Anti-angiogenic agents — overcoming tumour endothelial cell anergy and improving immunotherapy outcomes

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Abstract | Immune-checkpoint inhibitors have revolutionized medical oncology, although currently only a subset of patients has a response to such treatment. A compelling body of evidence indicates that anti-angiogenic therapy has the capacity to ameliorate antitumour immunity owing to the inhibition of various immunosuppressive features of angiogenesis. Hence, combinations of anti-angiogenic agents and immunotherapy are currently being tested in >90 clinical trials and 5 such combinations have been approved by the FDA in the past few years. In this Perspective, we describe how the angiogenesis-induced endothelial immune cell barrier hampers antitumour immunity and the role of endothelial cell anergy as the vascular counterpart of immune checkpoints. We review the antitumour immunity-promoting effects of anti-angiogenic agents and provide an update on the current clinical successes achieved when these agents are combined with immune-checkpoint inhibitors. Finally, we propose that anti-angiogenic agents are immunotherapies — and vice versa — and discuss future research priorities.

Immunotherapies, in particular immune-checkpoint inhibitors (ICIs), have become a pillar of cancer treatment that have enabled unprecedented clinical benefits in a variety of malignancies, including durable responses in patients with malignancies that are aggressive, metastatic and/or previously difficult to treat[1–5], although typically only a subset of patients derive benefit[1–7]. Neither a low immunogenic capacity of tumour cells[8,9] nor the differential expression of immune checkpoints[10–12] alone can fully explain this effect. Thus, tumours presumably use additional mechanisms to evade immune destruction[13] and interest in using novel combinatorial treatment strategies to enhance antitumour immunity is increasing[14,15].

Angiogenesis is an important immune evasion mechanism. While tumour-induced angiogenesis is crucial for the outgrowth of solid tumours[16], mounting evidence shows that ongoing angiogenesis contributes to immune evasion through the induction of a highly immunosuppressive tumour microenvironment (TME)[17,18]. For example, VEGF (mostly VEGFA) is a key stimulator of angiogenesis[19] but also affects immune responses by inhibiting dendritic cell (DC) maturation[20] and by increasing the intratumoural numbers of both regulatory T (T_{reg}) cells[21,22] and myeloid-derived suppressor cells (MDSCs)[23]. VEGF has also been found to inhibit T cell development and function[24,25] and to promote T cell exhaustion through the upregulation of immune checkpoints[26]. Similar immunosuppressive effects of other pro-angiogenic factors, including angiopoietins, HGF and PDGF, have been extensively reviewed elsewhere[27]. Furthermore, ongoing angiogenesis has been found to hamper leukocyte infiltration[28–30]. In addition to the effector function of immune cells, their access to the tumour parenchyma is a prerequisite for an effective antitumour immune response and therefore for the efficacy of cancer immunotherapies[31,32]. Indeed, an 'immune-excluded' tumour phenotype has been described in which T cells were present in the tumour stroma but not in its parenchyma[9,33]. This phenotype correlates with a lack of clinical benefit from cancer immunotherapies, especially ICIs; conversely, 'immune-inflamed' tumour phenotypes[34] are characterized by a rich $CD8^+$ and $CD4^+$ T cell infiltrate within the tumour and its stroma[9] and correlate with a favourable prognosis[35,36] and benefit from ICIs in patients with a variety of cancers[37–39].

Blood vessels are the pivotal point of entry for circulating leukocytes and, thus, the presence of an aberrant tumour vasculature might explain immune-excluded tumour phenotypes. Owing to sustained pro-angiogenic signalling, the tumour vasculature is morphologically abnormal (tortuous, disorganized, excessively branched and leaky), which results in aberrant blood perfusion and oxygenation[40,41]. This abnormal morphology in turn leads to hypoxic areas and a low pH in the TME, which are known to be immunosuppressive[42]. In addition, tumour endothelial cells undergo a phenotypic change characterized by the expression of immune inhibitory molecules, non-adhesiveness and an unresponsiveness to inflammatory cytokines, which creates a barrier for immune cells. This phenomenon, referred to as tumour endothelial cell anergy, has been majorly underreported in the literature. Mechanistically, the exposure of tumour endothelial cells to VEGF and other angiogenic growth factors inhibits their activation by pro-inflammatory cytokines, such as TNFα, IFNγ and IL-1 (REFS[28–30]), leading to the decreased expression of endothelial adhesion molecules (EAMs). These molecules are required for leukocyte adhesion, extravasation and subsequent infiltration into the tumour parenchyma and, thus, their downregulation impedes antitumour immune responses and promotes immune evasion[28–30]. Therefore, the angiogenic tumour vasculature establishes a physical barrier for circulating leukocytes. Hence, the use of angiogenesis inhibitors to promote leukocyte infiltration into the tumour is considered an effective strategy to improve the efficacy of ICIs[27,43–45]. Indeed, the clinical benefit from combining anti-angiogenic agents with various cancer immunotherapies is becoming clear. Such

approaches are already used in the clinic for selected tumour types[46–50] and several others are under study[27,43,44,51].

In this Perspective, we review the endothelial immune cell barrier and its involvement in immune evasion, providing novel insights into the way in which anti-angiogenic agents can promote antitumour immunity. We then present preclinical and clinical advances achieved with combinations of anti-angiogenic and immunotherapeutic agents. We highlight the previously overlooked mechanism of endothelial cell anergy, hypothesizing that it is a remnant of the immune-privileged condition necessary during embryo development, and postulate endothelial cell anergy as a vascular immune checkpoint. Together, this evidence contributes to a deeper understanding of the angiogenesis-induced immune evasion that is required to pave the way for improving combinations of anti-angiogenic agents and ICIs in order to increase the number of patients with a clinical response.

The endothelial immune cell barrier

The vasculature has an important role in enabling effective immune responses by facilitating and regulating the tissue infiltration of immune cells. In cancer, the angiogenic vasculature forms a barrier for immune cells through endothelial cell anergy and the expression of immunosuppressive molecules (FIG. 1).

The vasculature and immunity

Endothelial cells are active participants and regulators of inflammatory processes, changing their phenotype to facilitate various phases of immune reactions[52]. In non-inflamed tissues, the vascular endothelium is quiescent and non-adhesive and yet prevents coagulation, regulates blood flow and controls vessel-wall permeability. Moreover, the quiescent endothelium actively prevents leukocyte adhesion through the intracellular sequestration or transcriptional suppression of EAMs. Upon inflammation, EAM sequestration is relieved by the transport of P-selectin to the luminal cell surface and pro-inflammatory cytokines (such as TNF and IL-1) activate endothelial cells via AP1 and NF-κB signalling. These pathways activate the gene expression of EAMs, such as E-selectin, ICAM1 and VCAM1, and the secretion of chemokines[52] (BOX 1), initiating leukocyte extravasation and infiltration into the tissue[53,54] (FIG. 2a). While different EAMs promote essential steps in leukocyte extravasation, ICAM1 is not only required but is also sufficient for transendothelial migration into the extravascular space[55,56].

Tumour endothelial cell anergy

Early research from our laboratory demonstrated that, upon stimulation with pro-angiogenic factors, tumour-associated endothelial cells become anergic, losing the ability to respond to inflammatory signals and become incapable of upregulating EAMs[28,30]. This barrier to infiltration (FIG. 1) was found to promote tumour escape from immune destruction. The concept of endothelial cell anergy has been reviewed in the context of immunotherapy efficacy[57] and seems essential to the benefits of combining anti-angiogenic agents with ICIs, an approach that is being demonstrated clinically[46–50].

Concept of tumour endothelial cell anergy. Initial studies in the early 1990s revealed the repressed expression of multiple EAMs in the tumour-associated vasculature[58,59] accompanied by diminished T cell–endothelium interactions[60,61]. The hypothesis that tumours interfere with the mechanism of immune cell extravasation, thereby adopting a strategy of immune evasion, arose at the same time and was first tested by Piali et al.[62]. They demonstrated the suppression of VCAM1 expression (at the mRNA and protein level) on tumour-associated endothelial cells in preclinical models as well as in blood vessels in metastatic tumour specimens from patients with melanoma or small-cell lung carcinoma[62]. Concurrently, we showed that ICAM1 and ICAM2 expression was downregulated in blood vessels in tumour samples from patients with renal cell carcinoma (RCC)[30]. Importantly, we identified that the exposure to angiogenic growth factors induced such downregulation of ICAMs. Indeed, the stimulation of cultured endothelial cells with the pro-angiogenic factor bFGF decreased the mRNA and cell-surface protein levels of ICAM1, which resulted in the reduced endothelial adhesion of activated leukocytes. Additionally, the exposure of endothelial cells to bFGF decreased their responsiveness to pro-inflammatory cytokines, preventing the upregulation of EAMs[30].

The concept of tumour endothelial cell anergy was introduced in 1996 (REF.[28]). Both bFGF and VEGF were found to inhibit the pro-inflammatory cytokine-induced adhesiveness of endothelial cells by suppressing the expression of ICAM1, VCAM1 and E-selectin. Hence, we suggested that the exposure to pro-angiogenic factors makes endothelial cells 'anergic' to inflammatory stimulation, resulting in a lack of upregulation of EAMs and, thus, in reduced leukocyte adhesion and extravasation[28] (FIG. 2b). Accordingly, we proposed that endothelial cell anergy is a mechanism exploited by tumours to evade immune infiltration and destruction[28,30].

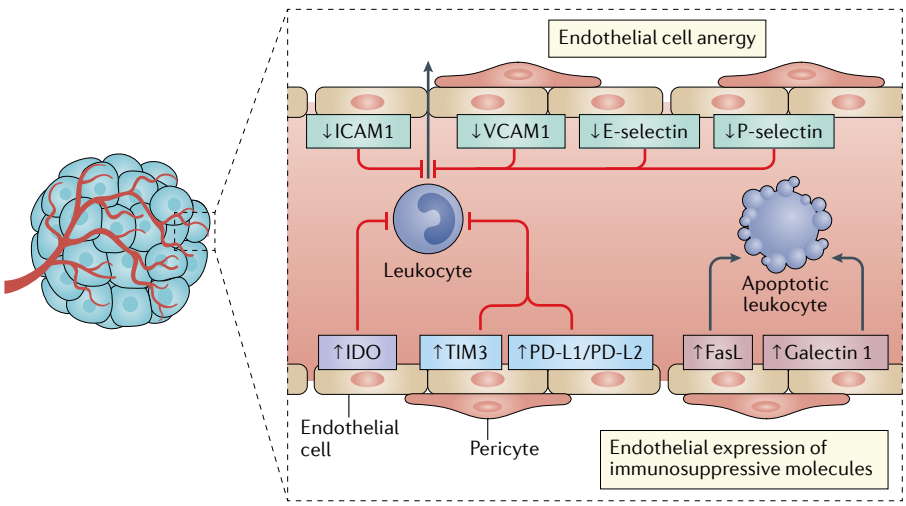

Fig. 1 | **The endothelial immune cell barrier.** Endothelial cells in the angiogenic tumour vasculature form a barrier for immune cells to infiltrate the tumour tissue. Immune cell infiltration is limited through pro-angiogenic factor-induced tumour endothelial cell anergy, characterized by a lack of endothelial adhesion molecules. In addition, endothelial cells express immunosuppressive molecules such as immune checkpoints that inhibit leukocyte function and molecules that induce the apoptosis of immune cells. Additional angiogenesis-mediated tumour immune-evasion mechanisms include the direct inhibitory effect of pro-angiogenic factors on immune cells and the presence of a hypoxic environment as a result of the tortuousness and leakiness of the tumour vasculature[27,43].

Establishment of mechanisms of endothelial cell anergy. Around the same time, Fukumura et al.[63] showed that TNFα induces leukocyte–vessel wall interactions to a lesser extent in tumours than in non-malignant tissues. Similarly, leukocyte adhesion was significantly reduced in pancreatic tumour vessels compared with those of the non-malignant pancreas, even when the tumour vessels were exposed to chemotactic substances resembling pro-inflammatory signals[64]. Until then, the only evidence for endothelial cell anergy came from in vitro studies and a detailed molecular mechanism behind this regulatory process, apart from the observation that TNFR expression was not altered by pro-angiogenic signalling[28], had not been provided. We developed in vivo models to demonstrate that the exposure of endothelial cells to bFGF or VEGF reduced ICAM1 and VCAM1 expression as well as leukocyte adhesion[29,65]. Moreover, poor leukocyte infiltration in human ductal breast carcinomas correlated with low levels of ICAM1 expression and a highly angiogenic tumour state[66]. Results from subsequent studies provided a better understanding of the mechanisms underlying endothelial cell anergy[67–72] (FIG. 3).

Melder et al. confirmed that the ability of natural killer (NK) cells to adhere to the endothelium was indeed inhibited by bFGF. VEGF, however, was found to promote EAM expression and NK cell adherence[73]. This ostensible discrepancy with the previously described effects of VEGF could be explained by the timing of assessment of VEGF-induced ICAM1 expression (24 hours). Indeed, previous reports demonstrated that endothelial ICAM1 is initially upregulated (0–48 hours) by pro-angiogenic stimuli before its expression drops (after 48 hours)[30]. This biphasic induction of ICAM1 by VEGF might relate to a requirement for endothelial cell migration early on in angiogenesis[74] or to VEGF initially acting as a pro-inflammatory cytokine (for example, during wound healing) before becoming anti-inflammatory. Both hypotheses remain to be tested.

Endothelial cell anergy is a vascular immune checkpoint. We favour the view that endothelial cell anergy is the vascular counterpart of immune checkpoints because both mechanisms share features related to immune regulation and opportunities for therapeutic intervention (FIG. 4a–e). Firstly, both endothelial cell anergy and immune checkpoints are key regulators of the immune responses required to maintain immune homeostasis. The expression of immune checkpoints, such as PD-1, is low in basal functional states, upregulated following TCR stimulation[75] and further upregulated upon chronic antigen stimulation[76,77]. Upon ligand interaction, intracellular signalling by PD-1 results in decreased proliferation, metabolic reprogramming and reduced cytokine secretion. In this way, the activity and function of T cells is inhibited, self-tolerance is maintained and tissue damage is prevented[78,79]. Similarly, endothelial cell anergy might be a naturally occurring process to coordinate and maintain immune homeostasis. This view is supported by the fact that physiological angiogenesis and immunosuppression are not independent processes and often occur simultaneously in response to the same stimuli[17,18]. The unresponsiveness to inflammatory stimuli, resulting in a lack of EAM expression, occurs in endothelial cells during embryonic development. A study by Nussbaum et al.[80], published in 2013, revealed the age-dependent expression of EAMs on and immune cell recruitment by umbilical cord-derived endothelial cells. Upon endothelial activation, the levels of E-selectin and ICAM1 and rolling of polymorphonuclear neutrophils were reduced on these cells in premature neonates compared with full-term neonates[80]. Importantly, during fetal development, wound healing is characterized by a paucity of inflammation, resulting in 'scarless' healing of the wound[81–83]. In addition, the placenta, which is formed by vasculogenesis and angiogenesis[84], creates an immunological barrier required to protect the fetus from rejection by its mother's immune system[85].

Moreover, during wound healing in adults, periods of inflammation are followed by a resolution phase characterized by restoration of the vascular network and formation of granulation tissue (which is key in wound healing). During this transition, inflammation and active immune responses need to be downregulated, whereas angiogenesis needs to be induced to properly regulate tissue restoration and maintain tissue homeostasis[83,86,87]. The numerous immunosuppressive roles of VEGF[27], the key inducer of angiogenesis[19], can be explained in this context. By contrast, crucial mediators of antitumour immune responses, including IFNγ, TNF and the chemokines CXCL9 and CXCL10, inhibit tumour angiogenesis[44]. Additionally, various immunosuppressive immune cells present during wound healing promote angiogenesis. These include MDSCs, specific subsets of DCs, regulatory NK cells, neutrophils, M2-like macrophages, regulatory B cells and T_{reg} cells[18,44,86,88,89]. Thus, angiogenesis and immunosuppression go hand in hand and we can argue that the physiological role of endothelial cell anergy is to prevent excessive immune responses and maintain immune homeostasis.

Secondly, both endothelial cell anergy and immune checkpoints are hijacked by tumours to evade immune destruction. The expression of immune checkpoints, usually tightly coordinated, is often dysregulated by tumours to provide resistance mechanisms[6,90,91]. For example, PD-L1 expression on the tumour cell surface is upregulated in many types of human cancers[91] as a result of oncogenic signalling or in response to interferons secreted during an antitumour immune response[90]. Hence, at the site of the tumour, checkpoint molecule ligation hampers T cell-mediated antitumour immune responses and results in a lack of tumour elimination[6,78,92] (FIG. 4b). Likewise, in the vast majority of human solid cancers, VEGF is overexpressed owing to numerous and diverse changes in genetic and epigenetic regulation and can be further increased in response to tumour-induced hypoxia[19], leading to endothelial cell anergy (FIG. 4d). Indeed, tumours have been described to mimic never-healing wounds, resulting in a highly vascularized and immunosuppressive TME that supports further tumour progression[86,93].

Lastly, both immune checkpoints and endothelial cell anergy can be targeted to improve antitumour immune responses. The blockade of immune-checkpoint molecule interactions results in the amelioration

Box 1 | Induction and function of endothelial adhesion molecule expression

Endothelial cells are activated upon binding of TNFα to its receptor TNFR1, expressed on the endothelial cell surface, or by similar pro-inflammatory cytokine-receptor interactions[52]. Upon stimulation, TNFR1 recruits TRADD, which in turn binds RIP1 and TRAF2. This signalling complex can subsequently initiate various kinase cascades that lead to the activation of NF-κB and AP1, both of which are transcription factors that, upon activation, migrate to the nucleus, where they initiate gene transcription of pro-inflammatory genes (such as the endothelial adhesion molecules E-selectin, ICAM1, ICAM2 and VCAM1), but also chemokines and cyclooxygenase 2 (REF.[52]). These adhesion molecules are involved in different steps of leukocyte extravasation: E-selectin and P-selectin are involved in the capturing and rolling of leukocytes, whereas ICAM1, ICAM2 and VCAM1 are involved in the later steps of rolling, crawling, arrest and transendothelial migration[53].

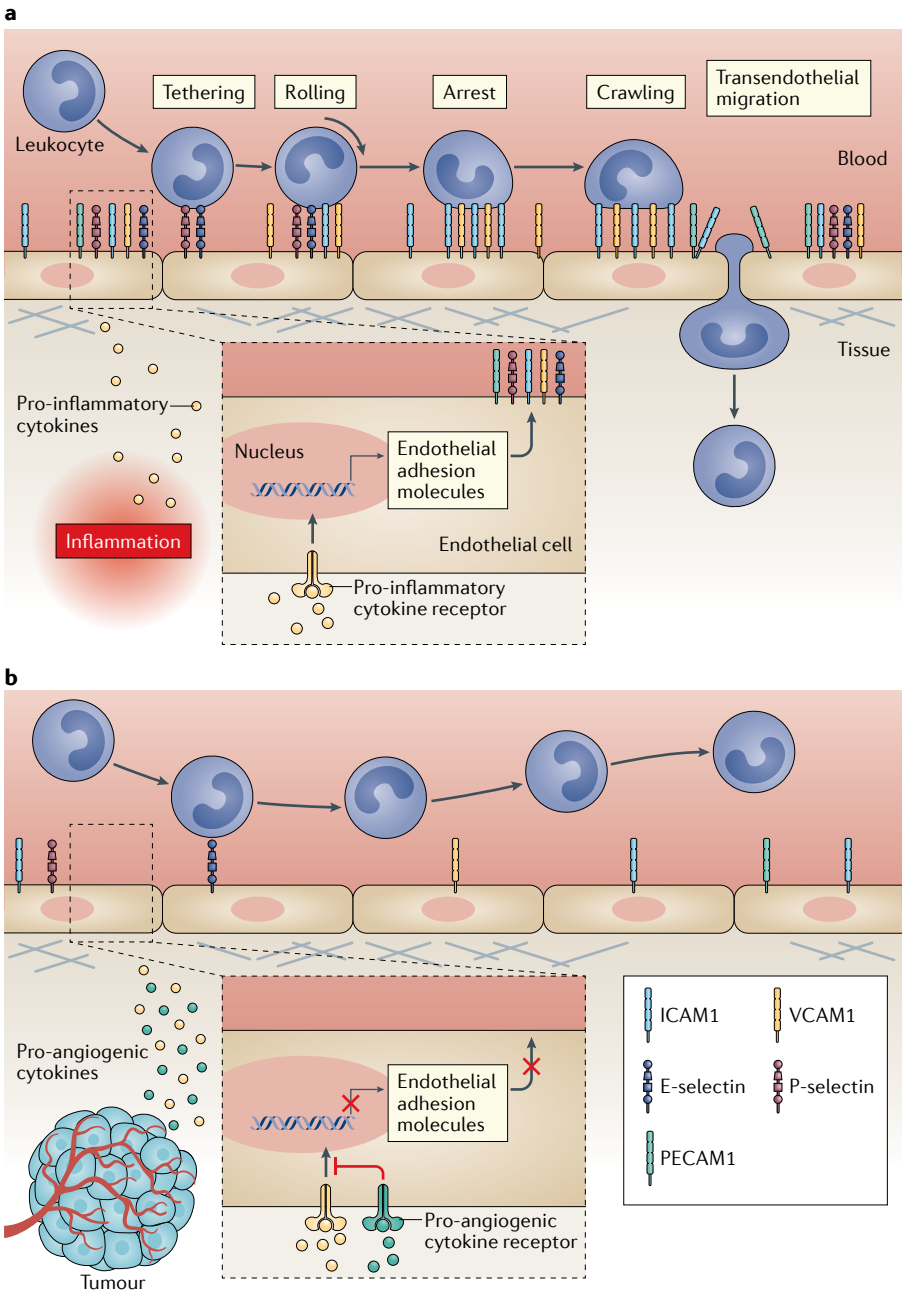

Fig. 2 | **Endothelial cell anergy.** The angiogenic growth factor-induced unresponsiveness of endothelial cells to pro-inflammatory stimuli results in a lack of expression of endothelial adhesion molecules (EAMs) and subsequent hampering of leukocyte extravasation. **a** | During inflammation, pro-inflammatory cytokines, such as TNFα, IFNγ and IL-1, interact with their receptors, resulting in EAM transcription and expression. The cell-surface expression of EAMs is required for leukocyte rolling, adhesion and subsequent extravasation. **b** | Tumour-secreted angiogenic growth factors, such as VEGF, bFGF and EGF, inhibit the inflammation-induced activation of endothelial cells, resulting in a lack of EAM transcription and expression. This process limits immune cell extravasation because leukocytes cannot sufficiently adhere to the endothelium.

of immune cell function[92]. For example, treatment with anti-PD-1 antibodies has efficacy in patients with non-small-cell lung cancer (NSCLC), RCC or melanoma by reinvigorating cytotoxic T cells[1,2,92,94–96] (FIG. 4c). Likewise, anti-angiogenic therapy can overcome tumour-induced endothelial cell anergy[16,68,97–101] (FIG. 4e). Thus, whereas immune checkpoints regulate the intensity and duration of the immune response through signalling in immune cells, endothelial cell anergy is a mechanism hardwired in the endothelium that also provides a checkpoint for immune responses. Hence, endothelial cell anergy is a vascular immune checkpoint.

Endothelial expression of immunosuppressive molecules. Various inhibitory molecules, expressed on endothelial cells of several tumour types, contribute to the endothelial immune cell barrier (FIG. 1). One of these molecules is the carbohydrate-binding protein galectin 1, which is overexpressed in various malignancies and is a feature associated with poor prognosis[102]. Galectin 1 facilitates tumour progression through its involvement in angiogenesis[103] but also through its important role in suppressing immunity[104]. Preclinical data indicate that, upon ligand interaction, galectin 1 expressed at sites of T cell development and maturation can induce the apoptosis of activated T cells[105], antagonize TCR-dependent signalling[106], inhibit the antigen-induced proliferation of T cells[107] and sensitize resting T cells to Fas-mediated cell death[108]. Galectin 1 is also overexpressed on tumour endothelial cells[103,109], resulting in limited infiltration by activated T cells[110–112]. Galectin 1 expressed by tumour cells also has an immunosuppressive role. In mouse models, the genetic silencing of galectin 1 in tumour cells resulted in enhanced antitumour immune activity[113,114]. Nambiar et al.[115] elegantly demonstrated that the secretion of galectin 1 by tumours can contribute to the endothelial immune cell barrier through the upregulation of PD-L1 and galectin 9 expression on the tumour endothelium, promoting immunotherapy resistance through T cell exclusion. Accordingly, blockade of galectin 1 increased the infiltration of T cells into the tumour, an approach that synergized with immune-checkpoint inhibition[115].

Fas ligand (FasL) was found to be selectively expressed in the vasculature of mouse and human tumours but not in that of non-malignant tissues[116]. The presence of FasL correlated with a lack of $CD8^+$ T cell infiltration and an intratumoural presence of T_{reg} cells. Indeed, FasL induced apoptosis in infiltrating $CD8^+$ T cells, whereas T_{reg} cells were unaffected owing to their higher expression levels of *CFLAR*, which encodes the anti-apoptotic factor c-FLIP. The attenuation of FasL expression levels led to an increase in the ratio between $CD8^+$ T cells and T_{reg} cells, resulting in tumour regression[116].

In the lymphoma endothelium, the expression of TIM3 inhibited the activation and T helper 1 polarization of $CD4^+$ T cells, thereby facilitating immune evasion and cancer progression[117]. The immune checkpoints PD-L1 and PD-L2 are also believed to be involved in the endothelial immune cell barrier. Various preclinical

studies showed that endothelial PD-L1 expression, induced upon IFNγ exposure, inhibits T cell activation[118–120] and promotes T_{reg} cell activation[121]. The latter can be blocked with anti-PD-L1 antibodies, thereby lowering the production of immunosuppressive cytokines[121]. By contrast, blockade of the PD-1–PD-L1 axis enhances the immunosuppression and proliferation of T_{reg} cells in tumours[122,123]. Establishing a definitive role for PD-L1 in endothelial cells would be of importance because the balance of PD-1 expression levels between $CD8^+$ T cells and T_{reg} cells in the TME has been suggested to be a predictor of the efficacy of anti-PD-1 antibodies in humans[123]. Nevertheless, the immunomodulatory role of endothelial PD-L1 expression in the TME remains under investigation. A study revealed that PD-L1 is expressed on endothelial cells in various malignancies and that this expression is correlated with the infiltration, proliferation and activation of immune cells and a poor patient prognosis[124]. Anlotinib, a novel tyrosine-kinase inhibitor (TKI) of various pro-angiogenic signalling proteins, inhibits PD-L1 expression on endothelial cells, resulting in enhanced $CD8^+$ T cell infiltration, an increased $CD8^+$ T cell to T_{reg} cell ratio, and suppression of tumour growth[124]. By contrast, other studies indicated that endothelial PD-L1 expression increases in response to treatment with the anti-VEGFR2 antibody DC101 (REFS[125,126]) or dual blockade of VEGFA and angiopoietin 2 (REF.[127]).

Indoleamine 2,3-dioxygenase can also be expressed by the tumour endothelium[128], which promotes immunosuppression of $CD8^+$ T cells through tryptophan depletion[128,129]. Moreover, the selective migration of T_{reg} cells into the tumour bed is promoted by VEGF and HGF through the upregulation of endothelial stabilin 1 (also known as CLEVER1)[130]. In conclusion, tumour endothelial cells can express a range of inhibitory molecules, thereby creating a barrier for immune cells to infiltrate into the tumour tissue.

Anti-angiogenesis promotes immunity

Anti-angiogenic agents, which frequently target the key angiogenic effectors VEGF and VEGFR, are widely used in the clinical management of cancer but have limited utility owing to their modest efficacy and the common occurrence of acquired resistance[131,132]. However, these agents also have additional effects that enhance immunity.

Overcoming endothelial cell anergy

Anti-angiogenic agents can overcome endothelial cell anergy and reinduce EAM expression, resulting in increased leukocyte infiltration into tumours. The anti-angiogenic cytokine platelet factor 4 was found to prevent bFGF-induced ICAM1 downregulation and to restore ICAM1 expression on endothelial cells. This model showed that bFGF limits TNFα-induced ICAM1 expression and platelet factor 4 inhibits this effect, thereby overcoming endothelial cell anergy[97]. The angiostatic agent SU6668 blocks the bFGF-induced inhibition of EAM expression and leukocyte transendothelial migration in vitro[99]. Another study revealed that treatment of endothelial cells with the angiostatic agent hPRL, a 16K fragment from human prolactin[133], increased the mRNA levels of ICAM1, VCAM1 and E-selectin in tumour-associated vessels, which improved leukocyte–endothelial adhesion and resulted in an increase of tumour infiltrating lymphocyte counts in vivo[98]. Moreover, the inhibition of angiogenesis with the angiostatic synthetic peptide anginex[134] was demonstrated to overcome endothelial cell anergy and to increase the number of infiltrating T cells in vivo[101]. DNA methyltransferase inhibitors and histone deacetylase inhibitors were found to have angiostatic activity[135,136] and reverted the epigenetic silencing of endothelial *ICAM1* (REF.[68]). Taken together, these studies show that anti-angiogenic agents can overcome endothelial cell anergy; we therefore suggest that such agents are inhibitors of the vascular immune checkpoint and, thus, immunotherapies by themselves. Promoting endothelial cell anergy alone or as an adjuvant to existing immunotherapies could be used as an immunotherapeutic approach.

Treatment strategies other than those involving anti-angiogenic agents can also increase EAM expression and immune cell infiltration, thereby promoting tumour immune destruction[137–140]. For example, mice with immune-excluded pancreatic adenocarcinomas were treated with Nano-sapper, a nanoparticle specifically designed to reduce physical hurdles in the TME and recruit cytotoxic T lymphocytes. Amongst others, ICAM1 and the EAM VE-cadherin were upregulated, resulting

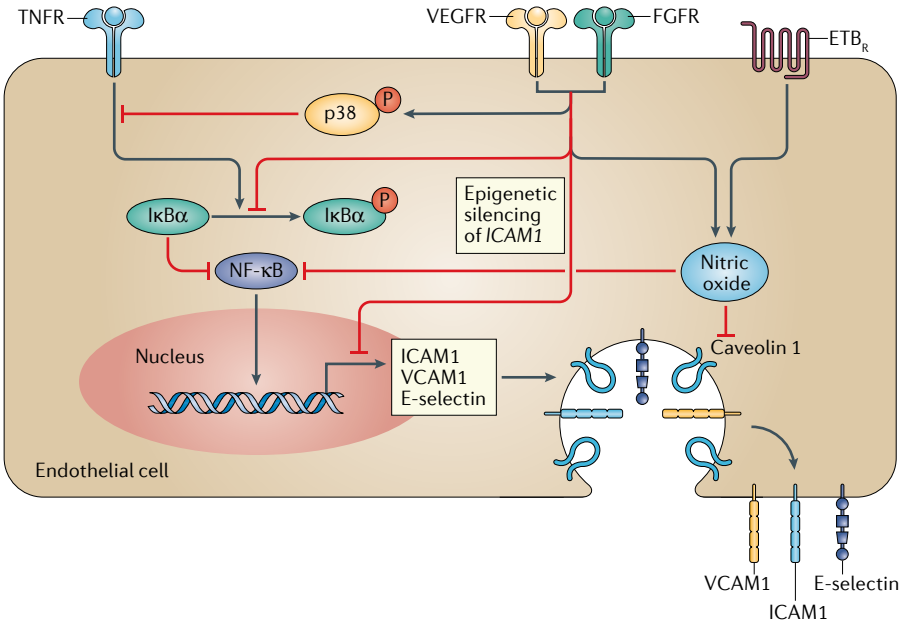

Fig. 3 | **Molecular mechanisms of endothelial cell anergy.** Pro-angiogenic signalling inhibits the transcription and cell-surface expression of endothelial adhesion molecules (EAMs) in several ways. The TNFα-induced activation of the NF-κB pathway, which regulates EAM expression, is inhibited by bFGF[67]. *ICAM1* was found to be epigenetically silenced in tumour endothelial cells through relevant histone modifications affecting the chromatin structure[68]. VEGF potently induces nitric oxide release from the vascular endothelium[204], which decreases pro-inflammatory cytokine-induced endothelial cell activation. The inhibition of nitric oxide production with small molecules in endothelial cells increases EAM expression and leukocyte adhesion[69,70]. Moreover, VEGF reduces the expression of caveolin 1 through stimulation of the nitric oxide pathway, resulting in abnormal clustering of ICAM1 and VCAM1 at the endothelial cell surface[71]. Caveolin 1 is a key nitric oxide-mediated regulator of caveolae, which concentrate a variety of signalling molecules important for cell function such as EAMs[205]. Finally, nitric oxide is a crucial downstream effector of ETB_R signalling, which affects ICAM1 mRNA and protein expression as well as ICAM1 clustering[72].

in enhanced cytotoxic lymphocyte infiltration[137]. Furthermore, endothelial cell anergy was overcome in mice with systemic thermal therapy, which involves a 6-hour core temperature elevation to 39.5 °C. This therapy increased the expression of the inflammatory cytokine IL-6, resulting in increased levels of EAMs, together with improved homing of adoptively transferred T cells into the tumour parenchyma[140].

Relief of immune cell suppression

Anti-angiogenic agents can reverse the direct immunosuppressive effects of pro-angiogenic factors on immune cells. Hence, numerous studies in mice and humans have shown that anti-angiogenic agents promote immunity. Bevacizumab, a VEGFA neutralizing antibody, has been shown to increase the number and activation of DCs[141,142] as well as the number of cytotoxic T cells[143,144] and to revert VEGF-induced T cell exhaustion[26]. Sunitinib, a TKI of VEGFR and other kinases, was reported to decrease the development and abundance of T_{reg} cells[145–147]

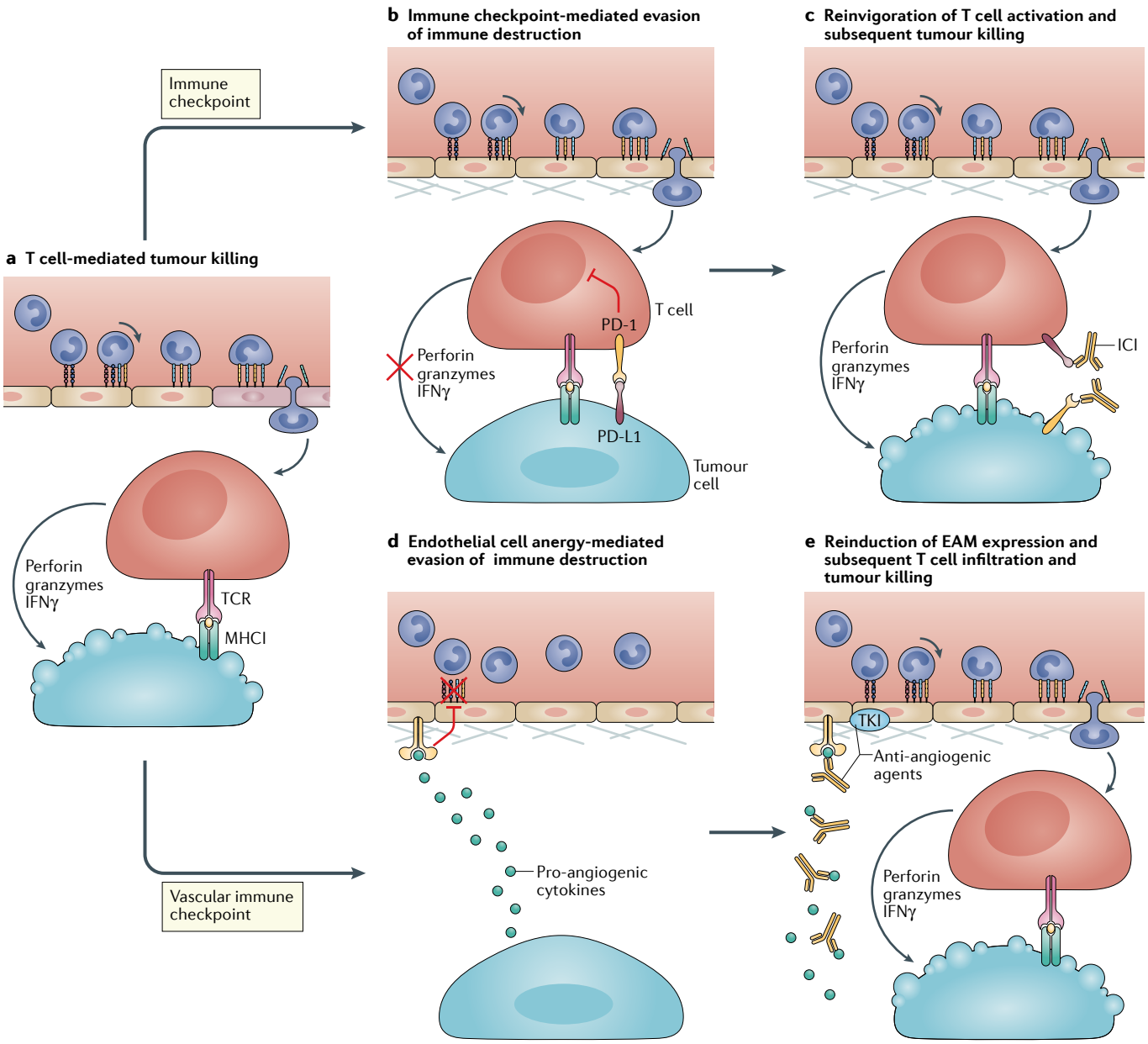

Fig. 4 | **Endothelial cell anergy is a vascular immune checkpoint.** Both immune checkpoints and endothelial cell anergy are pivotal regulators of immune responses. **a** | Circulating T cells usually infiltrate through the vessel wall into the tumour where, upon antigen recognition, they secrete effector molecules (such as perforin, granzymes and IFNγ) to kill tumour cells. **b** | Tumours hijack immune checkpoints (such as PD-1) by expressing their ligands (PD-L1). Upon interaction with tumour cells, T cells lose their ability to secrete effector molecules and kill tumour cells. **c** | This immune evasion mechanism can be inhibited by blocking immune checkpoint molecule interactions with monoclonal antibodies. **d** | Tumours impair the expression of endothelial adhesion molecules (EAMs), required for T cell adhesion and infiltration, by secreting pro-angiogenic cytokines that induce endothelial cell anergy. Insufficient EAM expression results in hampered T cell infiltration and subsequent evasion of tumour immune destruction. **e** | Agents targeting VEGF/VEGFR can overcome tumour-induced endothelial cell anergy, thereby reinducing EAM expression and promoting T cell infiltration and tumour cell killing. Thus, anti-angiogenic agents overcome this vascular immune checkpoint and can therefore be considered inhibitors of the vascular immune checkpoint. ICI, immune-checkpoint inhibitor; MHCI; TKI, tyrosine kinase inhibitor.

and reduce the number and function of MDSCs[145,148].

Vascular normalization

Although many anti-angiogenic therapies are used to exhaust or destroy the tumour vasculature, mounting evidence indicates that normalization rather than destruction of the tumour vasculature might be an effective anticancer strategy. Vascular normalization involves the judicious dosing of anti-angiogenic agents to reverse the abnormal phenotype of the tumour vasculature in order to improve blood flow and oxygenation — for example, by tightening endothelial cell junctions and improving pericyte coverage[149]. This reversion leads to the improved delivery of anticancer therapeutics and oxygen into the tumour and, thus, to the enhanced efficacy of various chemotherapeutic agents, radiotherapy and photodynamic therapy[149–151]. In addition, vascular normalization has been shown to improve immune responses through vessel maturation and by the relief of immunosuppression induced by hypoxia and/or VEGF[43,44]. For example, vascular normalization resulted in improved intratumoural T cell infiltration[152–154] and in polarization of tumour-associated macrophages towards a pro-inflammatory M1 phenotype[153]. Angiopoietin 2 is a pro-angiogenic factor that can be inhibited to normalize the vasculature[155]. In mouse models, the dual inhibition of VEGFA and angiopoietin 2 normalized the tumour vasculature and increased the efficacy of ICIs[125,127,156]. Moreover, vascular targeting of the cytokine TNFSF14 (also known as LIGHT) in mice can induce vascular normalization through improving pericyte contractility and maturation[157], which enhances endothelial barrier integrity and vessel function[158], thereby reducing immunosuppression through the relief of tumour hypoxia and intratumoural pressure. In addition to these effects, LIGHT induced tertiary lymphoid structures and high endothelial venules (HEVs) in tumours, which was associated with increased intratumoural leukocyte infiltration[158,159], as we describe below.

Of note, the extent to which overcoming endothelial cell anergy (with subsequent enhanced endothelial expression of immunosuppressive molecules) contributes to the immune-promoting effect of vascular normalization remains unclear (BOX 2). Only vascular normalization through LIGHT targeting has been shown to increase EAM expression[157,158], probably through the induction of inflammatory responses in endothelial cells[160].

Anti-angiogenics induce HEVs

In the past few years, several studies have revealed that anti-angiogenic agents, in some cases combined with ICIs, can drive antitumour immune responses through the induction of HEVs. HEVs are postcapillary venules comprised of high endothelial cells, typically found in secondary lymphoid organs, and mediate leukocyte extravasation from the blood directly into lymph nodes[161]. Allen et al.[125] showed that the combination of the anti-VEGFR2 antibody DC101 and an anti-PD-L1 antibody induced the formation of HEV-like blood vessels in mouse breast tumours and insulinomas, enabling T cell infiltration. Another study showed that the vascular targeting of LIGHT induced the formation of tertiary lymphoid structures and HEV in a mouse model of insulinoma, which resulted in increased T cell infiltration and in the elimination of tumours resistant to immunotherapy[159]. This strategy also enhanced the efficacy of ICIs, increased the presence of effector and memory T cell infiltrates, and improved survival outcomes in this model[159]. Similarly, the vascular targeting of LIGHT induced HEV formation and $CD3^+$ T cell accumulation in a mouse model of glioblastoma[158]. Triple combination therapy with LIGHT, an anti-VEGF antibody and an anti-PD-L1 antibody even further induced HEV formation and increased $CD4^+$ and $CD8^+$ T cell infiltration, with upregulation of granzyme B expression and downregulation of FOXP3 expression[158]. Nevertheless, two aspects that remain to be determined are how intratumoural HEVs are remodelled in response to tumour-secreted factors and to what extent they are involved in tumour cell dissemination[162].

Whether tumour-secreted factors lead to an anergic state in endothelial cells of the lymphatic system is unknown. Lymphatic vessels are involved in leukocyte drainage from rather than infiltration into tumour tissues and thus the relevance of this question might be limited. Of note, the expression of EAMs, such as ICAM1 and VCAM1, in lymphatic vessels is usually already limited[163].

Preclinical combination therapy

Several elegant preclinical studies have shown that the inhibition of angiogenesis, alone or combined with different immunotherapies, ameliorates antitumour immunity by increasing T cell infiltration. For example, anti-angiogenic treatment with sunitinib resulted in the improved infiltration of $CD4^+$ and $CD8^+$ T cells in colon tumours in mice[145] as well as in human RCCs[164]. Moreover,

Box 2 | **Is there a link between vascular normalization and the endothelial immune cell barrier?**

Vascular normalization is thought to improve antitumour immunity through the relief of hypoxia-induced and VEGF-induced immunosuppression[43]. Nevertheless, extensive research on whether this immune-promoting effect of vascular normalization also results from overcoming endothelial cell anergy is lacking to date. As described in the main text, several studies have demonstrated the reversal of endothelial cell anergy and the reinduction of endothelial adhesion molecule expression upon anti-angiogenic therapy[68,97–99,101]. Yet, whether appropriately low doses of anti-angiogenic agents would have a similar effect is unclear. This question also applies to the expression of immunosuppressive molecules in tumour endothelial cells. Some studies have shown that PD-L1 expression increased in tumours upon treatment with different anti-angiogenic agents[125,127], whereas others found that treatment with the tyrosine-kinase inhibitor anlotinib reduced PD-L1 expression in tumour endothelial cells and increased $CD8^+$ T cell infiltration, inhibiting tumour progression[124].

Besides these examples, however, two questions remain poorly investigated: (1) whether anti-angiogenic agents can, either in high doses or in vascular-normalizing doses, affect other immunosuppressive molecules expressed by the tumour endothelium; and (2) whether this modulation would promote antitumour immunity. As such, studies providing insights into the changes in the expression of endothelial adhesion molecules and immunosuppressive molecules on the tumour vasculature upon low-dose anti-angiogenic treatment could provide a theoretical foundation to identify appropriate combination therapies. Hence, the factors that mediate resistance (such as particular immunosuppressive molecules) and that maintain similar levels once the vasculature has been normalized could be addressed. For example, the effect of anti-angiogenic treatment on galectin 1 expression is unclear but this protein could be therapeutically targeted in patients with tumours retaining its expression while receiving anti-angiogenic agents. Moreover, the optimal anti-angiogenic drug concentrations and administration schedules to overcome endothelial cell anergy remain to be determined[206,207]. If vascular normalization relieves hypoxia and indeed overcomes endothelial cell anergy and represses the expression of immune inhibitory molecules by endothelial cells, judicious dosing of anti-angiogenic agents could be key to the clinical efficacy of immunotherapies.

the dual blockade of angiopoietin 2 and VEGFA with the bispecific antibody A2V in mouse models of various malignancies stimulated immunity by promoting antigen presentation as well as the extravasation, perivascular accumulation and activation of T cells[127]. Similarly, treatment with A2V and anti-CD40 antibodies led to the conversion of intestinal tumours in mice into an inflamed phenotype associated with T cell infiltration[165]. The combination of adoptive T cell therapies with anti-angiogenic agents in mice significantly increased T cell infiltration compared with either therapeutic modality alone[101,152]; similar results were obtained in studies with chimeric antigen receptor (CAR) T cells[166,167]. Furthermore, the inhibition of VEGFR2 signalling enhanced the infiltration of tumour-specific T cells into tumours of mice vaccinated against HER2 (REF.[168]) and promoted antitumour immunity in combination with anti-PD-1 treatment[169]. Of note, in most studies described above, increased T cell infiltration was associated with enhanced antitumour immunity and increased tumour cell death. Taken together, these studies provide evidence that anti-angiogenic agents can improve the intratumoural infiltration of effector T cells, thereby increasing the efficacy of immunotherapy. Some studies specifically assessed EAM expression[101,127] whereas others did not; however, the re-induction of EAM expression might have played a key role.

Combinations in non-angiogenic tumours

Owing to the assumption that the induction of an anergic endothelium is a feature of ongoing angiogenesis, the question arises of what happens in non-angiogenic tumour growth — that is, in tumours that rely on vessel co-option[170] or in those that apply vasculogenic mimicry[171]. Vessel co-option is a type of tumour growth in which tumour cells grow along the extravascular matrix and do not depend on the formation of a neovasculature and which often occurs in metastases[172,173]. Endothelial cell anergy has not yet been described in such tumours, although its presence can be assumed for several reasons. Firstly, the expression of angiogenic growth factors does not differ between tumours with co-opting vessels and angiogenic tumours[174]. As endothelial cell anergy is the result of changes in cellular signalling or metabolism and epigenetics (FIG. 3) rather than of changes in proliferative activity and neovasculature formation, it can occur in both tumour types. Secondly, tumour tissues with co-opting vessels (for example, liver metastases) have been described to have low levels of immune cell infiltration[175,176]. Although EAM expression was not studied in these reports, a low vascular adhesiveness has been suggested. Thirdly, angiogenesis inhibitors regulated ICAM1 expression independently from their effect on endothelial cell viability[100], suggesting that the reversal of endothelial cell anergy can be achieved in non-angiogenic co-opted vessels.

Vasculogenic mimicry is another form of non-angiogenic tumour growth[177,178] in which tumour cells mimic endothelial cells to form blood vessels. In vasculogenic structures, leukocyte–vessel wall interactions are rare, probably because of the absence of EAMs in the lining tumour cells[179]. We have described that vasculogenic tumour cells, despite trying to 'masquerade' as endothelial cells, do not develop a sensitivity to angiogenesis inhibitors[180] and maintain low levels of ICAM1 expression[181]. Therefore, in contrast to vessel co-option, vasculogenic mimicry might preclude the benefit from combinations of angiogenesis inhibitors and immunotherapies. Thus, this effect should be considered during analyses of the clinical benefit of these therapeutic approaches. Fortunately, vasculogenic mimicry is quite a rare process in most tumours, even in metastases[179].

Clinical implications

Both anti-angiogenic agents and ICIs have emerged as major anticancer therapeutic classes for various malignancies, but the efficacy of either treatment modality alone is limited by the development of resistance, a lack of responsiveness and often severe adverse effects[13,131,182]. As previously illustrated, numerous preclinical studies have provided evidence that angiogenesis-induced immunosuppression can be exploited to improve immunotherapy. Hence, the addition of anti-angiogenic agents to immunotherapies is currently considered an attractive treatment approach that is currently being addressed in clinical trials[17,27,43,45]. At present, >80 different combinations of anti-angiogenic and immunotherapy agents are under evaluation, the majority of which involve ICIs (Supplementary Table 1). The increased interest in this combination approach over the past few years is well demonstrated by the fact that half of these studies have been initiated since 2018. As a result of these trials, the FDA has approved five combinations of ICIs and anti-angiogenic agents for the treatment of RCC, NSCLC, hepatocellular carcinoma and endometrial carcinoma[46–50] (TABLE 1).

The first of these approvals was based on the results of IMpower150, an open-label multicentre phase III trial that evaluated the combination of the anti-PD-L1 antibody atezolizumab plus bevacizumab and chemotherapy in previously untreated patients with metastatic NSCLC. Patients with *EGFR*-mutated NSCLC are already known to not benefit from ICIs; thus, this study involved a majority of patients with wild-type *EGFR* or *ALK*. Furthermore, a subgroup of patients within this population harboured an effector T cell gene signature, which was defined by the tumoural mRNA expression of PD-L1, CXCL9 and IFNγ (TABLE 1). From all patients, including those with *EGFR* or *ALK* genetic alterations, those with low levels or no expression of PD-L1 in tumours, or those with low effector T cell gene signatures, progression-free survival (PFS) was longer in those receiving bevacizumab plus atezolizumab and chemotherapy compared with those who did not receive bevacizumab[46].

The second approval was based on the results from KEYNOTE-426, an open-label phase III clinical trial in which previously untreated patients with advanced-stage clear-cell RCC received either sunitinib or the anti-PD-L1 antibody pembrolizumab plus the VEGFR TKI axitinib (TABLE 1). A greater clinical benefit was observed in the pembrolizumab plus axitinib group, regardless of tumour PD-L1 expression[47]. The FDA subsequently approved avelumab (another anti-PD-L1 antibody) plus axitinib as a first-line therapy for patients with advanced-stage RCC. This approval was based on the multicentre, open-label, phase III JAVELIN Renal 101 trial in which this combination was also compared with sunitinib[48] (TABLE 1).

The fourth approval was based on the results of IMbrave150, a multicentre, open-label, phase III trial that compared atezolizumab plus bevacizumab versus sorafenib in previously untreated patients with locally advanced or metastatic hepatocellular carcinoma. Better overall survival and PFS outcomes were observed in patients receiving the combination[50] (TABLE 1).

Finally, the combination of pembrolizumab plus the multi-target TKI lenvatinib received accelerated approval for patients with previously treated advanced-stage and unresectable endometrial carcinoma that was neither characterized by mismatch repair deficiency nor by microsatellite instability, who progressed during prior therapy. This approval was based on data from the

Table 1 | **Clinical trials that led to the FDA approval of anti-angiogenic agent and ICI combinations**

Agents tested (experimental arm versus control arm)	Cancer type	Approval date	Status and results (experimental arm versus control arm)	Trial registration number (ref)
Atezolizumab + bevacizumab + carboplatin + paclitaxel versus bevacizumab + carboplatin + paclitaxel	Advanced-stage non-squamous NSCLC	6 December 2018	Phase III (active, not recruiting) 1,202 patients randomized 1:1:1[a] Patients with wild-type *EGFR* or *ALK* (86.5%): mPFS 8.3 months vs 6.8 months (HR 0.62, 95% CI 0.52–0.74; *P* <0.001); mOS 19.2 months vs 14.7 months (HR 0.78, 95% CI 0.64–0.96; *P* = 0.02) Patients with wild-type *EGFR* or *ALK* and high effector T cell gene signature (37%): mPFS 11.3 months vs 6.8 months (HR 0.51, 95% CI 0.38–0.68; *P* <0.001); mOS NA	NCT02366143 (REF.[46])
Pembrolizumab + axitinib versus sunitinib	Advanced-stage RCC	19 April 2019	Phase III (active, not recruiting) 861 randomized 1:1; 12-month OS 89.9% vs 78.3% (HR 0.53, 95% CI 0.38–0.74; *P* <0.0001); mPFS 15.1 months vs 11.1 months (HR 0.69, 95% CI 0.57–0.84; *P* <0.001); ORR 59.3% vs 35.7% (*P* <0.001)	NCT02853331 (REF.[47])
Avelumab + axitinib versus sunitinib	Advanced-stage RCC	14 May 2019	Phase III (active, not recruiting) 886 patients randomized 1:1 Overall population: mPFS 13.8 months vs 8.4 months (HR 0.69, 95% CI 0.56–0.84; *P* <0.001); ORR 51.4% (95% CI 46.6–56.1%) vs 25.7% (95% CI 21.7–30%) Patients with PD-L1$^+$ tumours[b] (63.2%): mPFS 13.8 months vs 7.2 months (HR 0.61, 95% CI 0.47–0.79; *P* <0.001); ORR 55.2% (95% CI 49–61.2%) vs 25.5% (95% CI 20.6–30.9%)	NCT02684006 (REF.[48])
Pembrolizumab + lenvatinib	Advanced-stage solid tumours	17 September 2019	Phase I/II (active, not recruiting) 108 patients; ORR at week 24: 38.0% (95% CI 28.8–47.8%)	NCT02501096 (REF.[49])
Atezolizumab + bevacizumab versus sorafenib	Untreated locally advanced or metastatic HCC	29 May 2020	Phase III (active, not recruiting) 501 patients randomized 2:1; 12-month OS 67.2% vs 54.6% (HR 0.58; 95% CI 0.42–0.79; *P* <0.001); mPFS 6.8 months vs 4.3 months (HR 0.59, 95% CI, 0.47–0.76; *P* <0.001)	NCT03434379 (REF.[50])

HCC, hepatocellular carcinoma; HR, hazard ratio; mOS, overall survival; mPFS, median progression-free survival; NA, not available; NSCLC, non-small-cell lung cancer; ORR, objective response rate; OS, overall survival; RCC, renal cell carcinoma. [a]Patients in the third arm received atezolizumab plus carboplatin and paclitaxel (with results not presented). [b]PD-L1 positivity was defined as ≥1% of immune cells staining positive within the tumour area of the tested tissue sample.

single-arm, multicentre phase I/II trial KEYNOTE-146/Study 111 (REFS[49,183]) (TABLE 1).

In all these trials, acceptable safety profiles were reported that were consistent with those of the individual drugs[46–50]. Notably, a clinical trial is under way to study the effect of VEGF inhibitors plus ICIs on hypertension and cardiovascular disease in patients with RCC or melanoma through assessment of changes in blood pressure and blood vessel function (NCT03709771).

Subsequent studies have investigated what factors might determine benefit from combinations of anti-angiogenic agents and ICIs. In patients with RCC, PD-L1 assessment on tumour cells did not enable the prediction of PFS with avelumab plus axitinib nor sunitinib[184]. By contrast, another study described that the atezolizumab plus bevacizumab combination improved PFS in patients with RCC with high effector T cell gene signatures and high PD-L1 expression on immune cells[185]. This contradiction, together with further opposing results from other studies[46–48], indicates that the positive predictive value of PD-L1 expression in RCC might be limited. Furthermore, both studies revealed that tumour mutational burden did not predict PFS in either treatment group[184,185]. Notably, in clear-cell RCCs, an abundant CD8$^+$ T cell infiltration can correlate with short PFS and overall survival durations[186] or lack a correlation with outcomes[187]. Poor outcomes were seen in patients with CD8$^+$ T cell infiltrates characterized by poor cytotoxicity and high levels of immune checkpoints, in which abundant T_{reg} cells and dysfunctional DCs were proposed to play an important role[186]. Several other studies are currently assessing the association between the immune-related or angiogenesis-related signatures of tumours and the immunological determinants of clinical benefit to hopefully identify predictive biomarkers for combination therapy.

Among many ongoing clinical trials testing combinations, a phase II study in patients with advanced-stage cervical cancer is worth mentioning (NCT03816553)[188]. Patients with this malignancy and disease progression after first-line chemotherapy have limited remaining treatment options. Monotherapy with the VEGFR2 TKI apatinib is associated with an objective response rate of 14.6–15.4% in these patients. In this study, however, the combination of apatinib with the anti-PD-1 antibody camrelizumab resulted in an objective response rate of 55.6% (95% CI 40.0–70.4%), with 2 complete and 23 partial responses[188]. Therefore, more approvals of anti-angiogenic and immunotherapy combinations are expected in the near future.

Future directions

The fact that anti-angiogenic agents can improve immune infiltration into tumours is widely acknowledged. Immune-inflamed tumours[9] respond better to ICIs; thus, an attractive approach to induce the sensitivity to immunotherapy is to use anti-angiogenic agents to turn immune-excluded tumours (with only a stromal leukocyte infiltrate[9]) into immune-inflamed tumours. Whether or not 'cold' tumours, characterized by very low numbers of leukocytes in the tumour and stroma[9], can be transformed into 'hot' tumours sensitive to immunotherapy

remains to be determined. In addition, adequate priming by antigen-presenting cells and antigen recognition in the context of MHC class I expression on tumour cells are key steps towards effective antitumour immune responses[32]. Hence, the combination of anti-angiogenic agents with tumour antigen vaccines, DC vaccines or chemotherapy might enhance immune responses and improve the efficacy of immunotherapies. Of note, the sequence and timing of treatment administration will be of importance.

Fully understanding the crosstalk between immunity and the vasculature is a remote situation although, clearly, crucial considerations regarding scheduling, dosing and sequencing should be made. In the previously described clinical trials, ICIs were given once every 2–3 weeks intravenously, with concurrent administration of an anti-angiogenic agent administered orally every day (axitinib or sunitinib) or intravenously together with the ICI (bevacizumab). Importantly, anti-angiogenic agents and ICIs have antitumour activity during the different stages of antitumour immune responses; thus, their spatial and temporal mode of action might differ. In other words, while anti-angiogenic agents can enable the infiltration of immune cells, ICIs exert their effects either in the tumour or at a systemic level on leukocytes (in lymph nodes, spleen or blood), depending on the expression of immune checkpoints. Similarly, part of the clinical success of combining antibodies targeting PD-1 or PD-L1 and CTLA4 (REFS[2,5]) is attributed to their different spatial and temporal mode of action, which facilitates the continuity of the different steps of the antitumour immune response[92]. Hence, we hypothesize that giving patients anti-angiogenic agents before ICIs would be beneficial to enable immune cell infiltration in the tumour before their effector functions are enhanced by ICIs. This concept is supported by a study in which Jaini et al.[189] showed that concurrent treatment with sunitinib and a breast cancer antigen-targeted vaccine inhibited immunogen priming owing to a decrease in the number of $CD11b^{+}CD11c^{+}$ antigen-presenting cells. This effect was avoided when sunitinib treatment was scheduled outside of the priming phase of the immune response[189]. Alternatively, in the case of cancer vaccines, ICIs might specifically have a stimulatory effect in the priming phase of the immune response and thus administering ICIs during the first vaccination as well as later on in the effector phase of the immune response might be necessary.

Furthermore, the dose of the anti-angiogenic agent and the period of time after which the vasculature is normalized and EAM expression is reinduced should be evaluated to optimize combinations of anti-angiogenic agents and ICIs. As previously described, vascular normalization is a promising therapeutic strategy, often involving low doses of anti-angiogenic agents, to enhance drug delivery, relieve hypoxia and improve antitumour immunity; however, whether the doses used normalize EAM expression remains to be determined. Similarly, the time frame in which normalization of EAM expression on the tumour vasculature is seen and maintained is yet unclear and determining when and for how long the anti-angiogenic agents should be administered is important. Therefore, we want to emphasize the importance of assessment of EAM expression upon vascular normalization anti-angiogenic treatment both in preclinical and clinical studies (BOX 2).

Immunostimulation with anti-angiogenic treatment can also potentially be used to sensitize tumours with a low expression of PD-L1 to anti-PD-1/PD-L1 ICIs[125]. The increased T cell infiltration observed upon anti-angiogenic treatment has been associated with enhanced tumour PD-L1 expression[125,127,190]. This increase could be explained by the fact that PD-L1 expression can be driven by IFNγ secretion by infiltrating effector T cells, which is known to be an adaptive immune resistance mechanism[191]. Of note, this observation also raises the possibility that adaptive immunosuppression by tumours, such as through PD-L1 upregulation, might have hampered the clinical efficacy of anti-angiogenic treatment and might have blunted its immune-promoting effect[44,192].

Results of studies from the past few years suggest that immunotherapies can promote the efficacy of anti-angiogenic agents by promoting vascular changes[27,44]. For example, a preclinical mouse study of breast cancer demonstrated that the depletion or inactivation of $CD4^{+}$ T cells decreased vessel normalization, whereas their activation by ICIs improved the normalization of the tumour vasculature[193]. Another study showed that ICIs normalized the tumour vasculature in mice through increasing pericyte coverage, even when used in combination with anti-angiogenic treatment[126]. Extensive research remains scarce, although we can hypothesize that immunotherapies could be used for the optimal efficacy of vascular normalization therapies, resulting in a strong and positive antitumour feedback loop. The confirmation of this hypothesis would add to the complexity of optimizing the dosing, sequencing and duration of anti-angiogenic and immunotherapy combinations.

Interestingly, the anti-angiogenic component of such combinations does not need to consist of a targeted agents, such as small-molecule TKIs or specific antibodies, and can be replaced by an immunotherapy directed against the tumour vasculature[194]. For example, CAR T cell approaches targeting VEGFR2 have been reported and are currently being evaluated in clinical trials involving patients with advanced-stage solid tumours[195,196]. Targeting CAR T cells to the tumour vasculature has many advantages such as the direct availability of target cells in the blood and the lack of a need for extravasation of the CAR T cells[197]. Moreover, vaccination strategies using RNA, DNA[198,199] and 3D-structured peptides[200] have been used to target VEGF signalling and are currently in development. Conjugate vaccines, aimed at inducing an antibody response, are another promising strategy in cancer vaccination. Together with antibody-based drugs, this approach is receiving attention from the pharmaceutical industry because it has several advantages, including the involvement of a polyclonal antibody response, long-term efficacy and superior cost effectiveness. A conjugate vaccine consists of a self-antigen fused to a foreign protein, which promotes recognition of the self-antigen as foreign, thereby inducing a strong anti-self-antigen response. Depending on the availability of a highly specific and selective target on the tumour endothelium, extremely high antibody titres could be induced against the tumour vasculature using such vaccines. These induced antibodies could mediate immune effector functions, such as antibody-dependent and complement-dependent cell cytotoxicity, reducing the need for a functional role of the target in the process of angiogenesis. Highly selective markers of the tumour vasculature, such as the extra domain A or B containing isoforms of fibronectin, were targeted with vaccines after conjugation to bacterial thioredoxin; this approach efficiently inhibited tumour growth[201]. A similar effect was observed after vaccination against the tumour endothelial-specific proteins Robo4 (REF.[202]) and CD99 (REF.[203]). Based on these results, we favour the view that vaccine-based immunotherapies used as angiostatic agents or vascular-targeting adjuvants to cancer

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(immuno)therapies have great potential in clinical oncology. Many tumour-specific vascular markers are expressed across cancer types and, thus, a limited selection of targets might be sufficient to treat the majority of cancers, thereby alleviating the need for personalized therapy. The combination with ICIs is expected to be highly successful.

Conclusions

Understanding how tumours escape immunity as well as the mechanisms of immunotherapy resistance can enable the design of efficient treatment combinations. The role of the tumour vasculature in immune evasion now provides a rationale for the combination of anti-angiogenic agents with ICIs, an approach that is being extensively tested in >80 ongoing clinical trials. Endothelial cell anergy is a process that has been mostly overlooked in the literature to date; herein, we propose that this process is the vascular counterpart of immune checkpoints. Both immune checkpoints and endothelial cell anergy play an important role in maintaining immune homeostasis by controlling the continuation and amplitude of immune responses. In addition, both mechanisms are exploited by tumours, thereby contributing to immune escape and resistance. Therefore, these processes are therapeutic targets to reverse and reinvigorate these rate-limiting steps in the cancer-immunity cycle.

We have reviewed how anti-angiogenics can be used to normalize the tumour vasculature and overcome the endothelial immune cell barrier and have described the successful results of clinical trials that led to several FDA approvals for various tumour types. This novel and fast-growing field has already accomplished many successes; thus, answering the outstanding questions regarding further development and application of these combinations is of crucial importance to understand and use their potential to the fullest (BOX 3).

Finally, immune-cell infiltration is necessary but not sufficient for the efficacy of ICIs and other immunotherapies and studies have shown that a therapeutic response to these agents is not always guaranteed in patients with inflamed tumours[9]. As such, we must realize that turning non-inflamed tumours towards a more inflamed phenotype is not the only obstacle to be tackled for effective immunotherapy. We have described other immune evasion mechanisms that can be present or might develop. While these considerations and challenges will take time to be studied and elucidated, promising research on anti-angiogenic adjuvant approaches to immunotherapy offers hope for the development of treatment strategies to improve the outcomes of patients with cancer.

Box 3 | **Outstanding questions and future directions**

How to ideally combine angiostatic therapy with immunotherapy?
Selection of the angiostatic and immunotherapy drugs of choice as well as the dose, schedule and sequence need to be determined through (pre)clinical testing. Making use of the spatial and temporal differences in the activity of each drug is expected to substantially improve clinical outcomes.

Can artificial intelligence guide decisions on drug combinations?
The number of immune-checkpoint inhibitors (ICIs) is increasing and that of angiostatic agents is substantial already. Anti-angiogenic drug selection for optimal combination can be achieved with artificial intelligence[206,207] and might be extended to find rational combination therapies with immunotherapies[208].

What is the best treatment approach: off the shelf or precision medicine?
Patient-tailored combinations might be highly effective in prolonging survival[208,209]. However, such strategies can be labour intensive and expensive and thus generic combination strategies might be preferable.

Can angiostatic agents enhance the effectiveness of immunotherapies beyond checkpoint inhibition?
Several clinical trials are evaluating combinations of anti-angiogenic agents with adoptive cell transfer or vaccination strategies (Supplementary Table 1). These studies are promising, although a better understanding of the immunological stage of the patient and of anti-angiogenic immunomodulation are probably needed to improve these combinations[210].

Are there valuable biomarkers for combination therapy?
At present, reliable biomarkers for responsiveness to either anti-angiogenic agents or ICIs are not available. Decisions regarding combination therapy are exceedingly complex and currently based on successful results from studies testing monotherapy and sometimes on the 'gut feeling' of decision-makers. Hence, the discovery of biomarkers that predict outcomes with combination therapy would be extremely important[211].

Is a third therapeutic modality needed?
Chemotherapy and radiotherapy can improve the efficacy of ICIs[212,213] and thus might improve effective combinations of angiostatic agents and immunotherapy. The use of multimodal therapies remains speculative (and the timing, dosing and toxicity would require careful consideration) but could lead to further improvement of cancer therapy.

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