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The mechanistic immunosuppressive role of the tumour vasculature and potential nanoparticle-mediated therapeutic strategies

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The tumour vasculature is well-established to display irregular structure and hierarchy that is conducive to promoting tumour growth and metastasis while maintaining immunosuppression. As tumours grow, their metabolic rate increases while their distance from blood vessels furthers, generating a hypoxic and acidic tumour microenvironment. Consequently, cancer cells upregulate the expression of pro-angiogenic factors which propagate aberrant blood vessel formation. This generates atypical vascular features that reduce chemotherapy, radiotherapy, and immunotherapy efficacy. Therefore, the development of therapies aiming to restore the vasculature to a functional state remains a necessary research target. Many anti-angiogenic therapies aim to target this such as bevacizumab or sunitinib but have shown variable efficacy in solid tumours due to intrinsic or acquired resistance. Therefore, novel therapeutic strategies such as combination therapies and nanotechnology-mediated therapies may provide alternatives to overcoming the barriers generated by the tumour vasculature. This review summarises the mechanisms that induce abnormal tumour angiogenesis and how the vasculature’s features elicit immunosuppression. Furthermore, the review explores examples of treatment regimens that target the tumour vasculature.
Introduction

Angiogenesis is the process of new blood vessel formation from existing vasculature and is abundant in growing tumours (1). As tumours increase in size, the accompanying increased demand for nutrients and oxygen upregulates the excess production of pro-angiogenic factors such as vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF) from tumour cells, termed the ‘angiogenic switch’ (2). However, the new vasculature often displays increased permeability, tortuosity, and immaturity, thus facilitating metastasis but not supplying the metabolic demands of the tumour (3, 4). These abnormal vessels are also poorly perfused due to a lack of mural cell recruitment and basement membrane coverage, leading to increased interstitial pressure (5, 6). These features ultimately form areas of hypoxia that generate an immunosuppressive environment by inhibiting effector T cell infiltration while upregulating the presence of immunosuppressive cells such as myeloid-derived suppressor cells (MDSCs) and regulatory T cells (Tregs) (1, 6, 7).

Importantly, the tumour vasculature can attenuate chemotherapeutic drug delivery or prevent the crucial formation of reactive oxygen species during radiotherapy (8). Therefore, treatments aiming to return the vasculature to a physiologically functional state termed ‘vascular normalisation’ are ideal areas of cancer research (6). Nonetheless, current anti-angiogenic drugs such as bevacizumab and sunitinib have shown variable successes in solid tumours, due to focusing largely on the effects of VEGF (9). However, the emerging use of nanoparticle technology in cancer therapy has shown promise in improving the efficacy of anti-angiogenic and chemotherapeutic drug treatments by enhancing their pharmacokinetic properties (9). Moreover, the ability to modify these nanoparticles allows for targeted delivery which can be employed to alleviate anti-cancer immunosuppression such as via endothelial cell regulation (10).

This review will therefore summarise the mechanisms leading to the immunosuppressive tumour microenvironment (TME) resulting from the generation of the tumour vasculature’s features. In addition, this review will discuss examples of past and novel treatments that target the aberrant tumour vasculature, promote normalisation, and improve traditional cancer therapies.

Angiogenesis and the structure of the tumour vasculature

Mechanisms maintaining blood supply in tumours

Tumours primarily induce new vessel formation via sprouting angiogenesis, forming most of the abnormal vasculature (Figure 1A) (11). Physiologically, angiogenesis is well-coordinated but due to the angiogenic switch, the
process is highly dysregulated. Secretion of pro-angiogenic factors such as VEGF-A by tumours initiates the process, weakening endothelial cell junctions and causing vessel-pericyte dissociation (11). Thereafter, proteases such as matrix metalloproteinases (MMPs) and cathepsins degrade the endothelial basement membrane (11, 12). VEGF-A then triggers angiogenic sprouting, creating tip cells that direct new vessel formation and induce stalk cell proliferation to form the vascular lumen (13–15). Lastly, pericytes are recruited by factors such as angiopoietin-1 (Ang-1) and platelet-derived growth factor-β (PDGF-β) to stabilise the new vessel (16).

Despite this, tumours have developed the ability to induce non-angiogenic neovascularisations by forming tubular structures composed of cancer cells termed ‘vascular mimicry’ (VM) (Figure 1B) (17). Tumours displaying VM have been increasingly linked to enhanced aggressiveness and invasiveness, demonstrated in a variety of cancers such as prostate, breast and gastrointestinal tumours (17). For example, in patients suffering from gastrointestinal tumours, VM was significantly more prevalent in tumours classified as high-risk than very-low/low-risk, where incidence was 39.5% and 5.9%, respectively (17). Currently, the criteria distinguishing VM from other vascularisation mechanisms necessitates that the vessels be composed of cells that are positive for periodic acid-Schiff (PAS) staining and negative for immunohistochemical detection of endothelial marker CD31 (18). Additionally, these structures must have erythrocytes present in the vessel lumen to be characterised as VM vascularisations (18). The mechanisms initiating VM remain to be fully elucidated, but evidence has suggested hypoxia/hypoxia-inducible factors (HIF) to be major inducers that could even result from anti-angiogenic therapy (19, 20).

‘Vessel co-option’ is an alternative mechanism for maintaining blood supply by utilising existing vasculature without inducing de novo vessel formation (21). This is often histologically identified by the presence of cancer cells surrounding structurally and architecturally functional vessels, contrary to the abnormal vasculature observed in angiogenesis (Figure 1C) (21). This has been observed in metastatic and primary tumours of the lung, brain and liver and may contribute to intrinsic and acquired anti-VEGF therapy resistance (22–24). This was observed in hepatocellular carcinoma xenografts, which displayed a statistically significant 51.7% increase in the proportion of co-opted vessels between untreated control and sorafenib resistant tumours (24). Similarly, lung metastasis models treated with sunitinib demonstrated altered growth patterns that promoted vessel co-option, further demonstrating an alternative mechanism of anti-angiogenic therapy resistance (25).

Features of the tumour vasculature

Tumour vessels are structurally and functionally heterogeneous with various cellular and molecular abnormalities (Figure 2). Mural cell coverage has been linked to promoting vessel stabilisation and endothelial cell survival while inhibiting endothelial cell proliferation (26). Recruitment of these cells is mostly mediated by PDGF-β secreted by endothelial cells but in situ hybridisation from mouse tumour models demonstrated that endothelial cells did not lack PDGF-β expression (26). This suggests that mural cell recruitment defects may be due to a lack of other contributing factors such as a decreased pool of mural cell progenitors (26). In addition, vascular hyperpermeability has been associated with upregulated angiogenesis and may generate aberrant vasculature with an increased dependence on VEGF-A for survival (5, 26).

As solid tumours develop in size past 1-2 millimetres, the radial diffusion distance from the associated blood vessels increases (1, 28). As the tumour is unable to receive adequate oxygen and nutrient supply, hypoxic areas form which promote angiogenesis (28). These hypoxic conditions stimulate HIF-1 and HIF-2 formation, which induces the expression of proangiogenic factors required for new vessel formation such as VEGF, PDGF-β, Ang-1 and Ang-2 (29, 30). However, the established imbalance of excess pro-angiogenic factors to anti-angiogenic factors continues generating aberrant vasculature that does not deliver sufficient oxygen supply. This perpetuates the presence of hypoxic and acidic areas, prompting these tumours to have poor prognoses (31).

VEGF, Ang-1 and Ang-2 are the main growth factors responsible for tumour angiogenesis indicated by their essentially restricted receptor expression on endothelial cells (32). When acting solitarily, Ang-2 can antagonise the stabilising function of Ang-1 by blocking the Tie2 receptor (33). This promotes vessel regression and remodelling; however, co-expression of VEGF and Ang-2 has been shown to have a pro-angiogenic effect on endothelial cells, suggesting a role in tumour vasculature formation (33). For example,
hepatocellular carcinoma specimens expressing both Ang-2 and VEGF demonstrated increased microvessel density, vessel destabilisation and tumour size (32, 33). Furthermore, hepatocellular carcinoma specimens positive for Ang-2 mRNA expression displayed an increased tumour microvessel area compared to their Ang-2 mRNA negative counterparts (32). In addition, Ang-2 overexpression was linked to endothelial cell aggregation, vessel leakage and decreased vessel lumen size which may lead to hypoxic regions, further promoting angiogenesis (34).

The tumour microenvironment and hypoxia induced extracellular matrix remodelling

The ECM is a major component of the TME and is primarily composed of collagen and fibronectin (35). However, alterations to the ECM composition can cause the release of sequestered pro-angiogenic factors that facilitate angiogenesis (36). Under hypoxic conditions, endothelial cells increase the deposition of ECM components such as fibronectin, collagen IV and laminin which have been demonstrated to promote endothelial cell proliferation, elongation, migration, and survival necessary for angiogenesis (36).

In response to HIF-1, fibroblasts display upregulation of collagen prolyl 4-hydroxylases such as P4HA1 and P4HA2 as well as collagen lysyl hydroxylases such as procollagen-lysine, 2-oxoglutarate 5-dioxygenase 2 (PLOD2) (35, 37). During 24-hour exposure to hypoxic 1% O2 conditions, mRNA expression of P4HA1, P4HA2, and PLOD2 in fibroblasts had increased over 5 times compared to fibroblasts exposed to 20% O2 conditions (35). These enzymes aid in collagen fibre formation that stiffens the ECM. P4HA1 and P4HA2 accomplish this by increasing collagen cross-linking while PLOD2 increases collagen deposition (35). This promotes cancer cell invasiveness and disrupts tumour endothelial cell alignment (35, 38). Experiments revealed these tumour endothelial cells exhibited increased constitutive Rho and ROCK activity, therefore increasing cytoskeletal tension which impedes endothelial cell mechanosensitivity (38). Tumour capillary endothelial cells displayed 2.5 times greater baseline Rho activity and 4 times greater baseline ROCK activity compared to normal endothelial cells (38). Increased ECM stiffness was suggested to trigger this, which
and aberrant vascular structures (38).

In addition, in response to hypoxia, ECM composition can, directly and indirectly, affect protease expression. When grown on a collagen I gel matrix compared to a Matrigel matrix, rat endothelial cells displayed upregulated MT1-MMP and MMP2 expression, possibly resulting from ligand binding or the aforementioned aberrant endothelial mechanosensory mechanism (36, 39). MMPs are vital during angiogenesis as they degrade the main components of endothelial basement membranes (40). These MMPs expressed by tumour cells, fibroblasts and endothelial cells can digest various ECM components, further promoting tumour invasion and metastasis (40, 41). Crucially, MMP2 and MMP9 expression is often upregulated in tumours and their degradation of components like proteoglycans can release bound bioactive components termed matrikines or matricryptins (42, 43). These matrikines induce MMP expression via positive feedback loops, such as canstatin release increases MMP2 and MMP9 expression in fibroblasts (42, 43). These MMPs promote vascular and tumoral invasion as well as release tumour necrosis factor-alpha (TNF-α) and soluble Fas ligand which inhibits tumour cell apoptosis (41). Data showed that MMP2 and MMP9 expression was positively correlated to tumour invasion depth, venous invasion and increased tumour size (over 4cm) (41). Crucially, MT1-MMP and MMP9 activity can release extracellular heparin-bound VEGF which can induce MMP2 and MMP9 expression from tumour cells, likely via VEGFR2 activation (44–46).

Cathepsins including cathepsin L, B and D are other ECM proteases exhibiting pro-angiogenic action and upregulation in many tumours (47–55). Upregulation of cathepsin L via fibroblast growth factor (FGF) and VEGF causes the enzyme to be extracellularly secreted as opposed to its normal function within lysosomes (47). In vitro studies showed cathepsin L’s paracrine interaction with endothelial cells increases endothelial invasion, migration and sprouting while cathepsin L inhibition suppresses angiogenesis in xenograft breast cancer models (47). Moreover, cathepsin L-induced galectin-1 has been shown to induce proliferation and migration microvascular endothelial cells, demonstrating a proangiogenic role of this lysosomal enzyme (55). Additionally, cathepsin B can degrade ECM laminin, collagen IV and fibronectin as well as induce pro-urokinase-type plasminogen activator (pro-uPA) activation in xenograft glioblastoma models (48, 56). This upregulates VEGF expression in tumours, thus inducing angiogenesis (48). Lastly, cathepsin D upregulation via HIF-1α is suggested to promote angiogenesis by activating cathepsin B and proteolytically releasing bFGF (57, 58). However, both Cathepsin D and L have been shown to enhance proliferation and migration of microvascular endothelial cells in a non-proteolytic manner, leaving its angiogenic mechanisms unresolved (50, 51, 57).

Tumour-associated macrophages (TAMs) are another component of the TME and have been shown to induce ECM remodelling by expressing a range of enzymes such as collagen prolyl 4-hydroxylases, collagen lysyl hydroxylases, MMPs (including MT1-MMP), a disintegrin and metalloproteinases (ADAMs) and cathepsins that aid angiogenesis (59). Besides expressing ECM remodelling enzymes, TAMs can also modify the enzymatic activity of other ECM remodelling cells such as by expressing procollagen c-endopeptidase enhancer (PCOLCE) to upregulate procollagen C–proteinase’s collagen maturation activity (59). This demonstrates TAMs unique ability to affect ECM remodelling in a manner typically associated with fibroblasts (59).

One of the most important components of the TME are fibroblasts which have a primary role in synthesising collagen and are essential in maintaining the ECM structure of associated tissues (60). However, cancer-associated fibroblasts (CAFs) which are located within or near tumours may have altered metabolism and function causing them to secrete factors, chemokines, and enzymes such as VEGFA, CXCL12 and MMPs that can promote angiogenesis (60). Preclinical evidence has also suggested that certain CAFs may have a role in reducing immunotherapy efficacy, making it another possible target of cancer therapy (60). Similar to CAFs, adipocytes can also secrete pro-angiogenic factors, chemokines and cytokines known as adipokines which include TNF-α, VEGF-A and FGF2 (37, 61). Additionally, adipocytes can support angiogenesis by releasing fatty acids during lipolysis which is upregulated by tumour-released factors. The resulting increased fatty acid availability promotes β-oxidation in endothelial cells, encouraging angiogenesis (37).

The Immunosuppressive role of the tumour vasculature

Tumour vasculature plays key roles in immunosuppression through various mechanisms, many of which have been reviewed elsewhere (62–64). In the following sections we describe mechanistic roles of endothelial cells and tumour vascular-associated ECM components in suppressing an anti-tumour immune response.

Endothelial adhesion molecules regulate immune cell infiltration

For successful T cell extravasation, endothelial adhesion molecules (EAMs) such as intercellular adhesion molecule-1 (ICAM-1), ICAM-2, vascular endothelial cell adhesion molecule-1 (VCAM-1) and E-selectin are expressed on endothelial cells in response to inflammatory cytokines such as...
TNF-α, IFN-γ and IL-1 (65). However, exposure of vessel endothelium to pro-angiogenic factors such as VEGF and bFGF can repress the inflammatory cytokine-induced expression of EAMs (65). For example, the addition of VEGF and bFGF to endothelial cells in vitro inhibited IL-10 induced ICAM-1 upregulation by 15% and 33%, respectively (65).

Murine tumour models also demonstrated that endothelial cells exposed to bFGF and long-term VEGF display reduced ICAM-1 and VCAM-1 expression as well as decreased expression of TNF-α induced CXCL10 and CXCL11 T-cell chemoattractants (1, 66). Evidence has shown that VEGF interferes with the NF-kB pathway by degrading the IkB component, thus reducing the expression of TNF-α and its downstream molecules (66). This was confirmed via RT-PCR analysis which revealed that endothelial cells treated with VEGF elicited a 60-95% reduction in TNF-α expression (66). Moreover, renal cell carcinomas (RCC) can also interfere with the NF-kB pathway mediated expression of TNF-α by increasing P38-MAPK activity (66). This phenomenon termed ‘endothelial energy’ causes an immunosuppressive reduction in immune cell infiltration, aiding tumour cell survival and inhibiting immunotherapeutic drug delivery (66, 67).

**Endothelial cells secreted extracellular vesicles in immunosuppression**

Tumour endothelium-secreted extracellular vesicles (EVs) could induce reprogramming of immune cells. For instance, Lopatina et al., 2020 observed an increase in secretion of TGF-beta and IL10 by PBMC and to increase Treg expansion (68). These EVs have been shown to carry specific proteins (e.g. TGF-beta 1) and RNA (long non-coding RNA MALAT1) that are responsible for Treg differentiation and immunosuppression (68–70). Additionally, the authors reported that these EVs induced differentiation of monocytes to immunosuppressive macrophage type M2 (68). Interestingly, endothelial cell-secreted EVs containing miRNA-222 have been shown to induce downregulation of ICAM-1 on endothelial cell surface, which in turn reduces transmigration of immune cells (71). miR-10a from EVs derived from endothelial was shown to inhibit inflammatory signalling in monocytes through the targeting of several components of the NF-kB pathway, including IRAK4 (72). Thus, anti-tumour immunotherapies against EVs in combination with anti-angiogenic therapies may be an important therapeutic mechanism in the future.

**Abnormal ECM remodelling and immunosuppressive cells**

Hypoxia-induced ECM remodelling in the TME alters the dynamic of physiological cellular and molecular crosstalk and processes such as migration and proliferation. In TME, ECM remodelling results in abnormal expression of various proteins such as collagen, fibronectin, versican, etc., that could result in immunosuppression. For instance, it has been shown that an excessive expression of versican, a chondroitin sulphate proteoglycan in the stroma of cervical cancer, was significantly associated with a low number of tumour infiltrating T cells, particularly CD8+ T cell, resulting in a reduced anti-tumour immune response (73). In contrast, TAMs, which share properties of M2 (immunosuppressive), are recruited in response to hypoxia via VEGFRI activation, observed by a positive correlation between macrophage index and VEGF expression intensity in breast carcinoma samples (44). Furthermore, high M2 macrophage ratios have been associated with a poorer prognosis as shown in non-small lung cancer patients which displayed a 20.6% decrease in overall survival rate compared to their low M2 ratio counterparts (74), suggesting an anti-tumour immune phenotype in hypoxic TME. This abnormal ECM remodelling also results in an immature phenotype consisting of an unstable vessel wall with a discontinuous basement membrane and an irregular endothelial lining. This abnormal vascular architecture is excessively chaotic and leaky, creating, as described above, a hypoxic TME. This hypoxic condition dysregulates signalling that alters the expression of endothelial cell surface adhesion molecules as well as mediates the presence of several immunosuppressive immune cell types such as immature dendritic cells, TAMs, tumour-associated neutrophils and MDSCs, leading to a further reduction in anti-tumorigenic immune cell population within the TME, which has been extensively reviewed elsewhere (75).

However, the reduced immune cell population in the TME does not exclusively result from inhibited leukocyte extravasation but also increased leukocyte apoptosis. Human and mouse cancer models show that tumour endothelial cells display an increased expression of apoptotic Fas ligand in response to VEGF-A upregulation (76). Despite that endothelial Fas ligand can act selectively on CD8+ leukocytes, Treg cells display resistance due to their increased expression of c-FLIP which inhibits Fas ligand-induced apoptosis. This consequently prevents infiltration of cytotoxic leukocytes while maintaining the immunosuppressive Treg cell population within the TME (76). This increased immunosuppressive cell population generated by the tumour vasculature includes TAMs, Tregs and MDSCs which aid the immunosuppressive profile of the TME (77–81). For example, pro-tumorigenic polymorphonuclear neutrophil myeloid-derived suppressor cells (PMN-MDSCs) can also suppress CD8+ leukocytes and induce pro-angiogenic MMP9 release (82). Furthermore, other pro-tumorigenic neutrophils such as N2 tumour-associated neutrophils been linked to poor prognosis and cancer.
demonstrated reduced SOX7 and SOX18 transcription factor in human umbilical vein endothelial cells (HUVECs) (83, 84). The tumour suppressor VEGF and bFGF as well as anti-angiogenic factors such as thrombospondin-1 (TSP-1) (83, 84). The tumour suppressor gene p53 regulates these factors and mediates cellular functions such as apoptosis, DNA repair, cell cycle development and angiogenesis inhibition (83). However, p53 is commonly mutated in cancers, thereby promoting pathological angiogenesis (83).

P53 knockout human colon adenocarcinoma cells displayed increased HIF-1α expression in comparison to p53 homozygous cells under hypoxic conditions (1% O2) (85). Regardless, no significant difference in p53 mRNA expression was detected, posing further investigations into the mechanistic pathway. These results indicated that p53 loss-of-function mutations prevent the functional ubiquitination and degradation of HIF-1α (85). In addition, p53 demonstrated a possible role in controlling HIF-1β expression via regulating microRNA transcription (86). RNA analysis has shown the presence of a p53 binding site 1,811bp upstream of the pantothenate kinase 1 intron which contains the encoding section for microRNA-107. Upon p53 binding, microRNA-107 levels increased and subsequently inhibited HIF-1β expression (86). Therefore, p53 mutations may lead to both increased HIF-1α and HIF-1β expression, thereby promoting angiogenesis by upregulating factors such as VEGF (86). Furthermore, p53 mutations have also been demonstrated to upregulate VEGF expression in human colon, bladder, and breast cancer surgical specimens as p53 acts directly on the VEGF promoter region (44, 84).

Additionally, the anti-angiogenic ECM glycoprotein TSP-1 is implicated in reducing cancer cell proliferation, survival as well as motility and is upregulated by p53 binding to the encoding THBS1 promoter region (84, 87, 88). This suggests that p53 mutations downregulating TSP-1 may further contribute to the formation of abnormal tumour vasculature (84, 88).

Contrary to tumour suppressor genes, oncogenes can enhance angiogenesis when their activity is upregulated via gain-of-function mutations. For example, PTPN11 encodes the tyrosine phosphate SHP2, which is often overactivated in tumour endothelial cells (89). Knockout or inhibition of Shp2 in human umbilical vein endothelial cells (HUVECs) demonstrated reduced SOX7 and SOX18 transcription factor expression, thus inhibiting cell motility, proliferation, and tubular vessel formation (89). This was supported by data comparing relative SOX7 levels in Shp2-knockout (Shp2KO) and Shp2 homozygous HUVECs which showed a significant reduction in Shp2KO HUVECs (89). Both transcription factors have similar roles and re-expressing SOX7 in Shp2KO HUVECs restored cellular functions while also increasing vessel branching and density (89). Furthermore, inducing SOX7 expression in Shp2KO mice decreased basement membrane and pericyte coverage, indicated by results showing that Shp2KO mice displayed a relative branching index reduction of 0.54 and a 0.58 (arbitrary units) increase in relative pericyte coverage when compared to Shp2 homozygous mice (89). These were quantitatively measured using computer image analysis. Additionally, Shp2KO endothelial cells displayed increased PDGF-BB expression, which aids vessel maturation and thus may be implicated in the observed disparity in pericyte and basement membrane coverage of Shp2KO mice (89). Finally, tumour mouse models lacking Sox7 expression within endothelial cells demonstrated reduced tumour growth, Treg penetration, endothelial VEGFR2 expression and aberrant vessel morphology (90). Evidently, SH2 and SOX7 pose as ideal targets to induce vascular normalisation (89, 90).

Regulating genes of the tumour vasculature

Dysregulations of the TME contributing to the angiogenic switch have been linked to distinct mechanisms including anomalous expression levels of HIF-1, pro-angiogenic factors VEGF and bFGF as well as anti-angiogenic factors such as thrombospondin-1 (TSP-1) (83, 84). The tumour suppressor gene p53 regulates these factors and mediates cellular functions such as apoptosis, DNA repair, cell cycle development and angiogenesis inhibition (83). However, p53 is commonly mutated in cancers, thereby promoting pathological angiogenesis (83).

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Therapeutics targeting the tumour vasculature

Vascular normalisation

Many treatments seek to improve the tumour vasculature by simultaneously removing aberrant vessels and stabilising those that are functional. This aims to restore the vasculature to a normal physiological state, via a process termed ‘vascular normalisation’ (28). Currently available anti-angiogenic drugs can be broadly divided into three groups. These are receptor tyrosine kinase (RTKs) inhibitors, anti-VEGF or anti-RTK antibodies and downstream angiogenic signalling inhibitors (91). Although monotherapeutic use of these drugs has displayed variable effectiveness and reached to FDA approval only in some specific tumour types (92), addition of these anti-angiogenic agents to standard chemotherapy or radiotherapy has shown promise in improving their efficacy (4, 6). The inconsistency in efficacy has been associated with the varying innate drug susceptibility of certain tumours and the development of acquired drug resistance after treatment (92). There are currently many FDA approved angiogenesis inhibitors used in cancer treatment targeting proangiogenic molecules, with the vast majority targeting VEGFR (93). These treatments include bevacizumab and ramucirumab, which target VEGF-A and VEGFR2, respectively and RTK inhibitors sunitinib, axitinib, sorafenib and lenvatinib (93). Furthermore, the combinational use of anti-angiogenic drugs and
immunotherapy [reviewed elsewhere (94, 95)] has been demonstrated to increase the efficacy of the latter, making it a promising target to replace cytotoxic treatments that often accompany quality of life reducing side effects such as alopecia, mucositis, and cardiotoxicity (4, 96). Importantly, these anti-angiogenic drugs continue to be utilised in clinical trials to explore their effects on cancer therapy. Some of these ongoing clinical trials are shown in Table 1.

**Anti-VEGF antibody therapy - bevacizumab**

Bevacizumab is an anti-VEGF IgG1 humanised monoclonal antibody, and the first anti-angiogenic drug approved for colorectal cancer treatment by the Food and Drug Administration (FDA) (4, 97). At the base of the angiogenic switch, VEGF acts as a highly potent pro-angiogenic factor, promoting endothelial cell proliferation and migration (98). However, bevacizumab use has been demonstrated to prevent VEGF from binding to its receptors, in particular VEGFR1 and VEGFR2, which activate multiple angiogenic pathways (99). For example, neuroblastoma xenograft models demonstrated that bevacizumab induced vessel regression, observed by a 75% decrease in vessel length 7 days after administration (100). This consequently improved tumour vascular architecture and structure (100). Additionally, 7 days after bevacizumab treatment, tumours displayed a 70% reduction in microvessel density, 60% reduction in tumour interstitial pressure and 48% reduction in blue dye extravasation representing reduced vessel

**Table 1 Clinical Trials Targeting Cancers and Tumours using Anti-angiogenic Treatment.**

| Drug/Treatment | Target | Phase | Application | (Estimated)Start Date -Predicted End Date | NCT Number/ Source |
|----------------|--------|-------|-------------|------------------------------------------|-------------------|
| SIBP04 + Paclitaxel + Carboplatin OR Avastin + Paclitaxel + Carboplatin | VEGF | Phase 3 | Non-squamous Non-small-cell Lung Cancer | Apr. 17, 2020 – Sep. 30, 2022 | NCT05318443 |
| Bevacizumab + Atezolizumab + Gemcitabine + Cisplatin OR Gemcitabine + Cisplatin | VEGF | Phase 2 | Combined hepatocellular carcinoma and Cholangiocarcinoma | Feb. 11, 2022 – Jan. 31, 2025 | NCT05211323 |
| Bevacizumab + STRO-002 | VEGF | Phase 1 | Ovarian Cancer | Mar. 22, 2022 – Jan. 2024 | NCT05200364 |
| Apatinib + Albumin-Bound Paclitaxel OR Bevacizumab + Albumin-Bound Paclitaxel | Anti-angiogenic (VEGFR2, VEGF) | Phase 2 | Triple-negative Breast Cancer | Jan. 14, 2022 – Jun. 1, 2024 | NCT05192798 |
| (Bevacizumab +) Chemotherapy OR Bevacizumab + Atezolizumab (+ Chemotherapy) OR Cetuximab + Chemotherapy | Anti-angiogenic (VEGF) | Phase 2/3 | Metastatic/Advanced Head and Neck Cancer | Dec. 16, 2021 – Dec. 15, 2027 | NCT05063552 |
| Bevacizumab + Ensartinib Carboplatin + Pemetrexed | VEGF | Phase 1 | ALK-Positive Lung Non-Small Cell Carcinoma | Mar. 18, 2021 – Sep. 23, 2022 | NCT04837716 |
| Bevacizumab + Riluzole + mFOLFOX6 | VEGF | Phase 1 | Metastatic Colorectal Cancer | Apr. 2, 2021 – Dec. 31, 2024 | NCT04761641 |
| Bevacizumab + Irinotecan sucroseolate | VEGF | Phase 2 | Ovarian, Fallopian tube, Primary Peritoneal Cancer | Mar. 16, 2021 – July. 1, 2024 | NCT04753216 |
| Bevacizumab + Atezolizumab | VEGF | Phase 2 | Resectable Liver Cancer | Feb. 10, 2021 – Dec. 31, 2027 | NCT04721132 |
| Avastin + Placebo + XELOX OR HLX04 + HLX10 + XELOX | VEGF | Phase 2/3 | Metastatic Colorectal Cancer | Mar. 10, 2021 – June. 30, 2025 | NCT04547166 |
| Bevacizumab + Brigatinib | VEGF | Phase 1 | ALK-Rearranged Non-Small Cell Lung Cancer | Mar. 9, 2020 – Nov. 1, 2023 | NCT04227028 |
| Bevacizumab + Osimertinib OR Osimertinib | VEGF | Phase 3 | Non-Small Cell Lung Cancer | Oct. 22, 2020 – Sep. 1, 2025 | NCT04185060 |
| Bevacizumab + Dasatinib + mFOLFOX | VEGF | Phase 1 | Gastrointestinal Cancer | Sep. 2, 2020 – Dec. 31, 2022 | NCT04164069 |
| Bevacizumab + Irinotecan + TAS-102 | VEGF | Phase 2 | Metastatic/Unresectable Colorectal Cancer | Dec. 27, 2019 – Apr. 22, 2024 | NCT04109924 |
| Bevacizumab/Nab-Paclitaxel + IPI-549 + Atezolizumab | VEGF | Phase 2 | Triple-Negative Breast Cancer and Renal Cell Cancer | Dec. 17, 2019 – Aug. 1, 2022 | NCT03961698 |

Table listing most recent active + not recruiting, recruiting, and enrolling clinical trials from clinicaltrials.gov (refer via NCT number) targeting cancers/tumours that employ the use of anti-angiogenic drugs that target VEGF. Clinical trials included were researched on the 21st July 2022. Anti-angiogenic drugs are listed in **bold.**
permeability, thereby improving overall tumour perfusion (100). Besides its anti-angiogenic effects, bevacizumab also exhibits cytotoxic action in chronic lymphocyte leukaemia (CLL) by upregulating pro-apoptotic Bad, Bax, and Akt proteins while downregulating anti-apoptotic McI-1 protein (99). This protein expression profile increases caspase 3 and caspase 9 activity, triggering CCL apoptosis (99).

Bevacizumab is often used to increase chemotherapy efficacy, which led to its FDA approval (100). Demonstrated in metastatic colorectal cancer patients, concurrent treatment of bevacizumab and irinotecan, fluorouracil, and leucovorin (IFL) chemotherapy significantly increased median survival duration by 30.1% compared to patients treated with IFL and placebo (97). Furthermore, median progression-free survival duration increased by 71.0% from 6.2 to 10.6 months with bevacizumab and IFL treatment compared to placebo and IFL treatment (97). Xenograft tumour models further demonstrated bevacizumab’s ability to augment the efficacy of chemotherapy (100). Combinational and delayed administration of topotecan to bevacizumab-treated mice elicited enhanced tumour volume reduction compared to their monotherapeutically topotecan or bevacizumab treated counterparts (100). These results were consistent with data showing that delayed chemotherapy administration to bevacizumab-treated mice improved intratumoral chemotherapeutic drug penetration as 3 days after bevacizumab treatment, topotecan tumour penetration increased by 22% compared to size-matched controls (100). Furthermore, combining bevacizumab with various chemotherapies (paclitaxel and ixabepilone) in patients with TP53 mutations improved PFS and OS compared to chemotherapy alone (101). However, cediranib, an anti-angiogenic tyrosine kinase inhibitor, demonstrated higher cell cycle abrogation and synergy with chemotherapy compared to bevacizumab in endometrial cancer models in vitro (102).

Receptor tyrosine kinase inhibitor therapy - sunitinib

Sunitinib is an FDA approved multi-RTK inhibitor used to treat RCCs, imatinib-resistant gastrointestinal stromal tumours (GIST) and advanced pancreatic neuroendocrine tumours (PNETs) (103, 104). VEGF and PDGF receptors are commonly overexpressed on tumour vessel pericytes and endothelial cells, causing increased angiogenesis. Since sunitinib can prevent ligand binding to VEGFRs, PDGFRs, c-Kit, Flt3 and RET kinases, it inhibits their activation and subsequent angiogenic effects mediated by them (105–107).

In vitro experiments using human lung microvascular endothelial cells (HLMECs) showed sunitinib treatment reduced endothelial cell proliferation and invasion due to sunitinib-mediated VEGFR2 inhibition, while in vivo experiments using RCC xenograft, mice presented a significant reduction in microvessel density, culminating in tumour growth inhibition (107). Furthermore, sunitinib-treated prostate cancer xenograft models demonstrated an average 0.15 reduction in nitroimidazole standardized uptake value (uptake based on radioactivity administered, radioactivity detected in volume of interest and body weight) measured via microPET analysis (108, 109). This correlated to decreased HIF-1α expression and thus indicates reduced tumour hypoxia (108). This was stipulated to result from sunitinib inhibiting VEGFR and PDGFR, preventing Akt and Erk1/2 signalling pathways that induce HIF-1α expression (108). As hypoxia is a known inhibitor of radiotherapy, subsequent investigations using in vivo mouse models demonstrated that sunitinib administration increased prostate cancer cell susceptibility to irradiation-mediated apoptosis (108).

Unfortunately, sunitinib has demonstrated variable results in its combinational efficacy with chemotherapy. The notable phase 2 clinical trial employing both sunitinib and gemcitabine to treat RCC, displayed an improved objective response rate (ORR) compared to monotherapies of either drug (110). However, other studies, such as those covering metastatic breast cancer, revealed no statistical significance in progression-free survival or response rate when sunitinib was excluded or used in combination with capecitabine therapy (111). Furthermore, the combination of both drugs increased toxicity risk in patients, which remains a current challenge in implementing sunitinib therapy (111, 112).

Interestingly, trials employing pre-treatment of sunitinib before chemotherapy in breast cancer patients, demonstrated a statistically significant 12.46% increase in vascular normalisation index (VNI) compared to pre-treatment with bevacizumab (113). The VNI biomarker is defined by vessel permeability, volume and collagen IV plasma levels (relative to basement membrane thinning) which allows for quantification of vascular normalisation (114, 115). The observed VNI therefore indicates anti-angiogenic pre-treatment may improve chemotherapeutic drug delivery, particularly in cancers where concurrent administration has not proved effective (113).

Besides sunitinib’s anti-angiogenic effects, sunitinib has also been shown to improve the immunosuppressive lymphocyte profile in the TME (116). In hepatocellular carcinoma mouse models, flow cytometry analysis revealed that tumour-bearing mice displayed an increased population of Treg cells compared to tumour-free controls (116). These Treg cells also presented with upregulated immunosuppressive IL-10 and TGF-β cytokine production, consequently inhibiting CD8+ T cell immune responses (116). In the presence of Tregs isolated from tumour-bearing mice, stimulated tumour-antigen-specific (TAS) CD8+ T cells exhibited reduced cell proliferation and IFN-γ production when compared to CD8+ T cells stimulated with Tregs isolated from tumour-free mice (116). Despite this, when
TAS CD8+ T cells were stimulated with Tregs from tumour-bearing mice treated with sunitinib, T cell proliferation and IFN-\( \gamma \) production was restored (116). This association was supported as sunitinib-treated tumour-bearing mice exhibited a significantly reduced Treg population in spleen, draining-lymph node, and liver samples compared to tumour-bearing controls (116). Crucially, IL-10 and TGF-\( \beta \) cytokine production was also restored to levels comparable to Tregs from tumour-free control mice, improving the CD8+ T cell-mediated cytotoxic response (116).

**Investigatory receptor tyrosine kinase inhibitors in clinical trials**

There are some investigatory RTK inhibitors that are not approved by FDA. Some of these agents include cediranib, motesanib and surufatinib which inhibit VEGFR isoforms. Cediranib and motesanib also inhibit platelet-derived growth factor receptors and cediranib and surufatinib additionally inhibit fibroblast growth factor receptor (FGFR1).

Inhibition of multiple RTKs is an advantageous feature in cancer treatment. A study by Bi et al., 2021 has shown that cediranib combination with paclitaxel had higher cell death while bevacizumab combination with paclitaxel treatment showing a small insignificant change in cell death in endometrial cancer cells (102). In phase III trials, cediranib had limited benefit in metastatic colorectal cancer (NCT00384176), non-small cell lung cancer (NCT00795340), and recurrent glioblastoma (NCT00777153) (117–119). In platinum-sensitive ovarian cancer (NCT00532194) cediranib combination with chemotherapy then cediranib maintenance therapy had a significant improvement in progression-free survival when compared to chemotherapy (median, 11 months vs 8.7 months) but it was also associated with greater toxicity (120). After a follow-up, median survival with cediranib combination with chemotherapy then cediranib maintenance therapy was higher by 7.4 months when compared to chemotherapy (27.3 months vs 19.9 months) (121). On the other hand, in platinum-sensitive ovarian cancer, comparison between cediranib combination with olaparib to chemotherapy had no significant improvement in progression-free survival (median, 10.4 vs 10.3 months) (122).

Motesanib and surufatinib are reviewed in Qin et al., 2019 (103). Furthermore, there is a need for further research to clarify the role of these newer RTK inhibitors in cancer treatment. Additionally, other agents such as fruquintinib (NCT02314819), nintedanib (NCT00805194), and anlotinib (NCT02388919) have obtained regulatory approvals other than FDA could be also potential agents requiring further research for defining their clinical efficacy in various cancer treatment with drug combinations.

**Challenges and resistance to anti-angiogenic therapy**

Despite that anti-angiogenic therapies have shown success in certain tumours, many cancers including breast, pancreatic and prostate cancers initially display or develop resistance after treatment (92). As demonstrated by bevacizumab and sunitinib, a large proportion of anti-angiogenic therapies target VEGF/VEGFR pathways, which are not exhaustive mediators of angiogenesis. Many other growth factors such as Ang-2, FGF, PDGF and TGF-\( \beta \) continue to support angiogenesis in cancers which display anti-VEGF resistance (123). Additionally, tumours can employ non-angiogenic mechanisms to obtain blood supply, thereby rendering anti-angiogenic therapy as ineffective. This was observed in sorafenib-treated hepatocellular carcinoma mouse models, which displayed drug resistance 38 days after initial treatment, identified by an increase in human choriongonadotropin rate of 28.3 mIU/mg day that represents an increase in invasive tumour growth rate (24). Later histological analysis revealed sorafenib early-resistant tumours displayed a 51.7% increase in dependence on co-opted blood vessel supply compared to control tumours (24). Drug-resistant tumours can also obtain blood supply via vasculogenic mimicry as displayed in RCC cell lines and xenograft mouse models (124). Tumours from sunitinib-treated RCC mice displayed characteristic VM biomarker alterations, including increased PAS staining and reduced CD31 expression when compared to control mice (124). Observed RCC tumour progression during sunitinib treatment has been linked to VM induction, possibly via sunitinib upregulating ER\( \beta \) that causes HIF-2\( \alpha \) production (124, 125).

Even though anti-angiogenic therapies have shown success, the transient time that vascular normalisation is elicited after treatment, termed the ‘vascular normalisation window’ poses a major challenge (3). Maximising this period is essential to improving chemotherapy and radiotherapy effectiveness, but it is difficult to monitor due to a lack of biomarkers and effective parameters needed for optimal dosing strategies (3, 62, 126). However, techniques such as blood-oxygen-level-dependent MRI and dynamic contrast-enhanced MRI can be used to non-invasively determine tumour hypoxia, vessel perfusion and vessel permeability (62, 127). The oxymoronic effects of anti-angiogenic therapy have also been described to cause undesirable vessel regression leading to hypoxia (128). Hypoxia mediated HIF-1\( \alpha \) expression, upregulates factors that can recruit immunosuppressive bone marrow-derived cells (BMDCs), causing a range of downstream effects. For example, these cells can express MMP9 to promote ECM degradation and increase VEGF bioavailability which supports angiogenesis (128).
Future perspectives – novel nanoparticle therapy

The discussed limitations of anti-angiogenic therapy are difficult to overcome, evident by drug resistance and toxicity observed in clinical trials (129). Notably, the aberrant perfusion and architecture of tumour vessels limits the delivery of drugs to the relevant site, and the normalisation window consistently hinders effective dosing that also prevents excess vessel regression (126, 129). Nanoparticles can potentially bypass these obstacles via specific tissue targeting and improving drug pharmacokinetics by reducing dosage and enhancing drug stability (9). Furthermore, therapeutic nanoparticles are highly diverse, consisting of inorganic, lipid and polymer formulations whose varying characteristics can be utilised (9).

Liposomal nanoparticles have shown some promise when combined with doxorubicin. Doxorubicin chemotherapy is often utilised in the treatment of breast cancer but cumulative dosing poses challenges in preventing toxicity induced cardiomyopathy (130). Evidence has shown that liposomal-doxorubicin formulations induced 64% fewer cases of toxicity induced congestive heart failure compared to conventional doxorubicin in a metastatic breast cancer clinical trial. Despite the decrease in cardiotoxicity, no statistically significant difference in patient survival time was observed (130). Moreover, improvements upon liposomal nanoparticles via pegylation can allow for prolonged systemic circulation time. Pegylated liposomal-doxorubicin, known as Doxil/Caelyx achieves this by surface coating the liposome-encapsulated drug with methoxy-poly-ethylene-glycol molecules, permitting immune evasion from the reticuloendothelial system (131).

Using nanoparticle technology to improve drug delivery in solid tumours has largely been aimed at taking advantage of the enhanced permeability and retention (EPR) effect elicited by the tumour vasculature (132). The hyperpermeability of tumour blood vessels is proposed to result from attenuated endothelial junctions and the presence of vesiculo-vacuolar organelles (VVOs) in microvessels that can transendothelial extravasation (133, 134). The EPR effect attributes this to increasing the extravasation of nanoparticles in tumour vessels, thus increasing drug accumulation in tumours while reducing drug accumulation in healthy tissues (132). However, the effect appears more prevalent in xenograft mouse models than in human tumours, leading to scepticism regarding its impact in clinical use (132).

Despite this, nanoparticle-mediated technology can be effective in cancer therapy by inducing other mechanisms including vascular normalisation (135). Gold nanoparticles (AuNPs) contain a gold core surrounded by a monolayer that can contain specialised ligands to improve delivery to target cells (136). For example, AuNPs were conjugated to folic acid ligands to improve their targeting to tumours as folate receptor overexpression is observed in many cancers (135). Furthermore, the structure of AuNPs is variable depending on their size, which could be used to improve their efficacy (136). For example, AuNPs have demonstrated the ability to reduce chaotic vessel architecture, permeability and hypoxia while increasing pericyte coverage and T cell infiltration (135, 137). These effects were associated with AuNP treatment upregulating the expression of the semaphorin 3A cytokine (SEMA3A) in gastric adenocarcinoma cells which can subsequently inhibit the TGF-β mediated SMAD2/3 signalling pathway in endothelial cells (135). Lastly, AuNP treatment has also been shown to downregulate the expression of pro-angiogenic VEGF-A in gastric adenocarcinoma cells, attenuating angiogenesis (135).

As previously discussed, cathepsin D and L play potent proangiogenic roles within the tumour microenvironment. In an attempt to inhibit this potent proangiogenic role of cathepsins D and L in the extracellular space, we tested effects of highly compatible graphene oxide (GO) in vitro (138). Our group showed for the first time that GO could be used as a strong inhibitor for cathepsins D and L in a time, dose and pH dependent manner. Using analytical tools such as Raman scattering system, Fourier Transform Infrared Spectroscopy (FTIR), water contact angles and surface energy, we demonstrated a strong bonding between the enzymes GO and an adsorption capacity of GO which resulted in denaturation of the enzymes functional active sites. The cationic and hydrophilic residues on the surface of GO mediates adsorption of the enzymes, resulting in denaturation and deactivation. The mechanistic aspects of protein adsorption and/or protein corona formation as a result of the interaction of proteins with graphene may involve electrostatic and hydrophobic interactions (139). However, further studies are required to examine this promising anti-metastatic effect of GO in cell culture and in vivo models.

Recent developments in nanoparticle-based formulations have harnessed huge attention in detecting and treating cancer. The integration and conjugation of nanoparticles with wide-ranging therapeutic agents or ‘classical’ chemotherapeutic drugs provides innovative approaches to release or activate (in response to external or internal sources such as light, ultrasound, pH) the therapeutic agent in a controlled, safe and targeted manner. Nanoparticles-based approaches offer several advantages over conventional delivery or treatment modalities such as enhanced delivery of nanoparticles due to EPR, improved pharmacokinetics, precise control over release mechanisms, surface modification with specific cellular or sub-cellular targeting ligands, tunability of size, shape, morphology and surface charge as well as the ease of functionalisation with biomolecules (e.g., RNA, antibodies, nanobodies and other.
Another advantage is the two-in-one function of nanoparticles where they can be used as a theranostics agent, a combination of diagnostics and treatment. Over recent years, more than 15 nanoparticles have clinically been approved for diagnostics and therapeutic purposes (140, 141). In comparison to conventional treatment options, nanoparticles offer unique physiochemical features which have the ability to deliver drugs to the key players of the TME. When nanoparticles interact with different components of the TME, they also have the ability to modify the immunosuppressive environment. For instance, their interactions with blood vessels can induce hypoxia. Nanoparticles encapsulated with immunosuppressive factors such as VEGF and TGF-β can release these factors in a sustained fashion thereby leading to an abnormal tissue dynamic (142, 143). Such critical roles of nanoparticles in the TME are beyond the scope of the article and we refer the reader to specialised literature on this (144, 145).

Furthermore, the TME plays a crucial role in the biodistribution and biological fate of nanoparticles, although more in-depth investigations are required to explore the role of the TME in targeting nanoparticles towards the site of action. In this review we mainly summarise the crosstalk between the immune element of the TME and local tumour vascular endothelial cells with a brief discussion on the role of emerging nanoparticles. Nanoparticles have the potential to affect the abnormal roles of the TME, which in turn can also minimise the burden of drug resistance development thereby significantly improving the therapeutic effects in a targeted fashion.

Conclusion

The tumour vasculature’s features and their complex interactions with the TME are established to perpetuate angiogenesis and suppress the immune response. Associated mechanisms include upregulating pro-angiogenic factor expression, reducing therapeutic drug delivery and repressing effector T cell infiltration into the tumour parenchyma. These barriers consequently reduce chemotherapy, radiotherapy, and immunotherapy success in solid tumours, emphasising tumour vasculature formation as an important therapeutic target. Currently, anti-angiogenic treatments that promote vascular normalisation are used, however, their efficacy has proved inconsistent in clinical trials, with many tumours displaying initial and acquired drug resistance. The ambiguous processes inducing resistance remain to be clarified but include tumours exploiting non-VEGF pro-angiogenic pathways, obtaining blood supply via non-angiogenic methods such as vasculogenic mimicry and ineffective utilisation of the normalisation window during treatment. Therefore, exploring alternative therapies combatting these obstacles remains essential to improving cancer treatment.

Progress has been made in this field, including nanoparticle therapy, however, improving the understanding of inducing vascular normalisation will allow treatments to further leverage the normalisation window and thus reduce the risk of developing drug resistance. Furthermore, many anti-angiogenic treatments continue to predominantly focus on targeting VEGF however developing other therapies that target additional pro-angiogenic pathways may allow for the successful treatment of a wider variety of tumours. Lastly, the importance of non-angiogenic mechanisms in acquiring tumour blood supply remains to be fully explored so that therapies targeting these mechanisms can be developed. Evidently, furthering the understanding of the tumour vasculature’s effect on angiogenesis, immunosuppression and treatment could vastly improve the field of cancer therapy.

Author contributions

ZI, TT, JY and MP drafted the manuscript. DZ and JY reviewed and edited the manuscript. NA contributed with discussions and critical revision of the manuscript. MP edited the manuscript and conceptualised, supervised and administered the project. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Cell Biol mother vessel formation.

Angiogenesis

Pathological features of vessel co-option versus sprouting angiogenesis. Biol Ther chemotherapeutic resistance.

Histol Histopathol therapies.

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