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Targeting the tumor vasculature to enhance T cell activity

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T cells play a critical role in tumor immune surveillance as evidenced by extensive mouse-tumor model studies as well as encouraging patient responses to adoptive T cell therapies and dendritic cell vaccines. It is well established that the interplay of tumor cells with their local cellular environment can trigger events that are immunoinhibitory to T cells. More recently it is emerging that the tumor vasculature itself constitutes an important barrier to T cells. Endothelial cells lining the vessels can suppress T cell activity, target them for destruction, and block them from gaining entry into the tumor in the first place through the deregulation of adhesion molecules. Here we review approaches to break this tumor endothelial barrier and enhance T cell activity.

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Introduction

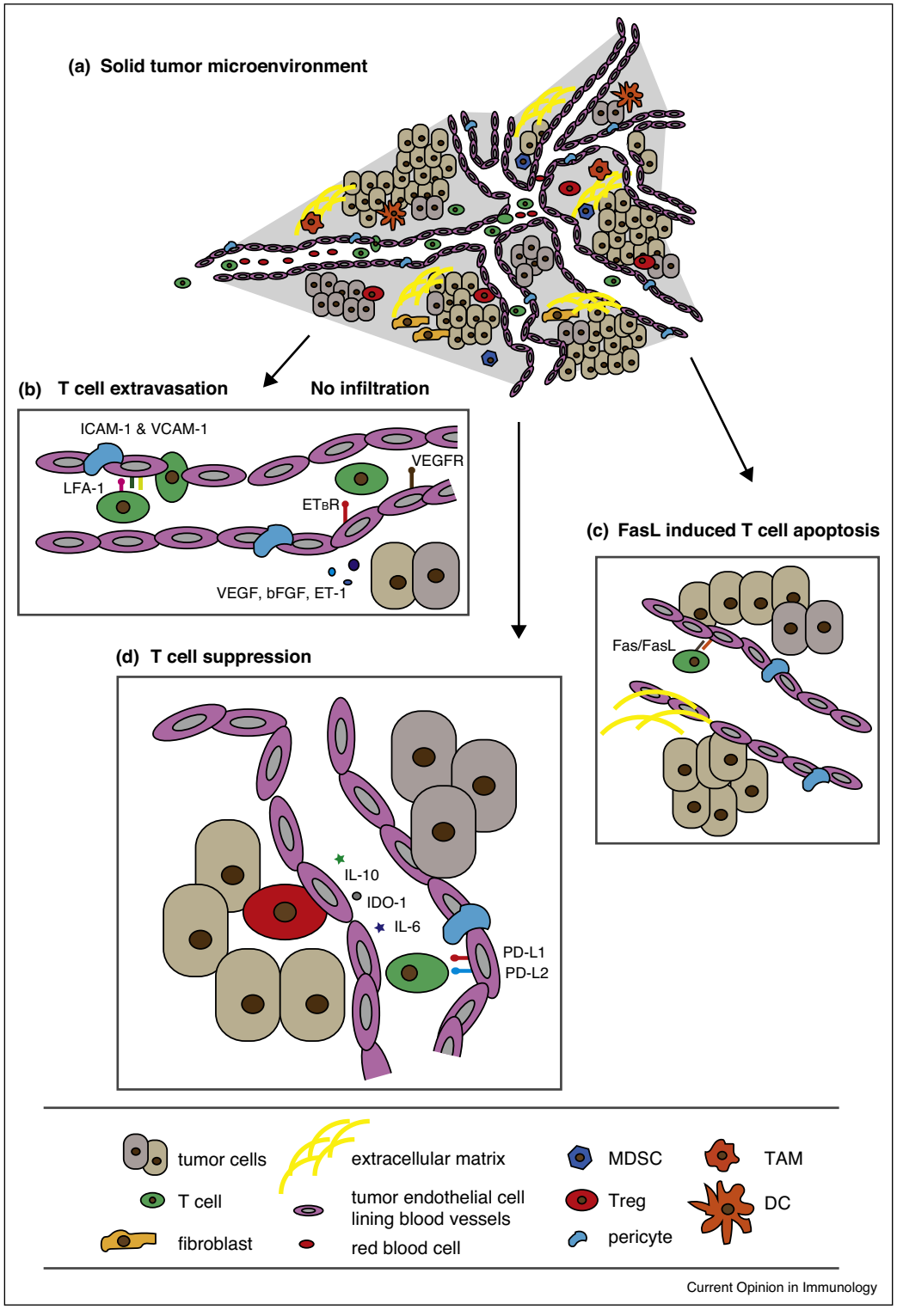
T lymphocytes play a key role in tumor immune surveillance through T cell receptor (TCR)-mediated recognition of tumor associated antigens that have been processed and presented as peptides (p) at the tumor cell surface by major histocompatibility complex (MHC) molecules [1]. Activated $CD8^+$ cytotoxic T cells are able to directly kill malignant cells upon TCR/pMHC engagement by mechanisms including perforin/granzyme secretion and FasL/Fas binding, and, along with $CD4^+$ helper T cells, can secrete various cytokines/chemokines to direct the activities of other immune cells [2,3]. Several clinical studies, including our own in epithelial ovarian cancer, have reported a positive correlation between patient survival and the presence of tumor infiltrating lymphocytes (TILs) [4–7]. Moreover, clinically significant anti-tumor activity has been achieved for dendritic cell (DC) vaccines [8,9] and for adoptive T cell therapies with TILs, and both TCR- and chimeric antigen receptor (CAR)-engineered T cells [10••,11•,12,13••,14••,15••,16,17]. In order to improve patient outcome, important research efforts have focused on optimizing the 'fitness' of vaccine-induced or transferred T cells, including their state of differentiation and phenotype for enhanced persistence, proliferation, homing, etc. [18] and their receptor qualities such as specificity and binding kinetics/affinity and avidity [19–21]. In addition, the characterization of different solid tumor microenvironments and the ways in which T cell activity is inhibited, so that it may be therapeutically reversed, is a field of intense study [22•,23–25].

Solid tumors are highly heterogeneous in nature, comprising divergent cancer cells and host stromal cells that are embedded within an extracellular matrix and nourished by an aberrant vasculature (Figure 1a). The dynamic interplay of tumor cells with their surrounding matrix and local cellular microenvironment composed of various immune cell infiltrates, fibroblasts, etc., affects gene expression and the patho-physiological characteristics of the tumor, including progression and response to therapies [26]. In general, T cells that reach the tumor bed after an initial priming in the tumor-draining lymph nodes or tumor stroma face a hostile environment, including the downregulation of MHC molecules and co-stimulatory ligands, as well as the upregulation of inhibitory receptors like programmed cell death protein ligand 1 (PD-L1) on tumor cells. They can also encounter immunosuppression by regulatory T cells (Tregs), myeloid derived suppressor cells (MDSCs), and tumor-associated macrophages (TAMs), as well as a plethora of soluble inhibitory factors such as IL-6, IL-10, arginase (Arg)1, and TGFβ, various metabolites like adenosine, depleted tryptophan levels as a result of indoleamine 2,3-dioxygenase 1 (IDO-1) activity, and low pH [23,27,28]. However, in many instances effector T cells do not gain entry into the tumor bed in the first place because they are functionally inhibited and physically blocked by the tumor vasculature. Here we review the mechanisms by which the tumor vasculature acts as a barrier to effector T cells, the so-called *tumor endothelial barrier*, and different therapeutic approaches being developed to 'break it' or 'normalize it' and enhance anti-tumor T cell activity.

The tumor vasculature is inhibitory to effector T lymphocyte responses

Tumor growth is critically dependent upon neovascularization to supply itself with nutrients. Although tumor

Figure 1



The tumor microenvironment and the tumor endothelial cell barrier. **(a)**. The tumor microenvironment is comprised of tumor cells, an aberrant vasculature lined by endothelial cells and supported by pericytes, stromal cells, an extracellular matrix, and a range of immune infiltrates including T cells, regulatory T cells (Treg), myeloid derived suppressor cells (MDSCs), tumor associated macrophages (TAMs), fibroblasts and dendritic cells (DCs). **(b)** T cell extravasation is dependent upon endothelial cell expression of intracellular cell adhesion molecule-1 (ICAM-1) and vasculature cell adhesion molecule-1 (VCAM-1). Tumor derived angiogenic growth factors such as VEGF and endothelin-1 (ET-1) signal through their cognate

blood vessels can be produced *de novo* from bone marrow-derived endothelial precursor cells, so-called vasculogenesis [29], or from tumor stem cells in a process called vascular mimicry, most are formed by the sprouting of pre-existing vessels, i.e., angiogenesis [30], promoted by an imbalance of proangiogenic factors in the microenvironment. Such factors are numerous and abundantly produced, including the most potent one, vascular endothelial growth factor-A (VEGF) [31,32], as well as angiopoietin, basic fibroblast growth factor (bFGF), platelet-derived endothelial growth factor (PDGF), transforming growth factor (TGF)-α, fibroblast growth factor (FGF), and placental growth factor (PGF). These soluble factors act on a range of tyrosine kinase receptors like VEGFR1, VEGFR2, PDGFRA and endothelial growth factor receptor (EGFR) to initiate signaling pathways leading to angiogenesis and other biological events [33]. Compared to normal vasculatures, tumor blood vessels are characterized by having slow and irregular blood flow, an oversized diameter that varies along their length, erratic branching (the vessels are tortuous), many dysfunctional microvessels, high red blood cell flux, permeability/leakiness due to oversized pores, low or absent pericyte coverage, high compression levels due to interstitial pressure, and the vessels may lack a basement membrane or have one that is unusually thick [34]. Overall these properties result in low oxygen supply causing a state of hypoxia and an accumulation of metabolic waste that can affect immune cell function in the tumor microenvironment [35] and trigger the release and activity of proangiogenic growth factors [36,37].

In addition, several mechanisms have been described for tumor endothelial cells (ECs) lining the vessels that specifically inhibit tumor immunity. For example, through the downregulation and/or declustering (i.e., deregulation) of intracellular adhesion molecule 1 (ICAM1) and vasculature cell adhesion molecule 1 (VCAM1), which are required for extravasation (a multistep process involving the adherence of leukocytes to ECs and their subsequent diapedesis), effector cells are unable to traverse the ECs into the tumor bed [38–40] (Figure 1b). Conversely, tumor ECs have been shown to promote Treg accumulation in the tumor through the upregulation of molecules such as common lymphatic and vasculature endothelial receptor 1 (CLEVER1) [41]. Like within the tumor itself, tumor ECs can selectively upregulate inhibitory receptors of T cell activation including PD-L1 and PD-L2 [42,43], TIM3 [44], B7-H3 [45,46•], B7-H4 [47], as well as IDO-1 [48–50] and other soluble inhibitory molecules such as IL-6, PGE_2, IL-10 and TGFβ [51–54] (Figure 1c). Moreover, tumor ECs can express the apoptosis inducing molecules TRAIL and FasL (Figure 1d), the latter of which we recently showed can selectively kill effector T cells while leaving Tregs unharmed [55,56••,57,58]. Thus, overall the tumor vasculature is inhibitory to the extravasation of effector immune cells into the tumor bed and it promotes a state of immunosuppression.

Strategies to break the tumor vasculature barrier and restore anti-tumor T cell activity

Monoclonal antibody and small molecules to block proangiogenic factors and their receptors to normalize the vasculature

The VEGF/VEGFR2 axis is critical for tumor growth as it promotes proliferation, survival, migration and invasion [59]. Moreover, it confers important immunomodulatory activities including the ability to inhibit DC maturation [60,61], and to promote the accumulation and activation of Tregs and MDSCs [62]. Consequently, VEGF expression negatively correlates with intraepithelial T cell infiltration, and it is associated with poor patient survival [4]. Not surprisingly, antiangiogenic molecules that target VEGF/VEGR2 are commonly used to treat cancer patients, and bevacizumab (Avastin), a humanized anti-VEGF monoclonal antibody (mAb) [63] was in fact the first antiangiogenic drug to be approved by the Food and Drug Administration for the treatment of various cancers including glioma and metastatic colorectal cancer [64,65]. Some therapies like bevacizumab that target proangiogenic factors not only inhibit the sprouting of new vessels [66], but can also 'normalize' the vasculature. Tumor vasculature normalization, first described by Dr. Rakeesh K. Jain and colleagues, is a transient correction of structural and functional defects of the tumor blood vessels that takes place when aberrant angiogenic signaling is blocked, temporarily enabling improved oxygen and drug delivery (e.g., chemotherapy) as well as immune cell infiltration [31,67–69]. In addition, many antiangiogenic drugs also promote overall immune responses; bevacizumab-based therapy of colorectal cancer patients, for example, has been shown to increase the peripheral B and T cell compartments [70], decrease the Treg compartment [71••], and enhance the functional maturation of DCs [72]. Small molecules targeting the tyrosine kinase domains of VEGFR and PDGFR, such as sunitinib and sorafenib, have also been approved for some advanced cancers [73] and can similarly lead to improved immune responses. A reduction in Tregs and MDSCs, for example, has been documented for metastatic renal cell carcinoma patients treated with sunitinib [74–76]. Despite initial clinical responses, however, resistance to such kinase inhibitors frequently occurs due to the redundancy of angiogenic pathways and protection of the tumor

(Figure 1 Legend Continued) receptors, VEGFR and ET_BR, respectively, to block the expression of adhesion molecules and inhibit T cell infiltration into the tumor bed. **(c)** The endothelium, under the influence of tumor-derived factors like VEGF, can directly inhibit T cell activation by upregulating inhibitory molecules such as PD-L1, PD-L2, IDO-1, IL-6, and IL-10, amongst many others. **(d)** Tumor endothelial cells can also express FasL which leads to apoptosis of Fas-expressing T cells.

vasculature through the recruitment of proangiogenic inflammatory cells [77].

Monoclonal antibody, small molecule and cytokine treatments to restore adhesion molecule expression

T cells are dependent upon both the expression levels and clustering patterns of ICAM-1 and VCAM-1 on ECs to extravasate into the tumor. Despite the presence of TNFα in the tumor microenvironment, a pleiotropic cytokine that can activate endothelium to promote T cell adhesion [78], several mechanisms have been identified that abrogate this effect. The expression, for example, of the proangiogenic factors VEGF and bFGF downregulate adhesion molecule expression [79,80]; this can be reversed with αVEGF and αbFGF mAb treatment [69]. Another critical signaling axis in tumors is endothelin-1 (ET-1, a 21 amino acid vasoactive peptide) and its G protein coupled receptors ET_BR and ET_AR. ET-1 driven signaling can activate proliferation, confer apoptosis resistance, induce VEGF expression, stimulate new blood vessel formation and promote invasion and metastasis, and it is now recognized as a common mechanism underlying the progression of many solid tumors [81–84]. Through the analysis of gene expression profiles for ovarian cancers we associated the presence of ET_BR with an absence of TILs, and we further demonstrated that ET-1/ET_BR binding stimulates nitric oxide production, consequently decreasing ICAM-1 clustering. Moreover, in preclinical models of ovarian and lung cancer we showed that ET_BR neutralization with the selective antagonist BQ-788 upregulates the expression and clustering of ICAM-1 to restore T cell adhesion, increase intra tumoral T cell infiltration, and significantly improve responses following vaccination as well as adoptive T cell transfer [85••]. ET_BR blockade probably also inhibits angiogenesis through suppression of tumor cell derived VEGF and the reduction of EC nitric oxide production [86,87].

Although TNFα treatment has been shown to exert potent anti-tumor effects in animal models [88], its systemic administration in phase II clinical trials yielded minimal anti-tumor responses and was prohibitively toxic [89,90]. New therapeutic approaches, however, have been designed to selectively deliver TNFα to tumor blood vessels. For example, TNFα coupled with the CNGRC peptide motif (NGR-TNF) that specifically interacts with CD13 (an aminopeptidase expressed by ECs of angiogenic vessels) [91], upregulates ICAM-1 and VCAM-1 expression as well as proinflammatory cytokines, enhances T-cell extravasation and intratumoral infiltration, improves the penetration of chemotherapies into the tumor and their efficacy, and enhances adoptive and active immunotherapies in various mouse tumor models [92,93,94•]. A similar compound, RGR-TNF, has been shown to augment active and adoptive immunotherapy in experimental pancreatic neuroendocrine tumors, in part by remodeling the vascular network with less $PDGFR\beta^+$ pericyte coverage, and in part through the polarization of tumor-resident macrophages to an M1 immunostimulatory phenotype [95•]. Another approach to upregulating tumor EC expression of adhesion molecules, including ICAM-1, VCAM-1 and E-selectin, is by αCD137 (4-1BB) agonist mAb therapy. CD137 is a costimulatory glycoprotein expressed on activated T cells, NK cells, DC cells, and, interestingly, also on human tumor blood vessels [96–98]. Thus, administration of αCD137 mAb not only directly heightens anti-tumor $CD8^+$ T cell activity by binding to their cell surface [99•,100–102], it also stabilizes the tumor vasculature.

Active immunization against tumor vasculature antigens

Given the genetic stability and accessibility of tumor ECs, as well as the fact that they express various angiogenic markers that are either not present or are expressed at low levels in normal vessels, the tumor vascular is an attractive target for immunization [103,104]. The majority of immunization strategies that have been developed and tested to date in pre-clinical models are against VEGF/VEGFR2, either in the form of protein pulsed DCs [104,105] or DNA vaccines [106–111]. Responses, including tumor EC destruction, and the inhibition of tumor growth and metastasis, are primarily $CD8^+$ T cell-mediated. Immunizations with both autologous and xenogeneic endothelium have also yielded encouraging preclinical responses [112,113]. More recently, a DNA vaccine administered with tetanus toxoid and targeting tumor endothelial marker 1 (TEM1), a protein which is expressed on tumor ECs [114–118], as well as tumor-associated pericytes [119] and fibroblasts [120], was shown able to enhance intratumoral infiltration of endogenous $CD3^+$ T cells as well as delay tumor progression in mice [121•].

CAR-T cell targeting of tumor vasculature antigens

Over the past few years, CAR T cells against tumor vasculature antigens, including VEGFR1, VEGFR2 and prostate specific membrane antigen (PSMA; glutamate carboxypeptidase II) [122], have also been assessed in various pre-clinical mouse models. CARs are hybrid receptors comprising an antigen-targeting moiety, typically in the form of a single chain variable antibody fragment (scFv), fused with a linker, a transmembrane domain, the intracellular signaling module of CD3ζ, and various combinations of co-stimulatory domains such as CD28, 4-1BB (CD137), and OX40 (CD134) [123]. Overall, these studies have demonstrated that vasculature-targeting CAR T cells can significantly delay tumor growth. The co-expression of IL-12 or IL-15 was also shown to enhance their efficacy through increased tumor infiltration, expansion, and *in vivo* survival of the transferred cells [124,125,126•]. Finally, the combination of CAR T cells against VEGFR2 and T cells specific for the melanoma tumor antigens gp100 and TRP-1 resulted in increased intratumoral T cells, synergistic eradication of established B16 melanoma tumors, and prolonged tumor-free survival [127••]. Thus, if the numbers of adoptively transferred

anti-VEGFR2 CAR T cells are carefully escalated in the clinic, and safety mechanisms like dual/combinatorial antigen recognition and split signaling [128•,129•], inhibitory CAR [130], or suicide gene incorporation [131–133], are implemented to minimize 'on target, off site' toxicity, an important consideration for this potent therapy [134], the transfer of T cell populations targeting both the tumor and its vasculature could prove highly beneficial for the treatment of advanced cancer patients.

Combinatorial treatment approaches

Administration of antiangiogenic drugs as single agents has produced only modest clinical responses with no long-term survival benefits [135–137]. Similarly, vasculature disrupting agents, drugs designed to destroy existing vessels by destabilizing microtubules etc. and create central tumor necrosis, have shown limited efficacy along with extensive toxicity, even in combination with chemotherapy (drugs that interfere with cell division by inhibiting genes involved in DNA replication or metabolism), in clinical trials [138]. When given in combination with chemotherapy, whereas, the antiangiogenic mAb bevacizumab (Avastin) led to a remarkable 5 month increase in survival time for colorectal cancer patients [139] likely because the anti-VEGF treatment normalized the tumor vasculature thus enabling enhanced oxygen and drug delivery [34]. For such outcomes, both timing and dosage of the antiangiogenic therapy is vital so that the accompanying drugs are administered when the vessels have normalized (normalization is a transient state of enhanced vessel perfusion) and so that the vasculature is not damaged to an extent that it is inhibitory to drug delivery, or leads to hypoxia and/or harms normal tissues.

Another successful combination that has been tested is vasculature targeting plus chemotherapy and active immunotherapy [140]. It is worth noting that conventional cytotoxic chemotherapeutics such as cyclophosphamide, when given at lower doses and more frequently, this is referred to as metronomic chemotherapy, can affect the endothelium and have anti-angiogenic properties themselves, through the upregulation, for example, of thrombospondin 1 (TSP1 is a component of the extracellular matrix and an endogenous inhibitor of angiogenesis [141]). Moreover, the efficacy of metronomic chemotherapy increases when administered in combination with antiangiogenic drugs [142–144]. In a recent trial we observed anti-tumor responses and clinical benefit for some patients with recurrent stage III/IV ovarian cancer vaccinated with autologous DCs pulsed with tumor cell lysate in combination with bevacizumab and oral metronomic cyclophosphamide [9,145]. Several trials are currently underway to investigate the potential benefits of combining the anti-angiogenic drugs bevacizumab and sunitinib with immune checkpoint blockade antibodies (anti-PD-1 and anti-CTLA-4) and with DC vaccines [146]. It should be mentioned, however, that some combination therapies have failed. For example, in a multi-institutional clinical trial, metastatic colorectal cancer patients were randomly treated with capecitabine, oxaliplatin, and bevacizumab with or without cetuximab (a mAb against EGFR), and unfortunately, the four-drug combination led to significantly shorter progression-free survival time and inferior quality of life [147]. A similar outcome occurred for metastatic colorectal cancer patients treated with panitumumab (another anti-EGFR mAb) along with folinic acid, fluorouracil, oxaliplatin and bevacizumab. Thus, careful pre-clinical assessment of drug combinations is critical.

Conclusions

There is strong evidence that tumor infiltration by T lymphocytes is associated with good patient prognosis for many types of cancer including colorectal, ovarian, breast and melanoma. The tumor vasculature, however, constitutes an important barrier to T cells by actively blocking extravasation into the tumor through the deregulation of adhesion molecules including ICAM-1 and VCAM-1 [85••], by suppressing them with inhibitory receptors like PD-L1 and molecules such as IL-6 and IL-10, and even by targeting them for death by FasL expression [22•,57]. Several therapeutic approaches have been developed to break the tumor endothelial barrier and have further been shown to act synergistically with active and adoptive immunotherapies. Given the heterogeneity of solid tumors (different immune cell infiltrates, stromal composition, level of vascularization, etc.), well-designed preclinical and clinical studies are warranted to identify optimal combinations of antiangiogenic drugs and immunotherapeutic treatments for each type. This will require extensive monitoring of changes in tumor vascularity and the patient's immunity pre- and post-treatment, determination of immune biomarkers to measure antiangiogenic responses, and continuous efforts to identify additional tumor EC targets. The optimal doses as well as the scheduling of the treatment modalities should be taken under consideration to avoid toxic side-effects and maximize clinical effectiveness. Overall there is strong evidence that targeting the tumor vasculature to enhance T cell activity improves patient outcome and tumor vasculature normalization may one day be a standard of care for the treatment of solid tumors.

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