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## Review

## The dark side of tumor-associated endothelial cells

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## ABSTRACT

Angiogenesis is a hallmark of cancer and a requisite that tumors must achieve to fulfill their metabolic needs of nutrients and oxygen. As a critical step in cancer progression, the ‘angiogenic switch’ allows tumor cells to survive and grow, and provides them access to vasculature resulting in metastatic progression and dissemination. Tumor-dependent triggering of the angiogenic switch has critical consequences on tumor progression which extends from an increased nutrient supply and relies instead on the ability of the tumor to hijack the host immune response for the generation of a local immunoprivileged microenvironment. Tumor angiogenic-mediated establishment of endothelial anergy is responsible for this process. However, tumor endothelium can also promote immune tolerance by unbalanced expression of co-stimulatory and co-inhibitory molecules and by releasing soluble factors that restrain T cell function and induce apoptosis. In this review, we discuss the molecular properties of the tumor endothelial barrier and endothelial anergy and discuss the main immunosuppressive mechanisms triggered by the tumor endothelium. Lastly, we describe the current anti-angiogenic therapeutic landscape and how targeting tumor angiogenesis can contribute to improve clinical benefits for patients.

## 1. Introduction

## 1.1. The angiogenic switch

The proliferation of every normal cell in our body is finely tuned by a network of growth-promoting and inhibitory mechanisms, in the form of soluble factors, physical stress and cell–cell interactions. When homeostatic control succumbs or is hijacked, the cell fails to continue as master of its own destiny within the tissue architecture. Unregulated proliferation and resistance to apoptosis represent two of the first critical events in tumor transformation; afterwards, tumor progressively evolves, driven by genomic instability, which in turn promotes acquisition of new functions and sculpts anti-tumor immunity in a process called “cancer immunoeediting” [1,2]. The contribution of the immune system to tumor evolution reflects a double-edged sword that restricts tumor growth in the elimination and equilibrium phases of cancer but eventually succumbs to tumor modulation by supporting cancer progression and metastatic spread during the escape phase. According to this hypothesis, tumors cannot be considered merely a mass of neoplastic and polarized stromal cells but rather a conductor, which exploits physiological immune regulatory mechanisms to generate peripheral and local immune tolerance, establishing a tumor-promoting

microenvironment that supports its own growth and ability to metastasize.

Neoplastic cells are characterized by specific hallmarks including undefined proliferation, evasion of growth suppressors, replicative immortality, resistance to cell death, invasive properties and ability to manipulate the local microenvironment by inducing angiogenesis and promoting immune system evasion [3]. Within blood vessel generation, vasculogenesis (assembly of the de novo vasculature assisted by recruitment of endothelial progenitors cells) and angiogenesis (sprouting of new vasculature from already established vessels, a process guided by proliferation of endothelial cells – ECs) are both active during organogenesis. Angiogenesis predominates in the adult and this term was introduced more than 200 years ago by the surgeon John Hunter to describe the growth of new blood vessels during tissue development in adult animals [4]. However, the players involved in angiogenesis and its role in promoting cancer progression were described quite recently by Judah Folkman [5] and then characterized by others [6,7]. Under physiological state, the endothelium is in a quiescent condition, maintained by a finely-tuned homeostatic process, that can be interrupted periodically (e.g. during female reproductive cycle), either by a reduction in angiostatic molecules or an increase in angiogenic factors according to the requirements of each body tissue. In each of these cases,

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after local basal membrane degradation, ECs change shape and begin proliferating by sensing a gradient of pro-angiogenic signals, invading the surrounding stroma and generating new capillaries. Primary angiogenic mediators include the vascular endothelial growth factor (VEGF) family proteins, fibroblast growth factors (FGF), platelet-derived growth factor (PDGF), placental growth factor (PIGF), angiotensin (ANG) 2, chemokines and cytokines such as chemokine (C-X-C motif) ligand (CXCL)8, tumor necrosis factor (TNF)  $\alpha$ , interleukin (IL) 1 $\beta$ , tumor growth factor (TGF)  $\beta$ , prokineticin (BV8), and matrix metalloproteases (MMPs). Angiogenesis is restricted by several molecules with angiostatic properties such as thrombospondin (TSP) 1, angiostatin, soluble VEGF1, endostatin, vasostatin, calreticulin, tissue inhibitor of metalloproteases (TIMPs), as well as cytokines such as interferon (IFN) $\gamma$ , IFN-induced cytokines binding CXCR3 (CXCL9/MIG, CXCL10/IP10, CXCL11/IP9) [8], and others [9]. The expression of angiogenic factors is finely tuned by local oxygen levels through hypoxia inducible factor (HIF). This protein is a heterodimer composed by HIF1 $\alpha$  and HIF1 $\beta$  able to activate the expression of genes through the binding to hypoxia response element (HRE) sequences placed in their promoter regions. Both subunits are constitutively expressed but HIF1 $\alpha$  is quickly hydroxylated under normoxic conditions [10], ubiquitinated and degraded after translation [11]. Once a tumor reaches the size of few millimeters, the simple diffusion of oxygen and nutrients from the surrounding tissue is not sufficient to support cell growth. This results in a condition of low oxygen concentration that stabilizes HIF1 $\alpha$  which can then enter the nucleus, dimerize with HIF1 $\beta$ , and trigger the expression of many angiogenic factors (VEGFs, PDGFB, PIGF, ANGPTs) [12], proangiogenic chemokines (stromal cell derived factor 1 $\alpha$  - SDF1 $\alpha$ /CXCL12 and sphingosine 1 phosphate) and receptors (CXCR4 and sphingosine 1 phosphate receptor) [13], the so called “angiogenic switch”. This process generates chronic endothelium activation, which results in continuous sprouting of new vessels supporting tumor growth [14]. Induction of angiogenesis represents an early event during tumorigenesis [15] for two biological reasons: an elevated proliferation rate requires high consumption of nutrients and oxygen and removal of toxic metabolic products, both duties performed by blood circulation. Furthermore, proliferative switches (e.g. RAF and RAS activation) induce activation of the angiogenic program [16,17]. VEGF members include VEGFA, VEGFB, VEGFC, VEGFD and PIGF and exert their angiogenic functions by interacting with the VEGF receptors (VEGFRs). VEGFRs are tyrosine kinase receptors (TKRs) that bind each of these ligands, triggering a tyrosine kinase-based signaling cascade: VEGFR1 is triggered by VEGFB and PIGF to induce haematopoiesis, monocyte migration, and EC metabolism, whereas VEGFR2 is activated following VEGFA binding to induce EC proliferation, survival, angiogenesis and vascular permeability. VEGFC and VEGFD are involved in activating lymphangiogenesis (proliferation of lymphatic endothelial cells – LECs) by triggering VEGFR3 [18]. FGF binds to FGFR and induces the proliferation and migration of ECs [19]. FGFR triggering activates mitogen activated protein (MAPK) and phosphatidylinositol-3 (PI-3) kinases through the adapter protein fibroblast growth factor receptor substrate (FRS2 $\alpha$ ). The latter also interacts with VEGFR2, inducing extracellular signal-regulated kinase (ERK1/2) mediated VEGFR up-regulation, potentiating VEGF-mediated ECs activation and suggesting a mechanism of cooperation between two pathways [20,21]. In alignment with these findings, FGF inhibition critically affects VEGFA-induced angiogenesis [22].

Another feature of tumor angiogenesis is the presence of polarized ECs, characterized by expression of growth factor receptors (e.g. EGFR) [23] and tumor endothelial markers (TEM) [24], up-regulation of angiogenic receptors and constitutive activation of survival PI3K-AKT signaling pathway [25,26], which confer augmented proliferation, migratory and drug resistance capabilities compared to normal ECs. These genetic and phenotypic differences result in morphological, structural and functional abnormalities of tumor-associated blood vessels. The abnormalities include enlarged vessels with severe branching,

multilayered and discontinuous EC alignment with defective coverage by pericytes and basement membrane, resulting in a dysfunctional vasculature characterized by low tissue perfusion, leakiness and poor blood flow [27].

The generation of an actively growing solid tumor mass with a high cellular proliferative rate significantly reduces the availability of O<sub>2</sub>, especially for the cells found within the inner tumor core. Tumor and stromal cells subsequently release augmented levels of pro-angiogenic factors, especially VEGFA, angiotensin (ANGPT) 2, and CXCL12, driven by the activation of HIF1 [28,29]. Another consequence of reduced O<sub>2</sub> availability is the metabolic change from oxidative phosphorylation to aerobic glycolysis, which results in dramatically enhanced glucose consumption and elevated accumulation of lactate with consequent acidosis of the tumor microenvironment. These hypoxia-dependent metabolic modifications are associated with drug resistance and support angiogenesis at both the tumor and stromal levels. Within tumor cells, nutrient deprivation and acidosis stabilize VEGFA mRNA [30], whereas tumor-derived lactate supports angiogenesis and tumor growth by activating ECs via an autocrine NF- $\kappa$ B-CXCL8 pathway that promotes migration and tube formation [31] and polarizes tumor-associated macrophage (TAM) towards a proangiogenic M2-like phenotype [32]. CXCR4 is expressed by many cell types including leukocytes, ECs, epithelial and cancer cells. CXCL12-CXCR4 interaction is directed involved in chemotaxis, invasion and recruitment of ECs to neoangiogenic niches to promote blood vessel sprouting [33]; moreover, the CXCL12-CXCR4 axis fosters angiogenic responses by activating AKT signaling and consequent VEGF synthesis in cancer cells [34]. Thus, blood vessel sprouting within tumors reflects a process in which tumor and stromal cells cooperate and synergize to sustain tumor growth and invasion.

Tumor stroma is composed of two main categories of cells, classified according to their origin surrounded by an extracellular matrix (ECM): bone marrow-derived, tumor infiltrating hematopoietic cells (predominantly leukocytes), and tissue resident cells, such as ECs, pericytes, adipocytes, fibroblasts and resident macrophages. Within the tissue-resident stromal cells, the most important angiogenic players are cancer-associated fibroblasts (CAFs) and pericytes. TGF $\beta$ -activated CAFs participate in tumor angiogenesis by directly secreting angiogenic factors such as VEGFA, bFGF, CXCL12 and by modifying the composition and stiffness of the ECM and the local interstitial pressure, collectively contributing to tumor progression [35,36]. Pericytes physically surround the vessels and play a crucial role in regulating endothelial proliferation and in promoting ECs survival and establishment of tight junctions [37]. However, cancer polarized-pericytes may support the survival of tumor blood vessels since their targeting in combination with anti-angiogenic inhibitors improves treatment efficacy compared to each single agent in a preclinical model of pancreatic islet carcinoma [38]. Within the tumor-infiltrating, bone marrow-derived leukocyte component, TAMs, granulocytes and myeloid derived suppressor cells (MDSCs) are the most abundant and representative angiogenic inducers. TAMs can either support or restrict blood vessel sprouting, according to the immune context and specific microenvironment to which they are exposed [39]. M2-polarized TAMs induce angiogenesis by directly releasing high amounts of growth factors such as VEGFA, VEGFC, PIGF, basic fibroblast growth factor (bFGF), platelet derived growth factor  $\beta$  (PDGF $\beta$ ), cytokines such as IL1 $\beta$ , or by producing membrane-bound or soluble proteases such as cathepsins or MMPs, which mobilize proangiogenic molecules sequestered in the ECM and remodel it supporting EC invasion [40–42]. Moreover, TAMs may indirectly promote angiogenesis by releasing inflammatory cytokines (IL6, CXCL8), which support the recruitment and activation of other myeloid subsets such as granulocytes and MDSCs [40]. Granulocytes and MDSCs are another main source of proangiogenic factors, especially VEGFA, bFGF, MMP9, BV8, whose expression is up-regulated following triggering of CSF3R and STAT3 activation [43]. In support of neutrophil and MDSC proangiogenic role, many researchers have

described how their depletion can correlate with impaired angiogenesis [44,45]. Tumor-infiltrating MDSCs have also been shown to contribute to tumor angiogenesis and have recently been implicated in tumor resistance to anti-angiogenic therapy [46]. CSF-1 regulates the tumor-mediated recruitment of monocytic MDSCs and pharmacologic blockade of CSF1R inhibits angiogenesis by reducing the expression of proangiogenic and immunosuppressive genes, restricting *in vivo* the growth of 3LL lung carcinoma tumor when used in combination with anti VEGFR2 [47]. T lymphocytes as well may either induce or impair tumor angiogenesis according to the cytokines they produce: T helper (Th)1 and IFN $\gamma$  cytokines act directly on the endothelium by inducing maturation of ECs and indirectly on TAMs by promoting their polarization towards M1 phenotype [48,49]. In stark contrast, Th2 cytokines support TAM skewing towards the M2 phenotype, whereas T regulatory (Treg) lymphocytes act directly on endothelium by secreting angiogenic factors [50], and on effector and Th1 T cells, by inducing their anergy [51]. More detailed mechanisms and players of cancer-dependent angiogenesis can be found elsewhere in recent reviews [52,53]; here we focus on the functional consequences of tumor endothelial dysfunction in fine-tuning the local immune response to favor cancer progression.

### 1.2. Leukocyte infiltration and prognostic value

The field of cancer immunotherapy began with the demonstration that the immune system is able to recognize and eventually control (especially in the early phase of carcinogenesis) tumor proliferation by innate and adaptive immunity. The first proof of concept, now widely accepted, was provided more than one hundred years ago by Dr. Coley who elicited tumor regression in inoperable sarcoma patients by local injection of bacterial toxins derived from *Streptococcus pyogenes* and *Serratia marcescens* [54]. The rationale of this approach originated from the observation that patients with unresectable sarcoma cancer experienced tumor regression after being accidentally infected by *Streptococcus pyogenes*; Coley hypothesized that cancer rejection was mediated by host immune response reactivation directed towards tumor. Then, other investigators provided clear preclinical and clinical evidence of host immune system involvement in tumor rejection. Dr. Gross showed that immune system can recognize spontaneous tumors induced by a carcinogenic drug in mice [55], 22 years prior identifying the cells involved in this process, namely T lymphocytes. Taken together, these findings show that tumors can be viewed as antigenic, even if often poorly immunogenic.

The vertebrate immune system is mainly divided into innate and adaptive immune responses that together works in tight synergy to resolve pathological situations (from pathogen infection to cancer development). The main difference between the two immunological arms relies in the target recognition mechanism. The innate immune response recognizes generic foreign motifs, while the adaptive immune response targets virtually any foreign antigen generating immunological memory. The innate arm of the immune system participates both as a first line defense and in the subsequent activation of the adaptive immune response by presenting antigens to B and T cells. Host immune-dependent tumor restriction is mediated by the activation of both arms of immune system; however, T lymphocytes are considered the main players owing to their ability to recognize specific tumor antigens, expanding in great numbers and killing antigen-expressing cells. Nevertheless, the ability of the host immune system to trigger a tumor-specific immune response fails to control tumor growth eventually, due to immune evasion strategies orchestrated by evolving tumors. Notwithstanding the encouraging preclinical results of many cancer immunotherapy approaches based on re-activating established, low frequency tumor-specific T cells or educating the host immune system to recognize and attack the tumor, clinical results do not reach expectations when translated into the clinic. Contributing to this lack of robust effectiveness, treatment efficacy widely varies among patients and tumor histology [56]. Failure of standard chemotherapy and new

immunotherapy approaches is in part explained by the impaired ability of these drugs and therapy to reach their intended target, owing to tumor-associated blood vessel dysfunction and the action of tumor-recruited stromal cells, which selectively inhibits cell infiltration and edits specific leukocyte subsets [57]. The critical role of tumor endothelium in either promoting or restricting the delivery of drugs and leukocytes in the tumor bed has been described by many research groups; the identification of tumor-infiltrating lymphocytes (TIL) within the tumor mass represents indeed a good prognostic factor for patient survival within different tumor types, such as colorectal [58], ovarian [59] melanoma, renal, breast, bladder, lung, prostatic, head and neck, lung and esophageal tumors [60]. Additionally, the composition, physical location and phenotype of CD3<sup>+</sup> T cells have become an important clinical attribute considered even more predictive than standard histopathology (*i.e.* tumor stage) and aptly named “immunoscore” [61]. The presence of an inflamed tumor with a high immunoscore is typical of immunogenic tumors, such as melanoma. However, about 40% of melanoma tumors do not harbor any TILs [62]. Even if there is a positive correlation between tumor mutation load and response to immunotherapy [63,64], higher numbers of mutations do not guarantee higher tumor immunogenicity since an immunosuppressive tumor microenvironment and defective antigen presentation machinery may limit the elicitation or maintenance of a tumor-specific immune response. Tumor endothelium directly and indirectly supports all these mechanisms of immune evasion triggered by tumor. More specifically, tumor endothelium plays a critical role in orchestrating the trafficking of unique leukocyte subsets (including T cells). Moreover, endothelium can directly present antigens to T cells affecting their activation status.

### 1.3. Endothelium directs leukocyte traffic to cancer

Leukocytes are recruited into peripheral tissues following extravasation from blood vessels; in a dysfunctional endothelial environment, such that exists in a tumor context, this process is severally impaired. The transmigration of leukocytes outside the vessel is a multi-step, finely controlled procedure which includes rolling, slow rolling, activation, adhesion strengthening, intraluminal crawling, paracellular and transcellular migration [65]. Many molecular actors involved during each of these phases are critical for efficient transmigration. During the rolling phase, leukocytes start interacting with ECs through the action of selectins: leukocyte-derived L-selectins and endothelial P-selectin bind to P-selectin glycoprotein ligand 1 (PSGL1) [66,67]. Leukocytes may interact with endothelial E-selectin through CD44 and E-selectin ligand 1 (ESL1)[68]. PSGL1-L-selectin mediated leukocyte–leukocyte interactions allows the capture of leukocytes not expressing ligands for either P or E selectins on the inflamed endothelium, in a process called secondary tethering. Leukocyte adhesion is promoted by selectin binding under normal conditions of blood flow, since cells detach when flow is stopped [69]. Leukocyte interaction on endothelium during rolling and adhesion steps is promoted as well by other mechanisms, such as integrin-mediated interaction with adhesion molecules; lymphocytes, for example, can roll *via* vascular cell-adhesion molecule 1 (VCAM1) interaction with very late antigen 4 (VLA4) [70]. Similarly, lymphocyte function-associated antigen 1 (LFA1) binding to endothelial intercellular adhesion molecule 1 (ICAM1) synergizes with selectins in lymphocytes rolling [71] *in vitro*. Rolling and slow rolling on endothelium is preliminary for leukocytes activation, which is triggered by the interaction of chemokines, either released or exposed on ECs after inflammatory stimuli, with cognate receptors expressed on leukocytes and result in integrin-dependent firm adhesion. These chemokines bind G-protein-coupled receptor (GPCR) on leukocytes, triggering an inside-outside signaling cascade that culminates with almost instantaneous integrin activation. The signaling cascade induced by GPCRs includes three steps: phospholipase C activation, GTPases activation and triggering of integrin structural changes through the interaction with actin binding proteins. For instance, CCL21, SDF,

macrophage inflammatory protein (MIP3) $\alpha$  and MIP3 $\beta$  induce arrest of rolling lymphocytes within one second under flow conditions through ICAM1 binding [72]. Platelets contribute to leukocyte activation by depositing CCL5 and CXCL4 on endothelium [73]. Chemokines can also synergize with leukocyte activation by heterophilic interactions; for example CXCL4 heterophilic interactions with CCL5 amplifies the adhesion of monocytes to endothelium. [74]. Shulman et al. [75] described the crucial role of activated endothelium-derived chemokines in supporting the transendothelial migration of Th1 and cytotoxic T cells, rather than rolling and adhesion steps. Interestingly, these chemokines are not necessarily exposed on the EC membrane, but stored in vesicles found beneath the plasma membrane and released during tight lymphocyte-endothelial synapse formation, guiding T cell migration [75]. Taken together, these observations suggest that the chemo attractant availability on the endothelium, expression levels of chemokines receptors on leukocytes, as well the affinity for their ligands, and the downstream complex signaling cascade, all contribute and consequently affect leukocyte activation and adhesion to the endothelium.

Leukocyte transmigration to the periphery includes overcoming the integrity of three barriers: endothelial cells, pericytes and the basement membrane. Before crossing the blood vessel, leukocytes crawl on endothelium seeking the best transmigration route. Strict interactions between adhesion molecules on ECs and cognate integrins on leukocytes and the association of cytoplasmic proteins trigger the generation of docking structures that promote and initiates transendothelial migration [76]. This process can be achieved by two main routes: paracellular migration, which consists of leukocyte crossing along endothelial cell–cell junctions and transcellular migration, in which leukocytes cross directly through EC body. The former is promoted by adhesion-molecule triggering, especially ICAM1, platelet endothelial cell adhesion molecule (PECAM)1, junctional adhesion molecule (JAM) 1, CD99, and endothelial cell selective adhesion molecule (ESAM). Transcellular migration is less common and supported by the generation of vesiculo-vacuolar organelles within ECs, which are membrane-associated gateways for leukocyte crossing through the EC body [77]. This process seems to be guided by the same adhesion molecules involved in paracellular transmigration, such as ICAM1, and requires the stabilization action of actin [78]. Leukocytes overcome pericytes and basement membrane barriers in regions where their presence is less consistent. For example, lower expression of laminin 10, collagen IV, and nidogen-2 in untreated murine cremasteric venules were associated with lower pericyte coverage and used as main migration routes by neutrophils [79]. This non-disruptive transmigration route, however, could be enhanced by inflammatory stimuli (such as IL1 $\beta$ ), which enlarge these transmigration areas by lowering the amount of basement protein coverage [79].

Given the multi-step, organization and complexity of the transmigration process, it is clear that during pathological processes, such as chronic inflammation and cancer, endothelium dysfunction can dramatically affect leukocyte migration into peripheral tissues. Tumors specifically exploit these regulatory mechanisms, such as endothelial expression of selectins, adhesion molecules, chemokines, as well as blood flow and shear stress to establish endothelial dysfunction with the purpose of generating a tumor microenvironment characterized by enhanced immune privilege status.

## 2. Molecular insights into “endothelial anergy”

Tumor-associated blood vessels, as mentioned above, are characterized by several structural and functional deviations that mirror poor functionality and consequent poor oxygenation. Hypoxia triggers the chronic release of proangiogenic factors, which polarize ECs thus establishing “endothelial anergy”. This state is characterized by endothelial unresponsiveness to inflammatory stimuli. Indeed, normal ECs become activated following triggering by inflammatory cytokines (e.g. TNF $\alpha$ , IFN $\gamma$ , IL1) and up-regulate selectins and adhesion molecules,

which in turn promote extravasation of leukocytes into the peripheral tissues. However, chronic endothelial stimulation with VEGF and bFGF dramatically affects this process, even in presence of TNF $\alpha$  [80]. Accordingly, VEGF axis blockade restores normal adhesion molecule expression and leukocyte-vessel interactions [80]. Preconditioning with bFGF is able to prevent ICAM up-regulation following IL1 or IFN $\gamma$  stimulation. Both bFGF and VEGF restrict ICAM1, VCAM1, E-selectin induction after TNF $\alpha$  challenge, whereas TGF $\beta$  pretreatment affects VCAM1 and E-selectin induction [81]. The mechanism of bFGF-mediated impaired up-regulation of ICAM1 following TNF $\alpha$  stimulation has been described *in vitro*: bFGF prevents the activation of nuclear factor kappa-light-chain-enhancer of activated B cells (NF- $\kappa$ B) triggered by TNF $\alpha$  by blocking the phosphorylation and degradation of I $\kappa$ B $\alpha$  (NF- $\kappa$ B inhibitor) and by activating p38 MAPK [82]. Dysregulated cytokine composition resulting in increased levels of IL4, IL10 and TGF $\beta$  are associated with reduced adhesion molecules expression on ECs [83]. Accordingly, elevated production of these cytokines is a common feature of several tumor types [59,84,85]. Clinical data confirm the negative role of proangiogenic factors in leukocyte recruitment by orchestrating the expression of adhesion molecules on tumor endothelium. For example, in breast tumor, the expression of VEGFC and VEGFD fosters bFGF down-regulation of ICAM1 on ECs [86]. Also, in ovarian cancer, the presence of TILs inversely correlates with VEGF levels [59]. In accordance with these results, the interruption of the VEGF axis by antibodies targeting VEGF or VEGFR2 or by using small molecules inhibitors of broad spectrum TKRs, such as sunitinib, sorafenib, and angiostatic peptides, improved leukocyte trafficking within tumors, increasing TIL infiltration and eventually tumor restriction in several preclinical tumor models [87–90]. Similarly, sunitinib-mediated VEGF signaling inhibition increases the expression of endothelial CXCL10 and CXCL11, in a NF- $\kappa$ B dependent manner, which results in a 18-fold higher concentration of TILs within B16 melanoma tumors [91]. Inhibition of signaling pathways upstream of VEGF expression, such as inhibition of RAF oncogene-driven angiogenesis, can indirectly restore TIL infiltration, improving the efficacy of ACT immunotherapy in a preclinical melanoma model [92].

Epidermal growth factor like domain (EGFL) 7 is a proangiogenic factor with unique features since it is physiologically expressed almost exclusively by actively proliferating ECs and act in paracrine and autocrine ways to support blood vessel development through Notch signaling [93]. However EGFL7 is also expressed in many tumors and cell lines and its expression levels correlates with worse prognosis and higher tumor grade in several cancers including malignant gliomas, hepatocellular carcinoma (HCC), and colon tumors [94–96]. EGFL7 expression is associated with decreased levels of adhesion molecules VCAM1, ICAM1 in preclinical models of lung and breast tumors and in breast cancer patients [97]. During acute inflammation, TNF $\alpha$  represses endothelial expression of EGFL7 by regulating its promoter activity in a NF- $\kappa$ B dependent manner. In contrast, EGFL7 maintains endothelium in an anergic state by preventing I $\kappa$ B $\alpha$  degradation and consequently NF- $\kappa$ B activation [98].

Nitric Oxide (NO) is a highly reactive free radical compound involved in a number of processes. In the tumor microenvironment, NO is often a crucial mediator of immune suppression on effector T cells [99–101]. However, NO plays a critical role in regulating blood flow, angiogenesis, and leukocyte-EC interactions [102]. NO synthase inhibition increases expression of E-selectin, ICAM1 and VCAM1 adhesion molecules on ECs, which in turn mediate higher leukocyte rolling and adhesion on endothelium [103,104]. Buckanovich and colleagues [105] identified an NO-dependent mechanism of adhesion molecule regulation and endothelial anergy triggered by the endothelin (ET) – ETBR interaction. ETs are a family of biologically active peptides that include ET1-4. ET1 was originally identified in venom snake and investigated for its potent vasoconstrictor action on ECs [106]. However, ETs and their receptors (referred as the ET axis) have been described in supporting pathological conditions as well, including cancer, by promoting

angiogenesis, vasculogenesis, proliferation, apoptosis and metastatic dissemination of tumor cells by activating paracrine and autocrine loops [107,108]. The ET1 axis is activated in many cancers, including breast, ovarian, renal, lung, cervical, glioma, prostate and colon cancers [108]. ETs bind their cognate receptors, named ETAR and ETBR, G-protein coupled receptors that triggers opposite action on ECs. ET1 is up-regulated both in patient-derived tumor specimens and *in vitro* in several models of cancer, and is able to promote angiogenesis by supporting VEGF synthesis in a HIF1-dependent manner [108,109]. ET1 binding to ETBR directly promotes angiogenesis *via* proliferation and differentiation on ECs by synergizing with VEGF, and ET1 blockade with a selective ETBR antagonist (BQ788) reverses this phenotype [110]. The transcription profiling of tumor endothelium isolated from ovarian cancer specimens (enriched by immunohistochemistry-guided laser-capture microdissection) identified genes, including *ETBR*, whose up-regulation significantly correlates with endothelial anergy and poor clinical outcome. The ET1 axis regulates ICAM1 expression on ECs through the paracrine release of NO, and ET<sub>B</sub>R or NOS inhibitors can reverse this phenotype. Accordingly, ET<sub>B</sub>R inhibition *in vivo* improves T cell homing to tumor and consequently, the efficacy of tumor vaccines and adoptive cell-based immunotherapies in ovarian and lung pre-clinical cancer models [105]. In the same transcriptional profiling study of tumor endothelium isolated from ovarian cancer patients, complement component 3 (C3) up-regulation significantly correlated with the presence of TILs. Expanding upon these studies, ET-1-dependent endothelial anergy can be reversed by transfer of adequate quantities of tumor-reactive T cells [111]. This process requires local production of C3, activation of the complement cascade with release of C5a, which in turn acts on ECs to express adhesion molecules promoting T cell transmigration [111]. Accordingly, both total and conditional C3 and C5aR1 deficiencies, and pharmacological C5aR1 blockade, critically affects T cell homing to tumor, rendering ACT immunotherapy ineffective [111]. Infiltrating tumor-specific CD4<sup>+</sup> and CD8<sup>+</sup> T cells release Th1 cytokines that activate ECs, triggering expression and activation of the complement cascade, resulting in the expression of adhesion molecules and promoting T cell infiltration in tumor [111]. However, the complement system is a complex component of the immune response, which embraces both innate and adaptive immunity with a clear role in fine-tuning host immune defenses against pathogens. The complement system is viewed as a global regulator of immunity and tissue homeostasis with the role of the complement system on tumor progression only beginning to be explored. A critical appraisal of the complement system has been summarized elsewhere and is currently envisioned having a dual function by both promoting or restricting tumor progression according to the immune context [112–114].

Soluble versions of adhesion molecules can also affect leukocyte transmigration efficiency by direct binding to their cognate receptors, in competition with membrane bound versions, or indirectly by promoting angiogenesis. Melanoma cell adhesion molecule (MCAM) or CD146 is a component of endothelial junctions with a role in cellular cohesion. It is expressed mainly by endothelial, perivascular cells and by a multitude of tumors; accordingly, CD146 is considered a poor prognostic marker in melanoma [115] RCC [116], and other tumors [117]. The rationale for this association relies on the role of CD146 in activating angiogenesis on tumor endothelium [118,119] and in synergizing with VEGF during cancer progression [120]. In accordance with these concepts, endothelial CD146 conditional deficiency or CD146 pharmacologic blockade impacts tumor growth by affecting angiogenesis [120,121]. However, tumor and ECs also produce a soluble version of CD146 (sCD146) with chemotactic and angiogenic properties [118]. Selective blockade of CD146 inhibits vascularization, growth and survival of CD146-positive tumors [122]. Endoglin is another protein expressed by ECs and a marker of tumor vasculature [123]. Endoglin interacts with VLA-5 expressed on leukocytes to promote adhesion to the endothelium. However, ECs produce an endoglin

soluble variant as well, which competes for binding to VLA5, impairing leukocyte transmigration [124]. Interestingly, serum levels of soluble endoglin correlates with cancer relapse and represents an early prognostic factor of metastasis development in colorectal, breast and other solid tumors [125].

Tumor endothelium can therefore express specific adhesion molecules to orchestrate the extravasation of selected leukocyte subsets with the purpose of sculpting the tumor microenvironment towards immune suppression. The common lymphatic endothelial and vascular endothelial receptor (CLEVER) 1 is a scavenging receptor constitutively expressed on lymphatic endothelium and TIE2<sup>+</sup> macrophages and synthesized in ECs during inflammation [126]. CLEVER1 relevance in cancer progression has been addressed in preclinical melanoma and breast cancer models. Both CLEVER1 conditional deficiency in an endothelial or macrophage context and selective antibody blockade in wild type mice reduce the accumulation of immune suppressive subsets (Treg and TAM) in breast and melanoma tumors, which in turn significantly reduces tumor progression [127]. In some cases, augmented expression of addressins on tumor endothelium can be responsible for preferential transmigration of Treg lymphocytes. Human pancreatic carcinoma endothelium up-regulates mucosal addressin cell adhesion molecule 1 (MAdCAM1), VCAM1, CD62E and CD166, which interact with their ligands  $\beta$ 7 integrin (binding to MAdCAM1 and VCAM1), CD62L (binding to E-selectin, MAdCAM1, and VCAM1) and CD166 (homophilic binding to CD166) exposed on Tregs, promoting their extravasation [128]. Disrupting these interactions by selective blocking antibodies critically impacts the efficiency of Treg transmigration through tumor-associated endothelium compared to other T cell subsets [128].

### 3. Angiogenic-derived mechanical dysfunction

#### 3.1. Shear stress and ECM stiffness

As previously described, tumor-associated blood vessels are characterized by irregular morphology and structure that reflect poor functionality, reduced flow, and leakage compared to normal blood vessels. Shear stress is a mechanical force that acts on ECs through CD31 and VE-cadherin and has an important role in regulating endothelial functions. High interstitial pressure and reduced blood flow within tumor blood vessels reduce shear stress and impair the rolling and adhesion of leukocytes on endothelium [69]. However, reduced blood flow also affects the expression of endothelial selectins and adhesion molecules exacerbating impairment of transmigration [129]. Finally, ECM composition contributes to endothelial dysfunction. ECM is composed of glycoproteins, proteoglycans and polysaccharides and regulates many cellular functions such as metabolism, proliferation, migration and survival by generating an environment with specific biochemical, physical and biomechanical properties [130]. Tumor-derived ECM is characterized by an elevated rigidity that reflects higher interstitial pressure due to tumor proliferation, cytoarchitecture modification of cancer cells, ECM composition and fibrosis [131]. Tumor and stromal cells react to ECM stiffness by integrin-mediated, mechanosensing structures. ECs may sense ECM stiffness as well. *In vitro* studies showed that hard substrates impair endothelial monolayer function compared to soft substrates, independent from inflammatory signals [132].

Vessel structure and function is deeply influenced by pericytes that physiologically and structurally surround ECs in the vasculature. Pericytes and vasculature mutually regulate each other. Activated and proliferating ECs promote pericyte recruitment by releasing PDGFB and expressing ANGPT receptor TIE2. Secreted PDGFB is anchored on ECs membrane and on ECM generating a chemical gradient which guides pericyte and vascular smooth muscle cells (vSMCs) chemoattraction towards EC [133]. Pericyte recruitment on sprouting blood vessel is supported by pericyte-derived ANGPT1 binding to endothelial TIE2

marker and by pericyte-derived NG2 proteoglycan [134]. Pericyte-EC interaction results in sprout stabilization by secretion of TIMPs, inhibition of proliferation, induction of survival and vessel maturation through ANGPT1-TIE2 binding [135,136]. During cancer progression though, ECs disrupt this molecular network in response to ANGPT2 up-regulation which competes with ANGPT1 for binding to TIE2 and by associating preferentially with TIE2<sup>+</sup> TAMs [137] which establishes a positive angiogenic stimulation loop with ECs [138]. However, pericytes may also support angiogenesis since inhibition of PDGFR signaling can lead to tumor blood vessel regression [139] underscoring a pivotal role pericytes play in promoting EC survival [140]. Disappointing results from clinical trials targeting PDGFR axis as single agent suggest that pericytes are not crucial elements in regulating vessel function. Other strategies that aim to enforce pericyte-ECs interaction by simultaneously antagonizing ANGPT2 and activating TIE2 showed tumor blood vessels normalization and increased perfusion, resulting in increased chemotherapy delivery and reduced tumor growth and metastasis in preclinical models of glioma, breast and lung cancers [141]. Cumulatively, these results support the concept that the presence of dysfunctional, irregularly branched and leaky blood vessels inversely correlates with the presence of TILs and that fluid extravasation does not contribute to T cell infiltration in the tumor bed. This observation is the result of dysregulation in the physiological and finely-tuned mechanical and molecular mechanisms of leukocyte-endothelium interactions and transmigration.

Hypoxia also drives the production and release of lymphangiogenic factors as well, specifically VEGFC and VEGFD, which promote the generation of new lymphatic vessels within tumor and, in turn, are associated with greater probability of metastatic dissemination, relapse and poor clinical outcome [142]. In this context, high interstitial fluid in solid tumors increases activation and secretion of TGFβ by CAFs thereby promoting local immune tolerance. Additionally, it increases also lymph drainage to adjacent lymph nodes where tumor-secreted factors and immature antigen presenting cells (APCs), such as immature dendritic cells – iDCs, previously exposed to tumor microenvironment, are recruited and activate naïve T cells in a tolerogenic manner to promote peripheral tolerance [143,144].

#### 4. Mechanisms of endothelium-mediated direct regulation of immune response

Transmigration is the principal and most characterized blood vessel-mediated mechanism that regulates the immune response. However, ECs may promote or impact T cell activation through several other strategies. ECs are peripheral, semiprofessional, non-hematopoietic APCs [145]. ECs constitutively express major histocompatibility complex (MHC) class I and II molecules, which can be up-regulated following inflammatory stimuli. ECs express antigen processing machinery, adhesion proteins, cytokines and co-stimulatory and co-inhibitory surface molecules such as CD137, CD40, OX40L, inducible T-cell costimulator ligand (ICOSL), programmed death ligand (PD-L1), PD-L2, FASL, TNF-related apoptosis-inducing ligand (TRAIL). However, they do not express CD80 and CD86 costimulatory molecules which explains why ECs cannot prime naïve T cells like professional APCs but only stimulate antigen-experienced T cells [146]. The balance between stimulating and inhibiting surface molecule expression defines T cell fate and tumors exploit this finely-tuned process to promote local immune dysfunction. FASL upregulation on tumor endothelium mediated by tumor-secreted VEGFA, IL10 and PGE2, has been associated with significantly lower CD8<sup>+</sup>/Treg ratio infiltration in human ovarian, breast prostate colon, bladder, renal cancers. This immune escape mechanism is promoted by endothelial, FASL-dependent selective apoptosis of effector T cells (but not Treg). VEGF or PGE2 pharmacological blockade restored effector T cell infiltration and tumor growth control by down-regulation of FASL on tumor endothelium [147]. Both tumor blood and lymphatic ECs can express B7-H1 (PD-L1), which is up-

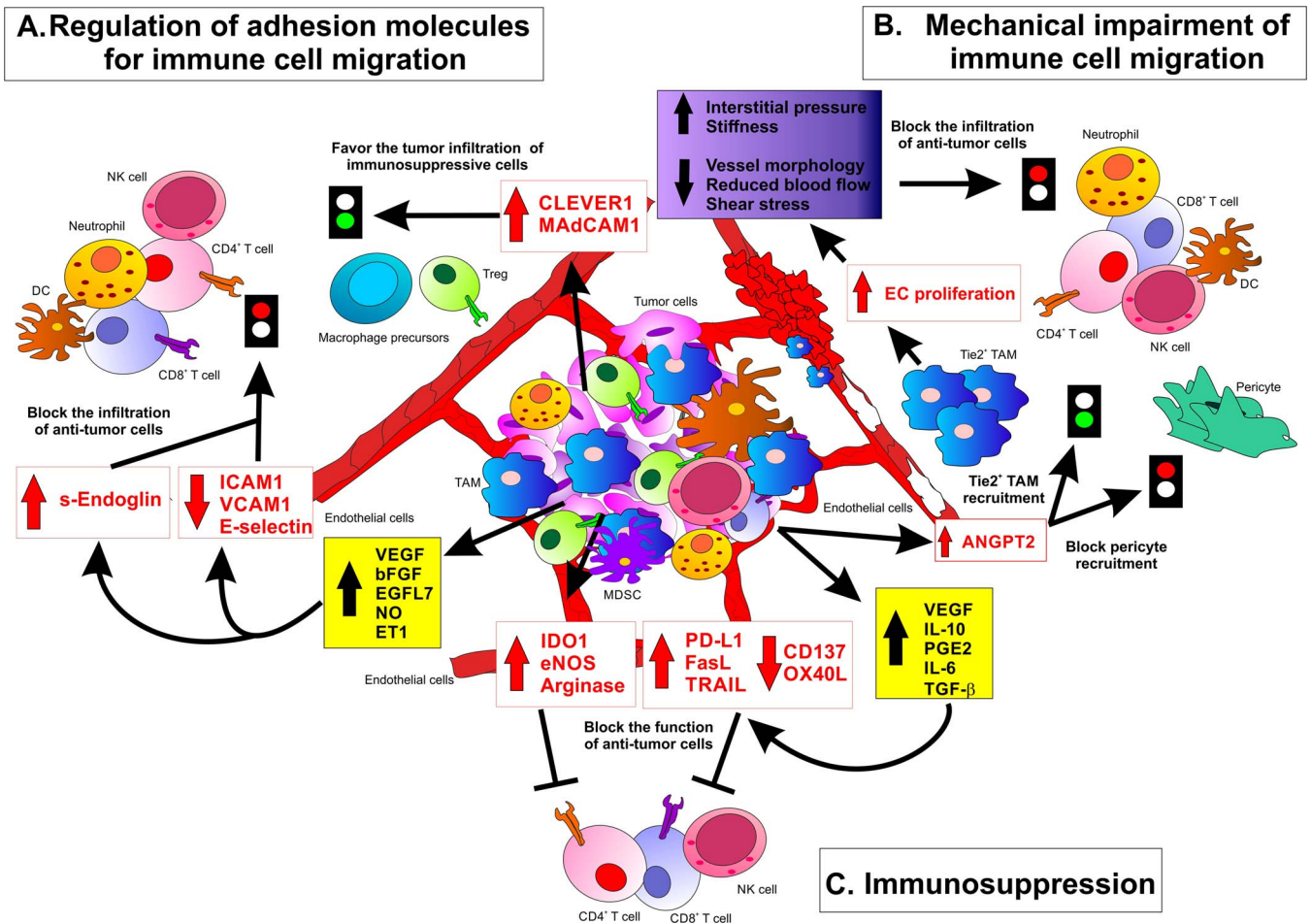
regulated under inflammatory conditions to refrain T cell activation [148,149]. PD-L1 belongs to the B7-family proteins and binds to PD-1 on activated lymphocytes to negatively control T cell activation. In a tumor context, PD-L1 induces peripheral (LEC) or local (EC) tolerance of tumor specific T cells [150,151] and pharmacological blockade of PD-1 axis increases endothelial cell co-stimulation of PHA-activated CD8<sup>+</sup> T cells *in vitro* [149]. B7-H3 and B7-H4 are inhibitory proteins related to the B7 family whose expression on tumor endothelium correlates with poor outcome in ovarian [152], renal cell [153,154] cancers. ECs express CD137 and its engagement activates ECs to produce adhesion molecules and co-stimulate T cell activity. However, cancer cells counteract this interaction by producing a soluble version of CD137 (sCD137), which antagonizes the membrane bound version of the receptor preventing T cell co-stimulation [155].

Additionally, ECs can interfere with T cell function by releasing soluble factors, especially cytokines, angiogenic factors [156], and immunomodulatory enzymes [157]. By releasing IL15, ECs may induce T cell proliferation and promote transmigration. IL15 derived from activated and resting umbilical vein ECs triggers and supports LFA1 integrin and CD69 up-regulation in T cells *in vitro* and in human rheumatoid arthritis *in vivo* preclinical models, respectively [158]. Moreover ECs can secrete IL6 in response to inflammatory stimuli skewing CD4<sup>+</sup> T cell polarization towards Th17 and Treg responses. This EC capability has been investigated in an allogenic model of microvascular endothelium (HMEC) and requires IL6 secretion and ICAM up-regulation in activated ECs to polarize CD4<sup>+</sup> T cell towards Th17 and Treg, respectively [159]. Preclinical *in vitro* functional studies reveal that tumor endothelial-dependent release of VEGF, PGE2, TGFβ and IL6 can promote T cell anergy, as well as reduce NK activity and macrophage phagocytosis [160]. Finally, ECs can produce enzymes such as endothelial nitric oxide synthase (eNOS), arginase and indoleamine 2,3-dioxygenase (IDO), which have direct negative immunoregulatory properties on T cells by depleting L-arginine and L-tryptophan amino acids [161]. IDO-dependent, DC-mediated T cell suppression is considered a potent tumor immunoevasion mechanism but its role in the endothelial context is less clear. Endothelial expression of IDO can promote endothelial tube formation and angiogenesis [162]. In renal cell carcinoma, endothelial-derived IDO seems to impair tumor cell proliferation and progression since its expression positively correlates with improved prognosis [162]. The mechanisms of tumor endothelium-mediated immune dysfunction are summarized in Fig. 1: the expression of endothelium-associated molecules (shown in red font) is modified by tumor-derived factors (listed in yellow filled squares), resulting in impairment of immune cell migration and increase of immunosuppression.

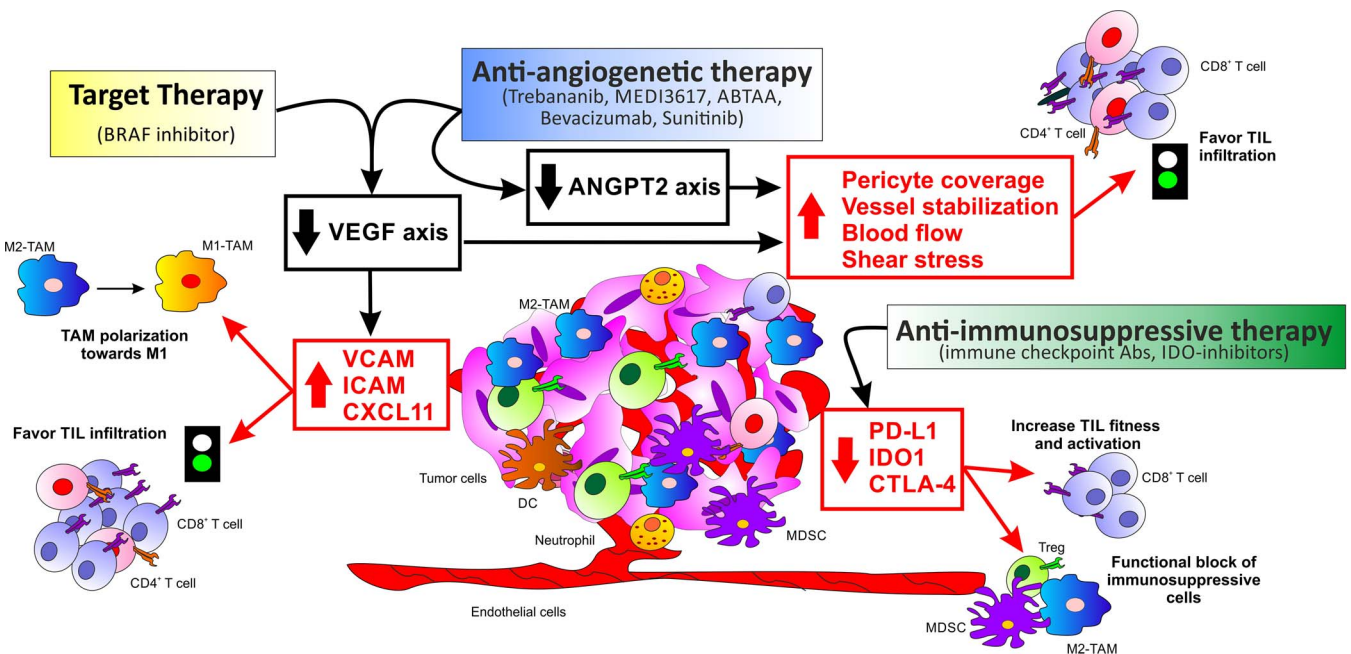
#### 5. Clinical implications of current anti-angiogenic therapies

Given the paramount role of angiogenesis in tumor progression, several therapeutic approaches targeting VEGF binding to their receptors or their activation status have been developed and tested in preclinical and clinical settings. Cancer anti-angiogenic therapy began with neutralizing VEGF antibody in preclinical models demonstrating *in vivo* tumor growth suppression [163]. Importantly, this tumor inhibitory effect relies on tumor vessel restriction rather than direct toxicity on tumor cells. Clinical trials using VEGF-blocking antibody bevacizumab for metastatic RCC patients [164] and subsequently for metastatic breast and prostate cancer patients [165] showed clinical benefit. Accordingly, bevacizumab combined with chemotherapy demonstrated encouraging clinical results improving overall survival for colorectal carcinoma patients [166]. Other therapeutic approaches targeting the VEGF-VEGFR axis soon afterwards were developed, such as VEGF2R-directed antibody or small molecule multi target inhibitors such as TKRs.

Since the demonstration of the antitumor effectiveness of bevacizumab, this antibody-dependent angiogenic blockade has been



**Fig. 1. Immune regulatory properties of tumor-associated endothelium.** The demand for greater nutrient and oxygen supply activates a hypoxic response within tumors that initiates and sustains the angiogenic switch. Tumor-secreted angiogenic factors and inflammatory cytokines (in yellow) act on tumor endothelium to regulate either directly (A) or indirectly (B) the expression of adhesion molecules and vessel functionality, which in turn affects and selects T cell extravasation in the tumor bed. Moreover, ECs regulate T cell activation and fitness (C) by modulating the expression of co-stimulatory and co-inhibitory molecules or by up-regulating enzymes that affect T cell metabolism.



**Fig. 2. Targeting of tumor blood vessels restores endothelium function.** Pharmacological blockade of the angiogenic axis restores adhesion molecule expression on endothelium and induces vessel normalization that promotes T cell migration within the tumor. Immune checkpoint inhibition can synergize with anti-angiogenic therapy by unleashing the cytotoxic potential of effector T cells.

approved for several other advanced malignancies, such as glioblastoma, RCC, ovarian, lung, breast, gastric and cervical cancer, often in combination with chemotherapy [167]. The clinical rationale for combining anti-angiogenic drugs with chemotherapy relies on the mechanism of action of anti-VEGFA therapy, which directly reduces tumor growth rate rather than inducing tumor regression [168]. Secondly, VEGFA blockade generates pruning of newly-formed and immature blood vessels (such as tumor-induced vessels) rather than established and stabilized blood vessels. This observation suggests anti-VEGF-dependent “normalization” on tumor vessels may consequently improve perfusion and extravasation of chemotherapeutic agents and immune cells within the tumor bed [169] (Fig. 2). Although a probable explanation for the observed increased efficacy of chemotherapy in combination with anti-VEGFA blockade, a definitive demonstration is lacking for anti-VEGF-dependent, augmented accumulation of drugs within tumors of patients.

Another debated point is whether anti-angiogenic therapies can unleash metastatic progression. This observation relies on the modest clinical results that anti-angiogenic therapy is achieving in overall survival benefit rather than progression free survival compared to preclinical studies [170]. One explanation is that preclinical and clinical settings are quite different. Mouse experiments are performed on localized primary tumor whereas phase 2 and 3 clinical trials are carried out on patients with late stage metastatic tumors so it is difficult to predict overall survival efficacy before entering the clinic. Moreover, many tumor-targeting therapies (such as radiation or chemotherapy) limit tumor progression but potentially increase pro-metastatic potential of cancer cells [170]. Tumor relapse and metastasis after initial tumor response have been extensively reported for anti-angiogenic therapies as well [171]. Moreover, as with many types of cancer treatments, it is very difficult to predict which patients will benefit on anti-angiogenic therapies; indirect impact of antiangiogenesis therapy on other tumor compartments of the immune system may compensate and bypass VEGFA blockade. Indeed, myeloid cells, especially MDSCs and TAMs, foster angiogenesis by VEGF dependent and independent mechanisms [49,99] and their targeting increases sensitivity to anti-angiogenic therapy [172–174]. On the other hand, anti-angiogenic therapy can enhance cancer immunotherapy strategies by affecting tumor-induced endothelial immune suppression. In accordance with this hypothesis, many research teams combine anti-angiogenic therapy with immunotherapy in preclinical models with encouraging results, supporting their translation to the clinic [175]. The combination of anti-angiogenic therapy with passive T cell administration or active tumor cell vaccination can improve T cell infiltration within tumor, delaying tumor growth and increasing survival in melanoma [90] colon [176] and breast cancer models [88,177].

Another successful preclinical approach to inhibit tumor angiogenesis and restore host immune function against tumor relies on educating the immune system to recognize and destroy tumor vasculature. This was demonstrated by employing a DNA vaccine strategy coding for endosialin (TEM1) fused to a minimized domain of the C fragment of tetanus toxoid (TT) [178]. TEM1 is a protein expressed on tumor blood vessels, tumor vessel-associated pericytes, tumor-associated myofibroblasts and tumor cells [178]. This strategy has several advantages; TEM1 is expressed on vessels of tumors with different histologies, which are more accessible to effector T cells, less prone to evade immune system or to evolve compared to tumor cells. Both prophylactic and therapeutic DNA vaccination with TEM1-TT was able to elicit a T cell response against tumor endothelium, which reduced tumor vasculature, increased T cell infiltration and affected tumor growth in colon and lung cancer models [179]. Interestingly, TEM1-TT vaccination is able to trigger a CD8<sup>+</sup> T cell response against tumor-specific antigens (epitope spreading) without impairing the angiogenic physiological processes [179]. Other preclinical approaches of tumor endothelial CD8<sup>+</sup> T cell-dependent targeting have been tested exploiting genetically engineered T cells with chimeric antigen receptors recognizing

VEGFR2 [180,181] and resulted in tumor growth inhibition and overall increase in survival in different tumor types. From a translational point of view, many clinicians are aiming at reversing angiogenic-dependent endothelial anergy to improve T cell infiltration in tumor and reactivate those T cells with checkpoint inhibitor blockade. Although most of these studies are still in early clinical trials, preliminary results are encouraging. In melanoma, the combination of bevacizumab with ipilimumab reverts endothelial anergy on tumor endothelium by up-regulating adhesion molecules E-selectin, ICAM1, and VCAM1, which results in higher TIL in tumor bed [182] and better clinical outcome. Higher TIL are induced as well in RCC patients after combined bevacizumab and atezolizumab therapy, which are associated with increased T effector markers, CX3CL1 and Th1 cytokine production within tumors. Taken together, these data suggest a mutual synergy of action between anti-angiogenic and checkpoint inhibitor blockade that can result in superior anti-tumor efficacy and clinical benefit for patients [183].

## 6. Conclusions

In this review, we highlighted the role of the tumor endothelium in sculpting immune responses contributing to the generation of a localized immune privileged microenvironment necessary for tumor outgrowth and spread to distal body organs. From this point of view, many anti-angiogenic strategies significantly reduce and/or inhibit tumor-derived blood vessel proliferation and/or growth improving overall survival. These treatments delay tumor growth rates rather than causing tumor shrinkage [184] and clinicians and researchers question which piece of the puzzle is missing. Preclinical and clinical research has already provided some answers such as angiogenic factor redundancy [185] and compensatory mechanisms triggered by the recruitment of suppressive myeloid cells [46]. However, the complexity of tumor microenvironment remains unresolved in its detailed intricacies; tumors of different histology are generally characterized by abundant angiogenesis (hallmarks of cancer), yet even within the same histological classification, tumor progression and survival changes according to the immune system’s ability to recognize and kill tumor cells as well as by immune evading strategies. Thorough characterization of the tumor microenvironment and increased understanding of tumor-promoted immunosuppressive mechanisms will provide more answers as well as more questions. Improving the molecular knowledge of tumors is the first step on this route. Many factors other than endothelial anergy may affect T cell infiltration and function, tumor fate and clinical outcome, such as the chemokine and cytokine milieu within the tumor microenvironment. For example, hypoxia-driven secretion of CCL28 promotes T-reg recruitment [50], while CX3CL1 (fractalkine) increases T cell homing to tumors [186], and these chemokines are considered negative and positive prognostic factors, respectively. Cytokines directly limit or stimulate T cell function and polarize both T lymphocytes and myeloid cells in a tumor-promoting or tumor-restricting manner [187]. Even the same molecule or pathway may induce positive or negative effects on immunity, depending on the cellular context; CCL2 promotes both recruitment of T cells and monocytic MDSCs and its post translational modifications induced by reactive nitrogen species (RNS) results in dysfunctional homing of T cells in favor of monocytic MDSCs [188]. HIF1 $\alpha$  can be considered a main driver of angiogenesis. However, HIF1 $\alpha$  and VEGFA expression in CD8<sup>+</sup> T cells drives their tumor infiltration and cytotoxic properties, whereas their absence increases tumorigenesis. Moreover, an inverse correlation was found between VEGFA expression and CD8<sup>+</sup> T cell infiltration in breast cancer patients, suggesting yet another mechanism of angiogenic regulation of immunity [189]. According to this study, classic histological classification of tumors is considered obsolete and clinicians should start thinking more in terms of molecular classification (genetic fingerprinting) and the tumor immune context rather than tumor type and grade. For example, the genomic analysis of 456



pancreatic ductal adenocarcinomas (PDACs) defined 4 PDAC subtypes which drive different evolution and clinical outcome [190]. Accordingly, another group unveiled the role of specific genetic alterations in combination with the loss of suppressor gene *Pten* in defining tumor progression and stromal cell composition in prostate cancer. Surprisingly, *Pten* loss or in combination with *Trp53*, *Zbtb7a* or *Pml* mutations, generated tumors with totally different immune infiltrates ranging from “cold” (non-inflamed tumor) to deeply inflamed and heavily leukocyte infiltrated tumors [191]. Tumor genetic instability and somatic mutations may trigger T cell responses against neoantigens [192,193] that can be fostered by immune checkpoint inhibition therapy. However, even in the case of highly immunogenic melanoma, not all patients respond equally to checkpoint inhibition therapy. Lastly, the nature of mutations can distinguish tumor fate. In support of this hypothesis, a genetic mutational analysis performed on 10,000 human tissues from 30 different cancer types classified tumors into two types according to the kind of mutation: mutator-type associated with response to immunotherapy and chromosome instable, which is associated with immunoevasion [194]. Thus, genotypic and immunophenotypic characterization may improve patient stratification and move the current treatment paradigm one step forward towards patient-tailored anti-cancer therapy. Understanding the molecular circuitry and major players for different histologically distinct tumor types that contribute to tumor endothelial dysfunction with the aim of reversing the tumor endothelial anergic state is a lofty goal and carries high potential for development of novel therapeutic strategies that target the tumor endothelium.

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