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ORIGINAL ARTICLE

Overcoming physical stromal barriers to cancer immunotherapy

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Abstract

Immunotherapy has emerged as an unprecedented hope for the treatment of notoriously refractory cancers. Numerous investigational drugs and immunotherapy-including combination regimens are under preclinical and clinical investigation. However, only a small patient subpopulation across different types of cancer responds to the therapy due to the presence of several mechanisms of resistance. There have been extensive efforts to overcome this limitation and to expand the patient population that could be benefited by this state-of-the-art therapeutic modality. Among various causes of the resistance, we here focus on physical stromal barriers that impede the access of immunotherapeutic drug molecules and/or native and engineered immune cells to cancer tissues and cells. Two primary stromal barriers that contribute to the resistance include aberrant tumor vasculatures and excessive extracellular matrix build-ups that restrict extravasation and infiltration, respectively, of molecular and cellular immunotherapeutic agents into tumor tissues. Here, we review the features of these barriers that limit the efficacy of immunotherapy and discuss recent advances that could potentially help immunotherapy overcome the barriers and improve therapeutic outcomes.

Keywords Tumor endothelium · Extracellular matrix · Tumor microenvironment · Tumor infiltration · Combination regimen

Introduction

Immunotherapy has been the most highlighted class of cancer therapy in the recent years, spurred by the groundbreaking clinical successes with immune checkpoint inhibitors (ICIs) [1]. A few immunotherapies, such as recombinant interferon-α (IFNα) and interleukin-2 (IL-2), were approved for certain cancers decades ahead, but their moderate efficacy and/or severe systemic adverse effects dampened the earlier optimism [2, 3]. Immunotherapy regained explosive interests in the field of clinical oncology since the regulatory approval of ipilimumab, the ICI targeting cytotoxic T-lymphocyte associated protein 4 (CTLA4), based on tremendous therapeutic benefits shown in patients with advanced melanoma [1]. This was soon followed by the approval of other ICIs targeting the programmed cell death protein-1/programmed cell death ligand 1 (PD-1/PD-L1) axis, and chimeric antigen receptor T cell (CAR-T) therapies, further strengthening the enthusiasm towards cancer immunotherapy [4]. These innovative therapies exhibited surprisingly high rate of complete regression of the tumors and long-term disease control in responding patients, which have been rarely observed with conventional cancer therapies [5]. The achievement is primarily attributed to its inherent nature that directly works in concert with the host immune system. Specifically, anticancer immunity boosted by immunotherapy preferentially attack cancer cells and establishes immunological memory via the unique ability of T cells to remember the antigenic targets that they have encountered. This memory enables the immune system to quickly response upon re-encounter with the target, thereby generating a long-lasting and durable anticancer immunity even after the treatment is withdrawn [6]. However, a majority of cancer patients are unresponsive to this state-of-the-art therapeutic modality where only ~ 12% of patients across different types of cancer have been estimated to respond to ICIs [7]. Mechanisms of the resistance

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have been extensively investigated and currently thousands of clinical trials are underway to explore the combinations of immunotherapies or with other therapies, hoping to overcome the current limitation and to ultimately broaden the responding patient population [8, 9]. Among the proposed resistance mechanisms, physical barriers established in the tumor stroma significantly impact therapeutic outcomes by impeding the delivery of immunotherapeutic agents and the infiltration of immune cells into the tumor tissues [10, 11].

Chemotherapy and other cancer-targeted therapies necessitate their access from the site of administration into tumor tissues to act directly upon every single cancer cell to perturb proliferation or certain signaling pathways essential for survival [12, 13]. Thus, primary focus has been improving the delivery of drug molecules to, and their interaction with, cancer cells via various techniques, such as nanoparticle-based and/or ligand-directed delivery strategies [14]. Immunotherapy involves enhancing cancer cell susceptibility to immunosurveillance or augmenting immune cell activities [15]. The former approach includes ICIs targeting immunosuppressive molecules on cancer cell surfaces (e.g., anti-PD-L1 antibody or Ab), immunostimulatory cytokines (e.g., interleukin-12), or oncolytic viruses, which would be benefited by the aforementioned delivery strategies to enhance the benefit-to-risk ratio [16, 17]. On the other hand, the latter involves stimulation of the host immune system to generate tumor-specific immune responses (e.g., cancer vaccines) or unshackling immune cells by ICIs targeting immunosuppressive molecules on immune cell surfaces (e.g., anti-PD-1 and anti-CTLA-4 Ab) to boost immunological cancer cell death [6, 18]. Thus, efficient tumor infiltration of effector immune cells, rather than molecular agents, is critical to the success of this immunotherapeutic modality.

The immunological properties of the tumor microenvironment as well as the cellular and biochemical resistance mechanisms have been widely discussed elsewhere [19]. In this review, therefore, we primarily focus on the physical stromal barriers that impede delivery of immunotherapeutic agents and infiltration of native and engineered immune cells into the tumor tissues. We also discuss several strategies to improve intratumoral drug delivery and immune cell infiltration, which have displayed promises in preclinical and/or clinical investigations.

Stromal barriers that physically restrict immunotherapy

Tumor infiltration of immune cells is a universal and essential prerequisite for achieving clinically relevant immunotherapy, whereas a few subtypes additionally require direct access of non-cellular immunotherapeutic agents to cancer cells. Irrespective of specific modality, both molecular and cellular agents must breach the stromal barriers to gain an access to the tumor core to act upon cancer cells. To this end, we here provide a brief overview of the characteristics of primary stromal components present in tumor tissues, including abnormal vasculatures and dense extracellular matrix (ECM) (Fig. 1) [19–21].

Abnormal vasculatures

Angiogenesis during normal development and in healthy tissues is tightly regulated by the well-balanced levels of proangiogenic and antiangiogenic factors, leading to the formation of regular blood vessels. In many tumors, however, proangiogenic factors, including vascular endothelial

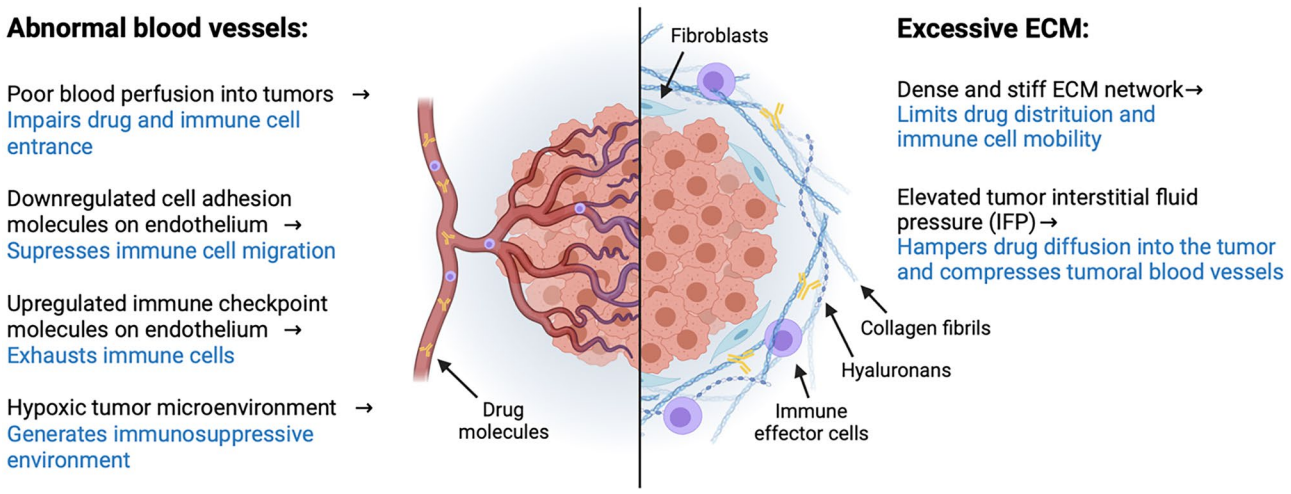

Fig. 1 Physical stromal barriers in tumors that limit the efficacy of immunotherapy. Abnormal vasculatures (left) and excessive extracellular matrix (ECM) build-ups (right) in solid tumors hamper the access of drug molecules and immune cells

growth factor (VEGF) and fibroblast growth factor, are overexpressed by cancer cells to support the tumor growth, which accelerates formation of aberrant blood vessels [22]. Specifically, tumor blood vessels are structurally tortuous and heterogeneously distributed throughout the tumor tissue. Moreover, tumor endothelium is porous, dilated, and leaky due to the loose connection of endothelial cells and the scarcity of mural cells (e.g., pericytes and vascular smooth muscle cells) which regulate homeostatic permeability of blood vessels [23]. Such irregular vasculatures lead to chaotic and dysfunctional blood flows, thereby resulting in poor tumor perfusion.

The abnormal vasculatures in tumor have profound clinical impacts on therapeutic outcomes of immunotherapy [20]. The poor perfusion significantly impedes uniform delivery of systemically administered drugs into the tumor tissues, limiting drug exposure to hypo-perfused areas [23]. We note that the porous and leaky tumor vasculatures have been widely believed to promote extravasation and accumulation of macromolecular and nanoparticle-based therapeutics in the tumor, a phenomenon known as the enhanced permeability and retention (EPR) effect [24, 25]. However, the role of the EPR effect in human on therapeutic delivery remains highly controversial and its existence appears to be highly sporadic at best [26, 27]. Similar to drug molecules, dysfunctional tumor vasculatures also hamper circulating effector immune cells from reaching the tumor tissue and ultimately limit their effectiveness on cancer cell eradication [28]. Moreover, proangiogenic factors alter the molecular signatures on tumor endothelium to further interfere with immune cell trafficking into the tumor. For example, cell adhesion molecules that mediate immune cell adhesion to, and transmigration across, the vessel wall, such as vascular cell adhesion protein 1 (VCAM1) and integrin ligands intercellular adhesion molecule 1 (ICAM1), are downregulated on tumor endothelium, thereby limiting immune cell extravasation into the tumor parenchyma [29–31].

Besides affecting immune cell trafficking, tumor endothelium upregulates Fas antigen ligand (FasL) and numerous immune checkpoint molecules, including PD-L1, T-cell immunoglobulin, and mucin domain-containing protein 3 (TIM3), B7-H3, and B7-H4, to promote apoptosis and inactivation of immune cells, respectively [32]. The dysfunctional tumor vasculatures also indirectly contribute on immune cell inactivation by creating a hypoxic tumor microenvironment (TME) [33]. Tumor hypoxia is established by the high metabolic activity of cancer cells that entails excessive oxygen consumption as well as the limited tumoral blood perfusion arises from the impaired tumor endothelium [23], and creates immunosuppressive tumor environment in a multi-pronged manner [33]. Specifically, hypoxic TME promotes accumulation and secretion of immunosuppressive cells (e.g., myeloid-derived suppressor cells, regulatory T cells, and tumor-associated macrophages) and factors (e.g., VEGF and transforming growth factor β or TGFβ), respectively [34–37], and upregulation of immune checkpoint molecules on cancer cells [38, 39]. The cascade of these events in hypoxic TME collectively compromises the ability of effector immune cells to eradicate cancer cells.

Tumor extracellular matrix

ECM is a macromolecular network that occupies the extracellular space within various tissues and provides cells with physical and biochemical supports to promote cellular differentiation and homeostasis, as well as tissue morphogenesis [40]. ECM is composed of approximately 300 different matrix macromolecules and specific molecular composition varies highly with tissues and pathophysiological conditions [41]. The structure of ECM is highly dynamic, and it undergoes continuous remodeling through enzymatic or non-enzymatic posttranslational modifications [41]. In tumor tissues, cancer cell and cancer-associated fibroblasts increase deposition of ECM molecules and mediate abnormal ECM remodeling, which in turn leads to tissue stiffening [42]. Tumors are often inflamed [43], which activates stromal fibroblasts and induces their differentiation into myofibroblasts that secrete a large quantity of ECM proteins and promote tissue desmoplasia [44]. Moreover, several enzymes that catalyze the crosslinking of ECM components, such as lysyl oxidase (LOX) and lysyl oxidase-like 2 (LOXL2), are elevated in tumor tissues to increase the density and stiffness of tumor ECM [45].

The tightened tumor ECM restricts the distribution of drug molecules, which manage to escape the blood vessels, throughout the tumor tissue. Additionally, amply deposited ECM molecules within the tumor tissue attract a large amount of water molecules to increase the tumor interstitial fluid pressure (IFP) [46]. The elevated tumor IFP hampers drug transport into the tumor core and compresses the tumor blood vessels to suppress the drug perfusion from systemic circulation into the tumor tissues [47]. This is of particular concern for immunotherapy as most of the clinically approved immunotherapeutic agents (e.g., Abs and CAR-T cells) are markedly larger than conventional small molecule-based chemotherapeutic drugs. Likewise, endogenous immune cells, such as those primed by cancer vaccines, shares the identical fate. Many solid tumors that exhibit an immune-excluded phenotype, characterized by few intratumoral effector immune cells, are associated with poor prognosis and resistance against immunotherapy [19, 21]. Interestingly, histological analysis of relevant clinical samples revealed that effector immune cells are often concentrated in the ECM-rich stromal tissue delineating the tumor-host interface [48]. Salmon et al. have demonstrated that T cell mobility is suppressed within the high-density

ECM, indicating that the peritumoral ECM physically traps immune cells and restricts their access to the tumor parenchyma [48].

Individual macromolecular components of tumor ECM play significant roles on its barrier properties. Collagen is the most abundant structural protein element of the stromal ECM [49] and is overexpressed and deposited in various tumor tissues by cancer cells and cancer-associated fibroblasts, comprising up to 60% of the total tumor mass [46]. Hypoxia established in tumor ECM upregulates genes or signaling pathways involved in collagen synthesis (e.g., TGFβ) and maturation, including posttranslational modification (e.g., *P4HA* and *PLOD*) and crosslinking (e.g., LOX) of the collagen fibrils, thereby further tightening the ECM mesh [49–51]. LOX is overexpressed in most cancers [52], which contributes to hindered diffusion of chemotherapeutic agents within the tumor by increasing the collagen crosslinking density in tumor ECM [53, 54]. Likewise, excessive collagen build-up poses a physical barrier that restricts infiltration of drug molecules and T cells into the tumor parenchyma [55, 56]. Mariathasan et al. have demonstrated that overexpression of TGFβ in metastatic urothelial cancer patients is associated with entrapment of cytotoxic T cells in the collagen-rich tumor stroma and thus renders anti-PD-L1 Ab therapy ineffective [57]. Similarly, increase in the intratumoral collagen level has been shown to promote resistance to anti-PD-1/PD-L1 therapy via LAIR1-dependent $CD8^+$ T cell exhaustion [58]. Hyaluronan or hyaluronic acid (HA) is another major macromolecular component of tumor ECM [59, 60]. HA in ECM forms polyvalent bonds with other ECM constituents to generate complex mesh networks and is upregulated in many types of cancers, including melanoma, glioma, and breast/lung cancers, to promote tumor progression via multiple mechanisms [61–64]. It has been reported that the hydroscopic property of HA increases tumor IFP to hamper both transvascular migration and intratumoral distribution of blood-borne drug molecules and immune cells [10, 47].

Strategies to overcome stromal barriers to enhance outcomes of immunotherapy

Based on the accumulated understanding of the stromal barriers overviewed in the previous section, numerous strategies to breach those barriers have been preclinically proposed and explored to enhance the effectiveness of immunotherapy. Encouragingly, a few approaches have been reached clinical trials or even approved by the FDA for use in patients. Here, we attempt to provide a concise introduction to most widely employed strategies that seek to bypass or transiently modulate the stromal barriers. A graphical summary of the strategies to modulate the stromal barriers is shown in Fig. 2.

Local administration and strategies to prolong intratumoral drug retention

Local administration, such as intratumoral and peritumoral injection, is the most straightforward means to bypass the physical stromal barriers to gain a direct access to cancer cells, while providing favorable therapeutic index by minimizing systemic exposure and maximizing intratumoral drug concentration. However, its use has been confined to highly localized early-stage cancers, as advanced cancers are often considered systemic diseases [65]. This is of particular concern for conventional anti-cancer agents, such as chemotherapy and targeted molecular therapy. However, emerging evidence suggests that local treatment could be effective for cancer immunotherapy regardless of stages, including advanced and metastatic cancers [16, 66–72].

Unlike conventional therapies, immunotherapy does not require complete coverage of the tumor tissue to elicit systemic immune responses directed to cancer cells disseminated throughout the body. Several preclinical studies of local immunotherapy have demonstrated the abscopal effect [16, 66–72], a term initially used to describe unexpected immune-mediated regression of untreated distal tumor tissues after localized tumor irradiation [73]. Sagiv-Barfi et al. reported that combined local administration of toll-like receptor 9 (TLR9) agonist (i.e., CpG) and agonistic OX40 Ab stimulated tumor-specific adaptive systemic T cell immune response [66]. The therapy eradicated not only the tumor received local treatment, but also untreated remote tumor tissues in several preclinical models of cancers, including B lymphoma and metastatic breast cancer. Mechanistically, intratumoral CpG administration induced the expression of OX40, a costimulatory immune checkpoint molecule that promotes T cell immunity, and agonistic OX40 Ab in turn stimulated systemic tumor-specific T cell immune response to destruct distant tumor tissues. Likewise, intratumoral administration of TLR7 agonist (i.e., SZU101) has shown to evoke systemic anti-cancer immunity in preclinical models of mammary carcinoma [67]. Interestingly, Francis et al. have recently demonstrated using a poorly responsive preclinical B16F10 melanoma model that intratumoral treatment with anti-PD1 and anti-CTLA4 Abs provides remarkably greater anticancer activity in both the treated primary tumor site and in untreated distal tumors, and thus the survival, compared to the systemic ICI treatment (Fig. 3) [68]. Immunological analysis revealed greater frequencies of $CD8^+$ T cells in tumor and tumor-draining lymph nodes by intratumoral over systemic ICI treatments, underscoring the superiority of localized immunotherapy on stimulating tumor-specific systemic immunity by enhanced T cell priming.

Despite the clear benefits of local immunotherapy described above, locally administered immunotherapeutic

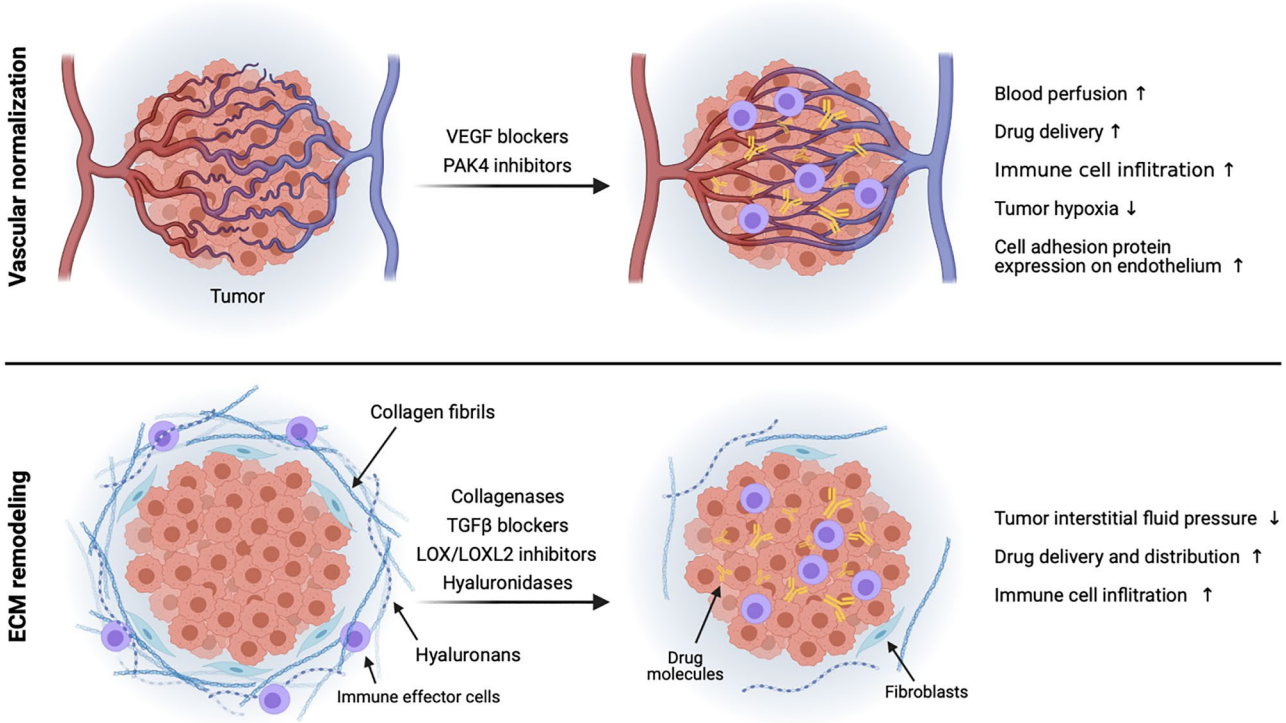

Fig. 2 Pharmacological strategies of normalizing the aberrant tumor vasculatures and ECM to improve immunotherapy. Vascular normalization (upper) and ECM remodeling (lower) could improve the delivery of drug molecules and infiltration of immune cells into the tumors

agents could rapidly escape the tumor tissue to enter the systemic circulation [74, 75]. Thus, strategies to prolong the intratumoral drug retention following local administration could markedly improve the therapeutic efficacy as well as therapeutic index. One of the relevant approaches involves incorporation of an ECM binding domain to immunotherapeutic agents to mediate their binding to tumor ECM, thereby enhancing tumor retention of the agents following local administration. Noor et al. fused a collagen-binding protein, lumican, to IL-2 and IL-12, which significantly extended their tumoral retention time and enhanced anticancer activity (Fig. 4A, B) [69]. Importantly, the prolonged tumoral retention of the cytokines augmented the abscopal effect while lowering the systemic toxicity, indicating strong and tumor-specific immune response. In another study, local treatment of anti-CTLA4 or anti-PD-L1 Abs conjugated with an ECM binding peptide (i.e., PlGF-$2_{123–144}$) derived from placenta growth factor-2 demonstrated similar results (Fig. 4C–E) [16], validating the effectiveness of the ECM binding strategies on localized immunotherapy. Use of conventional drug delivery systems that provide sustained and/or controlled payload release pose an additional means to enhance the retention of immunotherapeutic agents following local administration. For example, Park et al. have described a biodegradable HA-based hydrogel implant loaded with TLR7/8 or stimulator of interferon genes (STING) agonist that provides sustained payload releases [76]. The hydrogel implanted into tumor resection cavity prevented tumor recurrence and eliminated distant metastases in a preclinical model of 4T1-Luc2 mammary cancer by activating both innate and adaptive anti-cancer immunity. In contrast, such effects were not observed when TLR7/8 or STING agonist was administered systemically or locally in solution (i.e., no hydrogel), underscoring the critical role of the extended drug release on achieving meaningful therapeutic outcomes of localized immunotherapy. Likewise, other delivery systems, including nanoparticles, liposomes, and implants (e.g., nanofluidic drug eluting seeds), have demonstrated to provide sustained release of immunotherapeutic agents and thus to augment tumor-specific local and systemic immunity [77–80].

Vascular normalization

Rakesh K. Jain proposed the idea of normalizing the tumor vasculature by antiangiogenic therapy to improve drug delivery the tumor in 2001 [81]. The initial concept of antiangiogenic therapy was to preclude neovascular formation and/or abolish the previously established blood vessels, which fasts and suffocates the cancer cells to death [82]. However, accumulated evidence suggests that certain antiangiogenic therapy (e.g., VEGF inhibitors) transiently restore angiogenic homeostasis

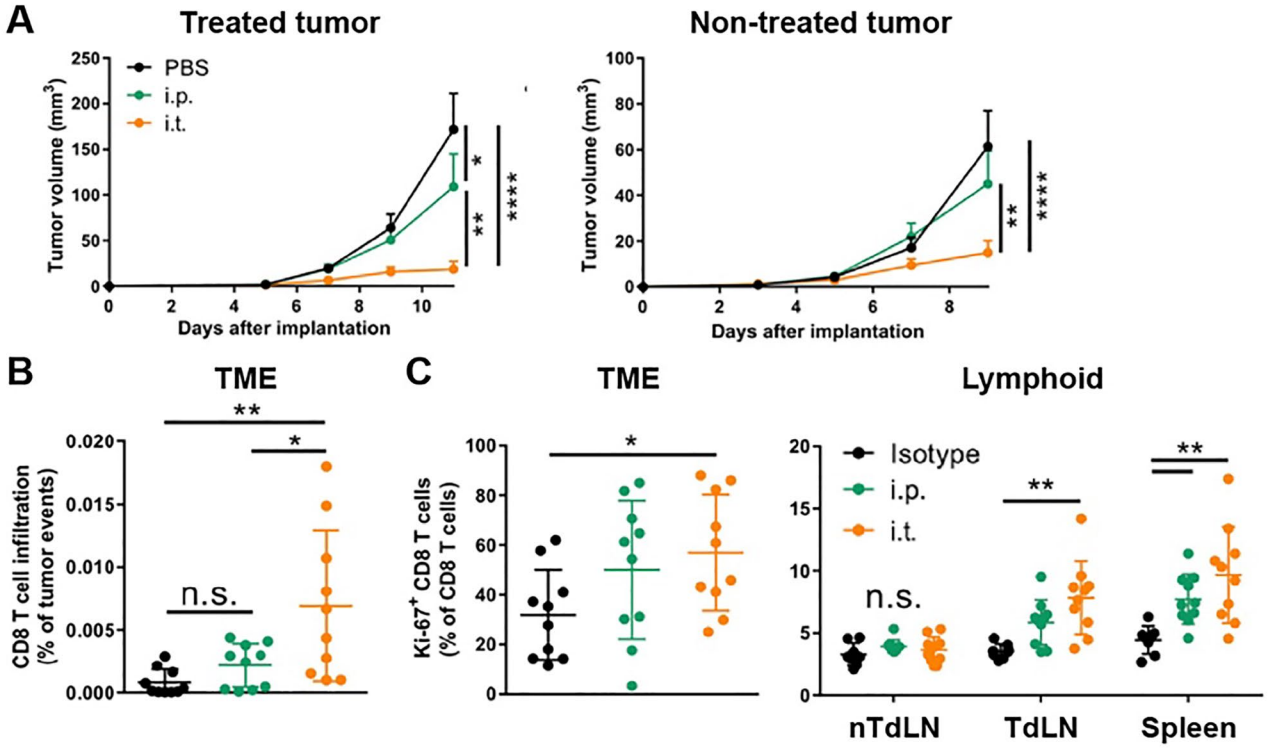

Fig. 3 Local administration of immunotherapy elicits tumor-specific systemic immunity. **A** Tumor growth curves of treated (left) and distant non-treated (right) tumors of a preclinical B16F10 melanoma model. Intratumoral (i.t.; yellow) injection of anti-PD-1 Ab plus anti-CTLA-4 Ab effectively suppressed the growth of both treated and untreated remote tumors. The degree of tumor growth suppression was greater with intratumoral administration as compared to systemic (intraperitoneal, i.p.; green) administration of anti-PD-1 Ab plus anti-CTLA-4 Ab. **B** Frequency analysis of CD8 T cells. Intratumoral injection, but not the intraperitoneal injection, of the immune checkpoint blockers (ICIs) increased the number of CD8 tumor-infiltrating lymphocytes. **C** Cycling CD8 T cell (Ki-67$^+$ CD8$^+$) frequencies were also increased in tumor as well as in tumor draining lymph nodes (TdLN) and spleen when ICIs were treated intratumorally. In spleen, intraperitoneal administration of ICIs also showed comparable increase of the cycling CD8 T cell frequency. nTdLN, non-tumor draining lymph node. Adapted with permission from [68]

to normalize aberrant structure and function of tumor vasculatures, thereby paradoxically enhancing oxygen supply to alleviate immunosuppressive tumor hypoxia [22]. Likewise, structural normalization of tumor endothelium by antiangiogenic therapy has been shown to promote uniform and enhanced delivery of systemically administered chemotherapeutic drugs to the tumor tissue [83, 84], by reducing tumor IFP as well as restoring the blood flow dynamics and patterns [22]

Normalizing tumor vasculatures with antiangiogenic therapy, especially with the inhibitors of the VEGF signaling pathway, has been also suggested as a promising means to improve the responses to immunotherapy [85]. In addition to the expected enhancement of the delivery of immunotherapeutic agents into and throughout the tumor tissue, vascular normalization improves intratumoral immune cell trafficking and rescues effector immune functions by reversing the immunosuppressive tumor hypoxia [86]. Numerous independent studies demonstrated enhanced therapeutic outcomes of different types of immunotherapy, including cancer vaccines, adoptive cell therapies, and ICIs, in many preclinical models when combined with blockade of VEGF/VEGFR signaling axis (e.g., anti-VEGF/VEGFR2 Abs, VEGFR tyrosine kinase inhibitors or TKIs) [87–93]. As a result, combination regimens of ICIs and antiangiogenic agents have been recently approved by the FDA for treating different cancers, including metastatic hepatocellular carcinoma and renal cell carcinoma (Table 1), and many more are currently under clinical investigation (Table 2) [11, 94]. In attempt to enhance the effectiveness of this strategy, the dual blockade of VEGF and angiopoietin-2 (ANG2) has been proposed, based on the finding that ectopic ANG2 expression destabilizes the blood vessels normalized by VEGF blockade [95–97]. Preclinical studies demonstrated improved and prolonged vascular normalization upon simultaneous inhibition of VEGF and ANG2, either by combination of a small molecule VEGFR TKI (i.e., cediranib) and anti-ANG2 Ab or anti-ANG2/VEGF bispecific Ab, as compared to the blockade of single molecules [90, 98]. Moreover, anti-ANG2/VEGF bispecific Ab enhanced the anti-cancer immunity mediated by PD-L1 blockade in several preclinical cancer models. However, a

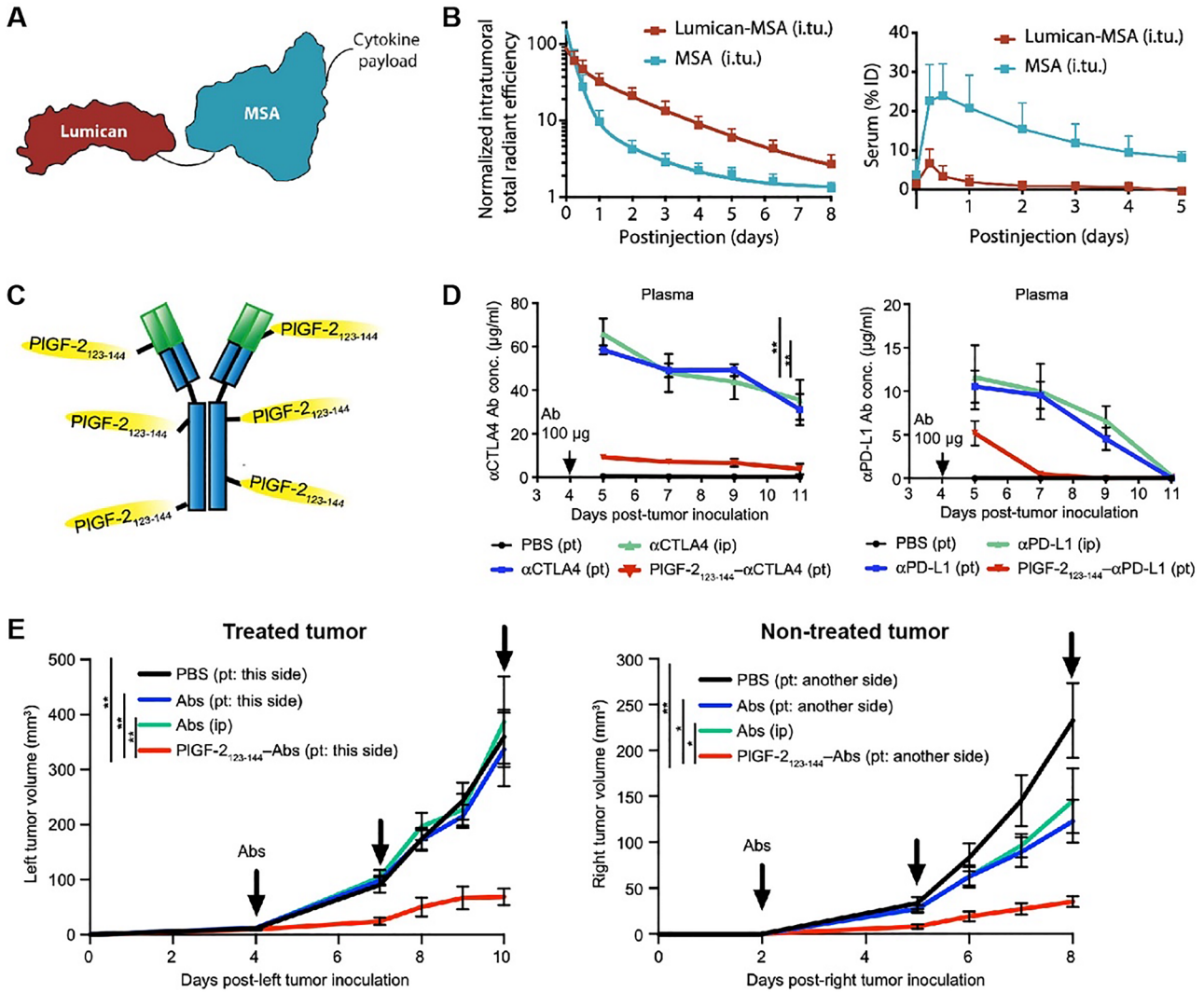

Fig. 4 Immunotherapeutic agents engineered to bind ECM demonstrate prolonged retention in tumor and lower systemic exposure following local administration. **A** Schematic of lumican-cytokine fusion protein that anchors to intratumoral collagen. MSA, mouse serum albumin. **B** Fluorescence-based quantification intratumoral (left) and serum (right) concentration profiles. Lumican-MSA fusion protein showed prolonged intratumoral retention (left) and lower degree of systemic dissemination when compared to MSA alone after intratumoral (i.tu.) administration into a preclinical apigmented B16F10 melanoma model. ID, injected dose. Adapted with permission from [69]. **C** Schematic of PlGF-$2_{123-144}$ peptide–conjugated Ab (PlGF-$2_{123-144}$–Ab) that binds to ECM proteins. **D** Plasma concentration profiles. PlGF-$2_{123-144}$ conjugation reduced systemic exposure of anti-CTLA-4 Ab (left) and anti-PD-L1 Ab (right) after peritumoral (pt) administration into a preclinical B16F10 melanoma model. The peritumoral injection of unmodified Abs demonstrated similar systemic concentration profile as compared to those injected intraperitoneally (ip). **E** Tumor growth curves of a preclinical B16F10 melanoma model. The peritumoral treatment of PlGF-$2_{123-144}$–anti-CTLA4 Ab plus PlGF-$2_{123-144}$–anti-PD-L1 Ab induced systemic anti-cancer immunity, evidenced by the significant tumor growth delay of untreated remote tumors, similar to the treated tumors in preclinical B16F10 melanoma model. Arrows depict the day of injection. Adapted with permission from [16]

phase 1 clinical study examining a combination regimen of anti-ANG2/VEGF bispecific Ab (i.e., vanucizumab) and anti-PD-L1 Ab (i.e., atezolizumab) failed to show improved therapeutic outcome compared to anti-PD-L1 Ab monotherapy in patients with recurrent ovarian cancer (NCT01688206; www.clinicaltrials.gov), leaving doubts on the clinical relevance of ANG2/VEGF dual inhibition to improving immunotherapy [99].

More recently, a distinct approach of normalizing tumor vasculatures by functionally reprogramming the tumor endothelium has been reported [100]. Besides the vascular abnormalities driven by angiogenic factors, tumor endothelial cells undergo genetic reprogramming, such as mesenchymal-like transcriptional activation, to mediate formation of abnormal vasculatures [101, 102]. Ma et al. identified p21-activated kinase 4 (PAK4) as the regulator of

Table 1 FDA-approved combination regimens of immunotherapy and antiangiogenic therapy

Immunotherapy	Modulator	Cancer type	Approval	Pivotal trial name (NCT number)
Nivolumab	Cabozantinib	Advanced renal cell carcinoma	2021	CHECKMATE-9ER (NCT03141177)
Atezolizumab	Bevacizumab	Unresectable or metastatic hepatocellular carcinoma	2020	IMbrave150 (NCT03434379)
	Bevacizumab + paclitaxel + carboplatin	Metastatic nonsquamous non-small cell lung cancer with no EGFR or ALK genomic tumor aberrations	2018	IMpower150 (NCT02366143)
Pembrolizumab	Axitinib	Advanced renal cell carcinoma	2019	KEYNOTE-426 (NCT02853331)
	Lenvatinib	Advanced endometrial carcinoma	2019	KEYNOTE-146/Study 111 (NCT02501096)
Avelumab	Axitinib	Advanced renal cell carcinoma	2019	JAVELIN Renal 101 (NCT02684006)

the mesenchymal-like transcriptional activation in human glioblastoma-derived endothelial cells by utilizing kinome-wide genetic screening (Fig. 5) [100]. In this study, genetic ablation or pharmacological inhibition of PAK4 normalized the aberrant vasculatures and restored the expression of cell adhesion proteins, including VCAM-1 and ICAM-1, on tumor endothelium to enhance intratumoral T cell infiltration and potency of CAR-T therapy. To this end, strategies to inhibit PAK4 could serve as an alternative or a complement to anti-VEGF therapy to normalize tumor vasculatures and ultimately improve the therapeutic outcomes of immunotherapy.

ECM remodeling

As introduced earlier, collagen and HA are most abundant and prominent macromolecular components of tumor ECM and thus have been most widely explored as primary targets to compromise the barrier properties of this stromal barrier. We here introduce recent strategies implemented and preclinically validated to enhance tumoral delivery of immunotherapeutic agents and/or therapeutic efficacy of immunotherapy, by modulating collagen or HA.

Collagen modulation

Enzymatic degradation of collagen by collagenase would perhaps be the most straightforward collagen-modulating means to reduce density and stiffness of tumor ECM. Indeed, numerous studies demonstrated improved tumoral delivery and intratumoral distribution of therapies, including therapeutic viruses and monoclonal Abs, by intratumoral or systemic collagenase treatment [103–106]. Of note, most of the relevant studies employed local administration, presumably due to its relatively short systemic half-life (< 30 min) [107], while delivery systems, such as liposomes and polymer-based nanoparticles, have been used to prevent premature degradation or clearance of collagenase prior to reaching the tumor tissue [107–109]. Salmon et al. showed ex vivo that mobility of T cells and their association with cancer cells were improved by collagenase in clinical samples of human lung cancer [48], suggesting that collagenase loosened the highly dense ECM in tumors and would likely improve the tumor response to immunotherapy. Alternatively, physical methods, such as focused ultrasound (FUS), can be used to transiently perturb the integrity of collagen matrix within tumor stroma. FUS, via deposition of high-density acoustic energy, has been shown to enhance tumor penetration and distribution of therapeutic agents, including Abs and nanoparticles, by disrupting collagen network and remodeling ECM within the tumor tissues [110–112]. Moreover, FUS has been shown to promote tumor infiltration of immune cells [113, 114], which may be at least partially attributed to the modulation of collagen and other macromolecular ECM components in the tumor tissue.

The stromal collagen continuously undergoes enzymatic and non-enzymatic turnover [46]. Therefore, besides breaking down the readily existing collagen, tumor ECM can be loosened by inhibiting de novo synthesis and/or stabilization (e.g., crosslinking) of collagen. It was previously shown that TGFβ blockade by anti-TGFβ Ab, an angiotensin receptor blocker (i.e., losartan), or anti-fibrotic agents (e.g., tranilast, pirfenidone) decreased ECM collagen content to subsequently improve drug perfusion and distribution in tumor tissues of preclinical models of breast, pancreatic, and brain cancers [115–119]. Likewise, Ab-mediated TGFβ blockade significantly increased the tumor infiltration of cytotoxic T cells and improved the outcome of anti-PD-L1 therapy by reprogramming the fibroblasts to reduce collagen synthesis (Fig. 6) [57]. Ongoing clinical trials of TGFβ blockers, including TKIs and Abs, in conjunction with immune checkpoint blockade clearly show clinical interests of TGFβ blockade-mediated stromal remodeling for improving the outcomes of immunotherapy (Table 3) [120]. As introduced earlier, upregulation of LOX in tumor ECM intensifies the crosslinking of collagen fibrils [52]. Thus, inhibition of the LOX family members, including LOX and LOXL2, has been considered a promising strategy to normalize the pathologically stiffened tumor

Table 2 Ongoing clinical trials investigating the combination of immunotherapy and antiangiogenic therapy

Immunotherapy	Modulator	Cancer type(s)	Highest status	NCT number(s)
Atezolizumab	Bevacizumab	Hepatocellular carcinoma, breast cancer, renal cell carcinoma, non-small-cell lung cancer, urothelial carcinoma, thyroid gland anaplastic carcinoma, pancreatic adenocarcinoma, ovarian cancer, fallopian tube cancer, peritoneal neoplasms, colorectal adenocarcinoma, intracranial melanoma, endometrial cancer, cervical adenocarcinoma, biliary tract cancer, melanoma	Phase 3	NCT04732286, NCT04732598, NCT02420821, NCT02366143, NCT03272217, NCT03181100, NCT03074513, NCT03394885, NCT03038100, NCT02873195, NCT03175432, NCT03526432, NCT02921269, NCT04677504, NCT04107168
	Cabozantinib	Neuroendocrine tumor, anaplastic thyroid cancer, adenocarcinoma, pheochromocytoma, paraganglioma, hepatocellular carcinoma, bladder cancer, non-small-cell lung cancer, renal cell carcinoma, prostate adenocarcinoma	Phase 3	NCT04400474, NCT04289779, NCT04471428, NCT04338269, NCT03170960, NCT03755791, NCT04446117, NCT02925234
	Ramucirumab	Non-small cell lung cancer (NSCLC)	Phase 2	NCT03689855
	Vorolanib	Extensive-stage small cell lung cancer	Phase 2	NCT04373369
	Derazantinib	Urothelial carcinoma, gastric adenocarcinoma	Phase 2	NCT04045613, NCT04604132
	Regorafenib	Colorectal cancer	Phase 2	NCT03555149
	XL092	Renal cell carcinoma, breast carcinoma, prostate cancer	Phase 1	NCT03845166
Pembrolizumab	Lenvatinib	Non-small cell lung cancer, renal cell carcinoma, hepatocellular carcinoma, glioblastoma, gastric cancer, ovarian cancer, breast cancer, melanoma, cholangiocarcinoma, urothelial carcinoma, head and neck carcinoma, thyroid gland carcinoma, neuroendocrine carcinoma, endometrial neoplasms	Phase 3	NCT04676412, NCT04704219, NCT03713593, NCT03797326, NCT04662710, NCT04519151, NCT04427293, NCT03820986, NCT03895970, NCT03898180, NCT04199104, NCT04171622, NCT03290079, NCT03884101
	Erdafitinib	Urothelial cancer	Phase 3	NCT03390504
	Sunitinib	Thymic carcinoma, renal cell carcinoma	Phase 3	NCT03463460, NCT03260894
	Axitinib	Renal cell carcinoma, soft tissue sarcomas	Phase 3	NCT02853331, NCT02636725
	Pazopanib	Renal cell carcinoma	Phase 3	NCT03260894
	Cabozantinib	Renal cell carcinoma, gastric adenocarcinoma, melanoma, cervical cancer, urothelial carcinoma, bladder cancer, head and neck carcinoma, oral carcinoma	Phase 2	NCT03149822, NCT04164979, NCT03957551, NCT04230954, NCT03534804, NCT03468218
	Ramucirumab	Non-small cell lung cancer, gastric adenocarcinoma, biliary tract cancer, head and neck carcinoma, transitional cell carcinoma	Phase 2	NCT04040361, NCT04632459, NCT02443324, NCT03650764, NCT04179110
	Pemigatinib	Lung cancer, gastric cancer, urothelial cancer, endometrial cancer, myeloma, cholangiocarcinoma, urothelial carcinoma	Phase 2	NCT04003610, NCT02393248
	Bevacizumab	Melanoma, non-small cell lung cancer, colorectal cancer, glioblastoma, ovarian carcinoma, fallopian tube adenocarcinoma, cervical cancer	Phase 2	NCT02681549, NCT02681549, NCT02563002, NCT03661723, NCT04361370, NCT02853318
	Regorafenib	Hepatocellular carcinoma, colorectal cancer, solid tumors	Phase 2	NCT04696055, NCT0365764, NCT02693535
	Futibatinib	Urothelial cancer	Phase 2	NCT04601857
	Sitravatinib	Urothelial carcinoma	Phase 2	NCT03606174
	Ziv-Aflibercept	Ovarian cancer, colorectal cancer, melanoma, renal cell carcinoma	Phase 1	NCT02298959
Nivolumab	Cabozantinib	Renal cell carcinoma, bone cancer, lymphoma, visceral cancer, hepatocellular carcinoma, non-small cell lung carcinoma, carcinoid cancer, breast cancer, thyroid gland carcinoma, metastatic soft-tissue sarcoma, prostate cancer, neuroendocrine carcinoma	Phase 3	NCT03793166, NCT03937219, NCT03878524, NCT01658878, NCT03899428, NCT04310007, NCT04197310, NCT03316586, NCT04551430, NCT03866382
	Sitravatinib	Renal cell carcinoma, urothelial carcinoma, non-small cell lung cancer	Phase 3	NCT03606174, NCT03680521, NCT03606174
	Sunitinib	Renal cell carcinoma	Phase 3	NCT02231749, NCT03729245, NCT03141177

Table 2 (continued)

Immunotherapy	Modulator	Cancer type(s)	Highest status	NCT number(s)
	Sorafenib	Hepatocellular carcinoma	Phase 3	NCT02576509, NCT04039607
	Bevacizumab	Renal cell carcinoma, kidney carcinoma, colorectal cancer, non-small cell lung cancer, glioblastoma, peritoneal cancer, ovarian cancer, fallopian tube cancer	Phase 2	NCT02210117, NCT03872947, NCT04008030, NCT02574078, NCT03452579, NCT02873962
	X-82	Thymic carcinoma, non-small cell lung cancer, refractory thoracic tumors, small-cell lung cancer	Phase 2	NCT03583086
	Regorafenib	Colorectal cancer, breast cancer, esophagogastric cancer	Phase 2	NCT04030260, NCT03878524, NCT04757363
	Lucitanib	Solid tumor, gynecologic cancer	Phase 2	NCT04042116
	Lenvatinib	Hepatocellular carcinoma	Phase 2	NCT03841201
	Axitinib	Renal cell carcinoma	Phase 2	NCT04540705
	Ramucirumab	Non-small cell lung cancer	Phase 2	NCT03527108
	Nintedanib	Non-small cell lung cancer	Phase 2	NCT04046614
	CEP-11981	Prostate carcinoma	Phase 2	NCT04159896
	Pazopanib	Renal cell carcinoma	Phase 2	NCT02959554
Ipilimumab	Cabozantinib	Non-small cell lung cancer, bladder cell adenocarcinoma, renal cell carcinoma, urethral carcinoma, prostate cancer, penile cancer, bone cancer, lymphoma, visceral cancer, melanoma, neuroendocrine carcinoma, thyroid gland carcinoma, soft-tissue sarcoma	Phase 3	NCT03468985, NCT03866382, NCT03793166, NCT03878524, NCT04091750, NCT04079712, NCT03914300, NCT04551430, NCT03937219, NCT04413123
	Sunitinib	Renal cell carcinoma	Phase 3	NCT02231749
	Lenvatinib	Renal cell carcinoma, hepatocellular carcinoma	Phase 3	NCT04039607, NCT04203901
	Bevacizumab	Melanoma, glioma	Phase 2	NCT01743157
	Nintedanib	Non-small cell lung cancer	Phase 2	NCT03377023
	Regorafenib	Colorectal carcinoma	Phase 1	NCT03377361, NCT04362839
	Sitravatinib	Renal cell carcinoma	Phase 1	NCT04518046
Durvalumab	Bevacizumab	Hepatocellular carcinoma, colorectal adenocarcinoma, pancreatic adenocarcinoma, glioblastoma, ovarian cancer	Phase 3	NCT03847428, NCT03376659, NCT03878524, NCT02336165, NCT04015739
	Cabozantinib	Gastric cancer, esophageal adenocarcinoma, hepatocellular carcinoma, colorectal cancer	Phase 2	NCT03824691, NCT03539822, NCT03899428, NCT03878524
	Bevacizumab	Breast cancer, gastric cancers, small cell lung cancer, ovarian cancer	Phase 2	NCT02734004, NCT04517526, NCT03737643
	Cediranib	Colorectal cancer, breast cancer	Phase 2	NCT02484404, NCT03851614
	Tivozanib	Hepatocellular carcinoma	Phase 2	NCT03970616
	Ramucirumab	Gastric cancer, non-small cell lung cancer, hepatocellular carcinoma	Phase 1	NCT02572687
Avelumab	Axitinib	Non-small cell lung cancer, urothelial cancer, adenoid cystic carcinoma, endometrial cancer, renal cell cancer	Phase 3	NCT03386929, NCT03472560, NCT03990571, NCT02912572, NCT02684006
	Bevacizumab	Pancreatic cancer, breast cancer, squamous cell carcinoma	Phase 2	NCT03329248, NCT03387085, NCT03387111
	Cabozantinib	Renal cell carcinoma	Phase 1	NCT03200587

ECM [46]. Indeed, the LOX blockade by anti-LOX Ab [121] or β-aminopropionitrile (BAPN) [122, 123], a small molecule-based pan-LOX family inhibitor, was shown to remodel tumor ECM and improve tumoral drug perfusion in several preclinical models, presumably by reducing the tumor IFP. In a preclinical lung cancer model, LOXL2 knockdown or ellagic acid-mediated blockade reduced the collagen deposition to increase cytotoxic T cell infiltration and decrease number of exhausted T cells, thereby sensitizing the tumor to PD-1/PD-L1 blockade [58].

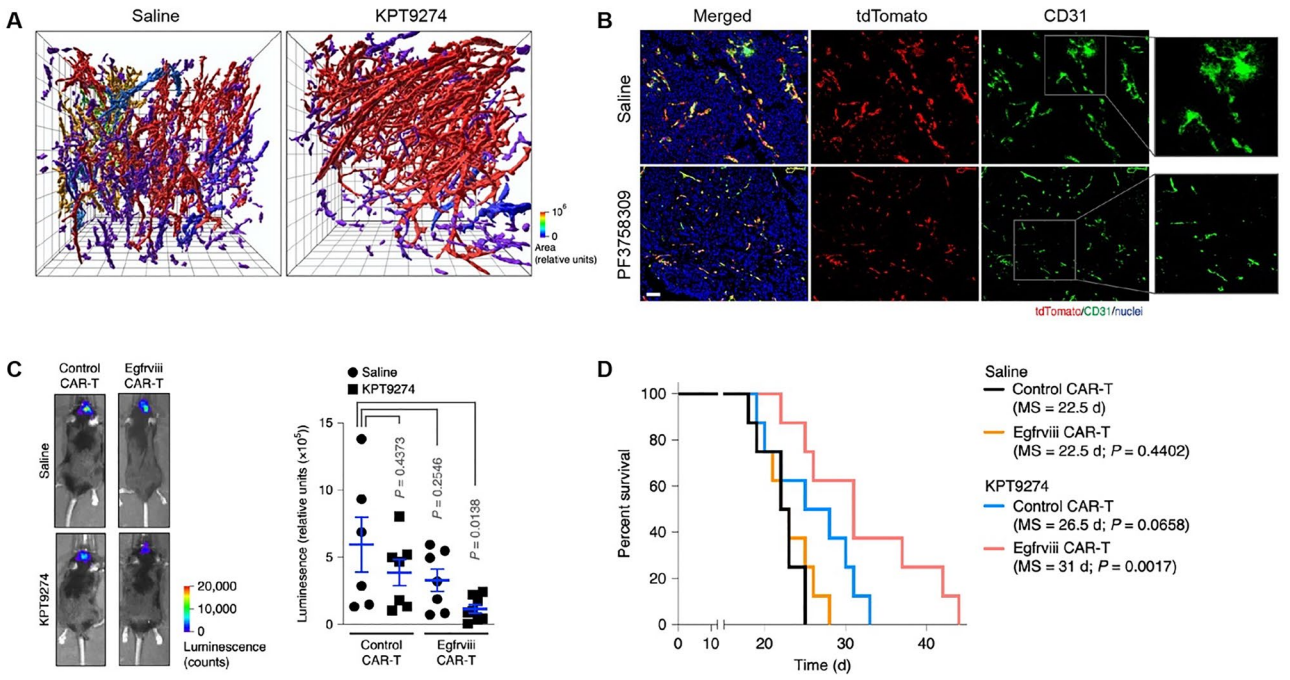

Fig. 5 Vascular normalization induced by the pharmacological inhibition of PAK4 inhibition enhances potency of CAR-T therapy. **A** 3D reconstructed tumor vasculatures in a preclinical glioblastoma multiform (GBM) model induced in *Rosa-LSL-tdTomato;Cdh5-Cre*^{ETR2} mice, in which tdTomato was specifically expressed in endothelial cells (EC). A PAK4 inhibitor, KPT9274, normalized the tumor vasculature of the GBM. Each grid, 100 μm. **B** Immunohistochemistry of the GBM tumor sections stained with anti-tdTomato (red) and anti-CD31 (green) Abs. Treatment with a PAK4 inhibitor, PF3758309, led to reduced EC abnormalities in the GBM model. Scale bar, 100 μm. **C** PAK4 inhibition using KPT9274 markedly sensitized EGFRvIII-overexpressing GL261 GBM model to CAR-T therapy. Tumor volumes were analyzed by bioluminescence imaging. Left, representative images; Right, quantitative results. **D** Survival of the GBM model. The combination KPT9274 and CAR-T therapy significantly increased the survival time of the animals. Adapted with permission from [100]

Hyaluronan breakdown

A number of studies have explored the plausibility of hyaluronidase-mediated HA breakdown as a method to improve intratumoral drug delivery. Multisite polyethylene glycol (PEG)-conjugated human recombinant PH20 hyaluronidase (PEGPH20 or pegvorhyaluronidase alfa) has been the most extensively investigated hyaluronidase evaluated for the ECM remodeling. In preclinical models of HA-rich prostate and pancreatic cancers, PEGPH20 was shown to decrease the HA content and IFP and to increase the blood perfusion, leading to enhanced anticancer effect of systemically administered chemotherapy [124]. More recently, PEGPH20-mediated stromal remodeling was shown to increase the tumor infiltration of effector T cells when combined with a cancer vaccine (i.e., GVAX), which resulted in improved survival of a mouse model of pancreatic ductal adenocarcinoma (PDAC) [18]. However, PEGPH20 failed to enhance the efficacy of combination of albumin bound-paclitaxel (i.e., Abraxane®) and gemcitabine in HA-high metastatic PDAC patients in a recently completed phase 3 clinical trial [125]. Nevertheless, clinical trials investigating the combination of PEGPH20 and different ICIs (e.g., atezolizumab and pembrolizumab) are in progress on patients with advanced gastric cancer, metastatic PDAC, and non-small cell lung cancer (NCT03281369, NCT03193190, NCT03634332, NCT02563548; www.clinicaltrials.gov) (Table 3). These clinical studies will reveal whether the hyaluronidase-mediated stromal remodeling is capable of sensitizing tumors to immunotherapy.

Other forms of hyaluronidase have also been reported to improve immunotherapy in preclinical models. Two independent groups demonstrated that non-PEGylated human recombinant hyaluronidase (PH20) enzyme anchored on extracellular vesicle (EV) membrane (PH20-EVs) effectively degraded HA and increased cytotoxic T cell infiltration into tumor tissues [126, 127]. In particular, Hong et al. showed that PH20-EVs, in combination with PD-1/PD-L1 axis blockade, evoked a strong and durable tumor-specific T cell immunity in preclinical breast cancer models [126]. Remarkably, PH20-EV exhibited three times greater enzymatic activity than a truncated and soluble form of PH20, presumably due to the native PH20 structure preserved on the EV surface [128]. Similar therapeutic outcomes were

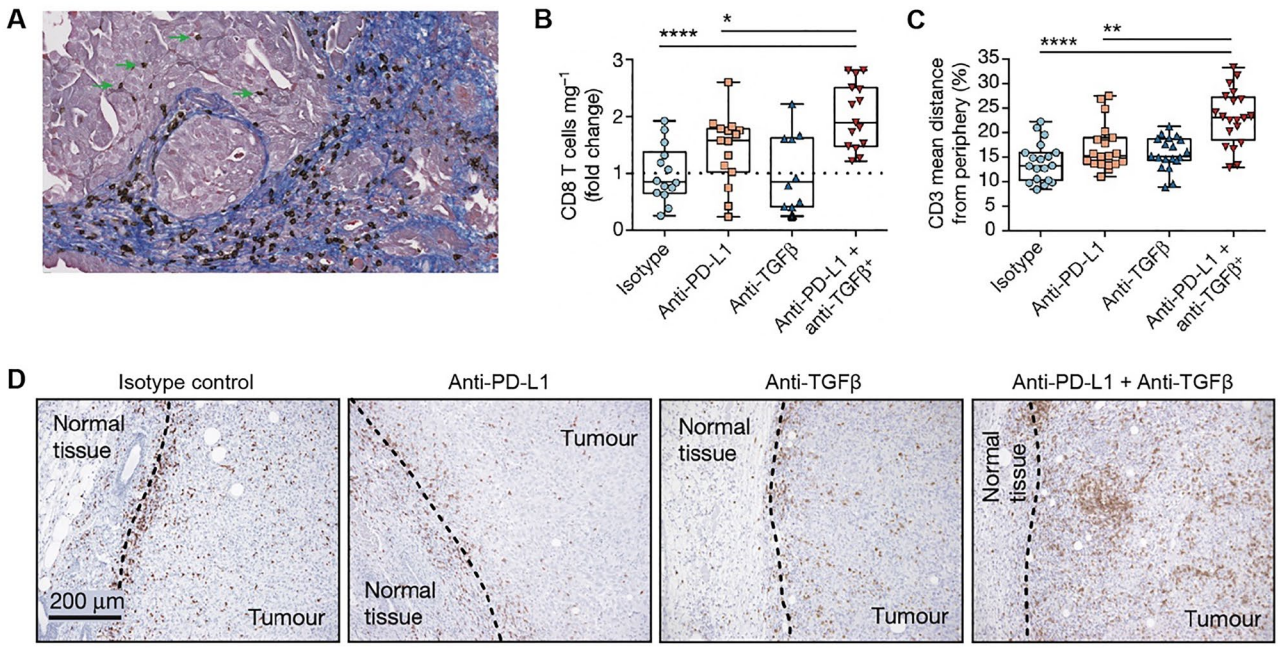

Fig. 6 TGFβ blockade increased T cell infiltration into tumor. **A** Combined CD8 immunohistochemistry and trichrome staining of tumor samples from a metastatic urothelial cancer patient. $CD8^+$ T cells (brown) were trapped within collagenous stroma (blue). Green arrows indicate rare $CD8^+$ T cells infiltrated into the tumor. **B** Quantification of CD8 T cells in the EMT6 murine mammary tumor analyzed by flow cytometry. anti-PD-L1 Ab in combination with anti-TGFβ Ab significantly increased the number of tumor-infiltrating $CD8^+$ T cells. **C** Immunohistochemistry-based quantification of tumor-infiltrating lymphocyte localization. Anti-PD-L1 Ab stimulated more deeper tumor infiltration of T cells only when used in combination with anti-TGFβ Ab. **D** Immunohistochemistry of CD3 (brown) in tumor periphery. T cells were primarily located in the interface of normal and tumor tissues in control, anti-PD-L1 Ab-treated, and anti-TGFβ Ab-treated groups, but the combined treatment with anti-PD-L1 Ab and anti-TGFβ Ab induced significant number of T cells to infiltrate into the tumor. Scale bar, 200 µm. Adapted with permission from [57]

Table 3 Ongoing clinical trials investigating the combination of immunotherapy and ECM-modulating agents

Immunotherapy	Modulator	Cancer types	Highest status	NCT number(s)
	TGFβ blockers			
Pembrolizumab	M7824[a]	Non-small cell lung cancer, thymic cancer, bladder cancer, laryngeal papilloma, rectal adenocarcinoma, colon adenocarcinoma, cervical cancer, kaposi sarcoma, urothelial cancer, bladder cancer, pancreatic cancer, breast cancer, prostate cancer, head and neck cancer, biliary tract cancer, colorectal cancers	Phase 3	NCT03631706, NCT04417660, NCT03833661, NCT03707587, NCT03436563, NCT04432597, NCT04303117, NCT04501094, NCT04235777, NCT04327986, NCT03620201, NCT04633252, NCT04247282, NCT04066491, NCT04491955
Durvalumab	Vactosertib	Urothelial carcinoma, non-small-cell lung cancer	Phase 2	NCT04064190, NCT04515979
	M7824[a]	Non-small cell lung cancer	Phase 2	NCT03840902
Spartalizumab	NIS793[b]	Pancreatic adenocarcinoma	Phase 2	NCT04390763
Cemiplimab	SAR-439459[b]	Solid neoplasm	Phase 1	NCT04729725, NCT03192345
	Hyaluronidase			
Atezolizumab	PEGPH20	Gastric adenocarcinoma, pancreatic adenocarcinoma	Phase 2	NCT03281369, NCT03193190
Pembrolizumab	PEGPH20	Non-small cell lung cancer, gastric cancer, pancreatic cancer	Phase 2	NCT02563548, NCT03634332
Nivolumab	rHuPH20	Neoplasmas by site	Phase 2	NCT03656718
BMS-986258	rHuPH20	Advanced cancer	Phase 2	NCT03446040
Durvalumab	VCN-01[c]	Head and neck carcinoma	Phase 1	NCT03799744

[a]PD-L1/TGFβ bispecific Ab

[b]anti-TGFβ Ab

[c]PH20-expressing oncolytic virus

accomplished by transgene expression of PH20 by intratumoral delivery of a polymer nanoparticle or adenovirus carrying SPAM1 gene that encodes PH20 [129, 130].

Conclusion

Cancer immunotherapy has revolutionized the cancer treatment since the revelation of groundbreaking clinical results with ICIs, but only a moderate fraction of patients respond to the treatment, necessitating a means to expand the responsive patient population [8, 9]. Conventional cancer therapies primarily focus on enhancing the ability to tackle individual cancer cells in a comprehensive and ideally selective manner and/or to reduce dose and dosing frequency to improve benefit-to-risk ratios, by using rationally engineered delivery systems. While these approaches are relevant to a few immunotherapeutic modalities, immunotherapy universally requires stimulation of tumor-specific immunity, trafficking of immune cells into the tumor tissues and modulation of immunosuppressive TME. Here, we have overviewed the pathologically altered stromal barriers that restrict the access of immunotherapeutic agents and immune cells to cancer cells embedded in the TME and strategies to bypass or normalize those barriers to improve the outcomes of immunotherapy.

Based on the optimistic proof-of-concept established by preclinical studies, we now have combination regimens of stroma-modulating agents and immunotherapy approved for clinical use and several under clinical investigation. However, as we have learned from long history of therapeutic development, clinical-to-preclinical correlation is not readily assumed due to the anatomical, physiological and metabolic variations among species. To this end, clinical studies should be carefully designed or adjusted to validate the clinical relevance of preclinically established or benchmarked strategies. Those include selection of dose, dosing form and schedule for stroma-modulating agents to maximize the effectiveness of immunotherapy while securing a desired therapeutic index. Perhaps, implementation of imaging techniques or other postresection measures that allow the monitoring of changes in tumor stroma or blood perfusion in the clinical trial design may provide critical information for making necessary adjustment for subsequent trials and patient use. We also note that rational combination of multiple strategies described above should be considered depending on the type and stage of target cancers. As introduced earlier, superior anti-cancer systemic immunity can be achieved by local over systemic administration of certain immunotherapeutic modalities. Therefore, combination of localized immunotherapy and systemic treatment with stroma-modulating agents may create synergy by facilitating the infiltration of locally primed immune cells into distal tumor tissues, including metastatic regions, to maximize the therapeutic outcomes. Advanced 3D in vitro models (e.g., organ-on-a-chip) based on human cellular components may promote the discovery or development of novel stroma-modulating agents or strategies and serve as a human-like testbed to reliably predict their performances in conjunction with immunotherapeutic modalities. Several tumor-on-a-chip models where human cancer and immune cells are embedded in collagen matrix have been developed and utilized to investigate T cell behaviors in TME [131, 132]. Likewise, vessel-on-a-chip models may provide an avenue to explore vascular modulatory strategies and/or their impacts on immune cell trafficking to, and behaviors in, tumors [133]. Despite the relatively short history, preclinical and clinical data regarding the performances of various immunotherapeutic strategies are explosively accumulating. Careful interpretation of the collected information and timely implementation of necessary adjustments would speed up a broader use of this state-of-the-art therapeutic modality.

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Declarations

Consent for publication Authors consent.

Conflict of interest The authors declare no competing interests.

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