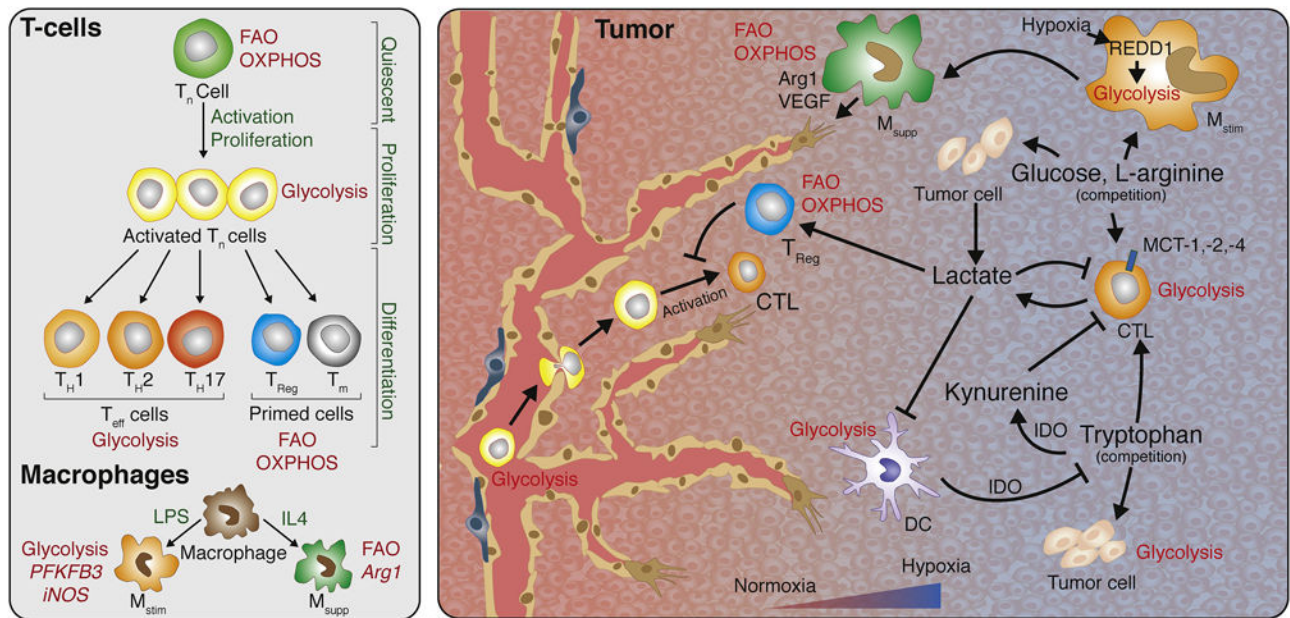


Figure 2. Metabolic competition in the tumor microenvironment.

Left: Metabolic changes during T-cell development, activation, and differentiation.

Quiescent T_n cells rely primarily on FAO and OXPHOS, but change to glycolysis for rapid proliferation when activated. Upon further differentiation T_H1, T_H2 and T_H17 cells remain glycolytic, while T_{Reg} and T_m cells switch back to FAO and OXPHOS. Unlike LPS stimulated M_{stim} macrophages that are characterized by a primarily glycolytic metabolism, IL4 stimulated M_{supp} macrophages are characterized by an oxidative metabolism. Right: Intratumoral competition for metabolites. Immune cell function in the tumor microenvironment is strongly regulated by the oxygen and nutrient availability. Poorly perfused areas can induce a hypoxic response, stimulating glycolysis and lactate dependent acidification. These environmental changes affect macrophage polarization and immune cell function. Lactate as by-product of glycolysis directly suppresses CTLs and DCs, but can be used as carbon source for T_{Reg} cells, promoting an immune suppressive tumor microenvironment. In addition to tumor cells, several immune cells including CTLs, M_{stim} macrophages (regulated by a REDD1 dependent hypoxia response), and DCs rely on glycolysis, making them compete for the available glucose. Other nutrients such as the amino acids L-arginine and tryptophan are also subjected to cellular competition. Several intratumoral cell types are L-arginine auxotroph, and both tumor cells and CTLs are depending on tryptophan for their function. Increased IDO activity in tumors results in tryptophan depletion and formation of the immune suppressive kynurenine, rendering the tumor microenvironment immunosuppressive. Arg1, arginase 1; CTL, cytotoxic T-lymphocyte; DC, dendritic cell; FAO, fatty acid oxidation; IDO, Indolamine 2,3-diogxygenase; IL4, interleukin 4; iNOS, inducible nitric oxide synthase; LPS, lipopolysaccharide; M_{stim}, Immune stimulatory macrophage, M_{supp}, Immune suppressive macrophage OXPHOS, oxidative phosphorylation; PFKFB3, 6-Phosphofructo-2-Kinase/ Fructose-2,6-Biphosphatase 3; T_{eff}, Effector T-cell; T_H, T-helper cell; T_n, naive T-cell; T_m, mature T-cell; T_{Reg}, regulatory T-cell; VEGF, vascular endothelial growth factor.