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Review

The evolving tumor microenvironment: From cancer initiation to metastatic outgrowth

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SUMMARY

Cancers represent complex ecosystems comprising tumor cells and a multitude of non-cancerous cells, embedded in an altered extracellular matrix. The tumor microenvironment (TME) includes diverse immune cell types, cancer-associated fibroblasts, endothelial cells, pericytes, and various additional tissue-resident cell types. These host cells were once considered bystanders of tumorigenesis but are now known to play critical roles in the pathogenesis of cancer. The cellular composition and functional state of the TME can differ extensively depending on the organ in which the tumor arises, the intrinsic features of cancer cells, the tumor stage, and patient characteristics. Here, we review the importance of the TME in each stage of cancer progression, from tumor initiation, progression, invasion, and intravasation to metastatic dissemination and outgrowth. Understanding the complex interplay between tumor cell-intrinsic, cell-extrinsic, and systemic mediators of disease progression is critical for the rational development of effective anti-cancer treatments.

INTRODUCTION

Our understanding of cancer has fundamentally evolved over the last decades. We now recognize that cancer is not simply a genetic disease but rather a complex ecosystem, involving a wide range of non-cancerous cells and their myriad interactions within the tumor. We appreciate that genetic alterations are necessary but not sufficient for cancer initiation and progression. The intricate complexity of cancer becomes evident upon microscopic examination of solid tumors, revealing that the tumor microenvironment (TME) is a highly structured ecosystem containing cancer cells surrounded by diverse non-malignant cell types, collectively embedded in an altered, vascularized extracellular matrix (Figure 1). The TME includes a rich diversity of immune cells, cancer-associated fibroblasts (CAFs), endothelial cells (ECs), pericytes, and other cell types that vary by tissue—such as adipocytes and neurons (Table 1). Initially, these host cells were viewed as bystanders of tumorigenesis. However, as a result of mechanistic studies, including in preclinical tumor models, TME cells and their secreted molecules are now considered to play critical roles in the pathogenesis of cancer and thus represent attractive therapeutic targets.[1] Depending on the organ in which the tumor arises, intrinsic features of cancer cells, the tumor stage, and patient characteristics, the cellular composition and functional state of the TME will differ, and various cells in the TME can be either tumor suppressive or tumor supporting. Before discussing the often intertwined and opposing processes that are characteristic of the TME, we will outline some fundamental principles underlying the formation and dynamic evolution of the TME during the different stages of tumorigenesis (Figure 1), which include heterotypic cell-cell communication and the importance of context dependency.

PRINCIPLES UNDERLYING THE FORMATION OF THE TME

Reciprocal communication between cancer cells and host cells

Cancer cells orchestrate a tumor-supportive environment by recruiting and reprogramming non-cancerous host cells and by remodeling the vasculature and extracellular matrix (ECM). This dynamic process depends on heterotypic interactions between cancer cells and resident or recruited non-cancerous cells of the TME. Recent advances in computational analysis and modeling using single-cell transcriptomic data, bulk tumor expression profiles, and spatial transcriptomics[2–4] have revealed a diversity of intercellular signaling networks in the TME. These atlases serve as powerful hypothesis-generating datasets to guide subsequent functional studies, which are revealing how complex intercellular interactions are integrated, leading to the formation and evolution of the TME. There are multiple mechanisms by which this intercellular dialogue is regulated, including through cell-cell contact and paracrine signaling.[5]

Contact-dependent communication is mediated by adhesion molecules, including integrins, cadherins, selectins, and immunoglobulin superfamily members, and also via gap junctions and tunneling nanotubes. As an example, aberrant glycan sialylation on cancer cells regulates numerous interactions, including with Siglec-expressing immune cells, promoting immune evasion and tumor progression[6] (Figure 2). Another well-known

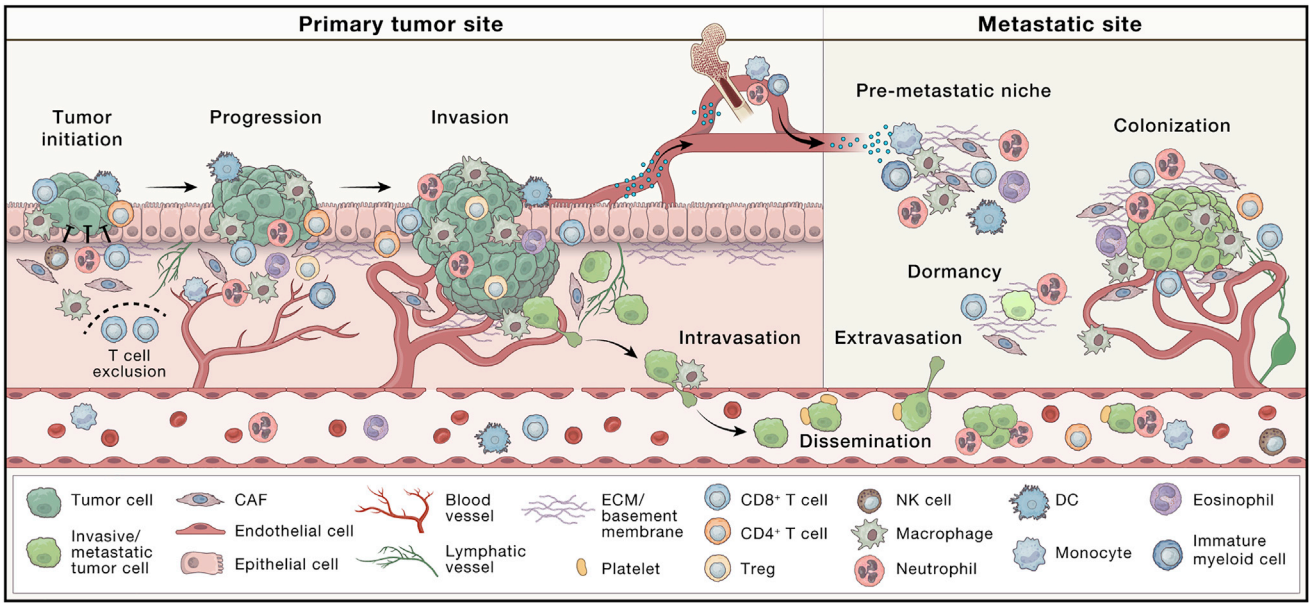


Figure 1. Microenvironmental regulation of primary tumor progression and metastasis

The evolving tumor microenvironment (TME) during all stages of cancer progression is depicted with key representative cell types shown. The TME includes diverse immune cells, cancer-associated fibroblasts (CAFs), endothelial cells, and the extracellular matrix (ECM), among others. These components may vary by tissue type and co-evolve with the tumor as it progresses. The normal tissue microenvironment can constrain cancer outgrowth through the suppressive functions of immune cells, fibroblasts, and the ECM. However, for cancer to advance, it must evade these functions and instead influence cells in the TME to become tumor promoting, resulting in increased proliferation, invasion, and intravasation at the primary site. Cells and factors of the TME also play a vital role in preparing the premetastatic niche, regulating cancer cell survival in the circulation, and promoting extravasation. During the metastatic stages, the TME helps to control metastatic cell dormancy, emergence from this state, and subsequent metastatic outgrowth. Additional molecular details can be found in Figures 2 and 3 and Table 1.

example of contact-dependent intercellular signaling in the TME is the PD-L1/PD-1 pathway (Figure 2). Cancer cells, but also tumor-associated myeloid cells, frequently overexpress the immune checkpoint protein PD-L1, which engages with the PD-1 receptor on adaptive immune cells to suppress immune surveillance. This illustrates how molecular insights into TME communication can have critical therapeutic value, as inhibiting the PD-L1/PD-1 axis via immune checkpoint blockade (ICB) has become standard-of-care treatment for an increasing number of cancers.[7]

Besides direct cell-cell contact, paracrine signaling through the release of cytokines, chemokines, growth factors, and proteases is critical for intercellular communication within the TME. These molecules are secreted in response to cancer-intrinsic features and cellular stress, and they can be derived from multiple cell types in the TME and exert direct and indirect actions on target cells through binding to their receptors or by ECM remodeling. The release of extracellular vesicles (EVs), including exosomes, is another paracrine mechanism that can modify the local environment[8] and even have far-reaching effects beyond the primary tumor site[9] (Figure 1). For example, preclinical studies revealed that melanoma-derived EVs educate bone marrow (BM) progenitors toward a pro-vasculogenic phenotype, fostering metastasis formation.[10] Cancer-derived PD-L1-expressing EVs can suppress T cell activation in draining lymph nodes, thereby promoting tumor progression and ICB resistance.[11,12] The importance of altered metabolic demands and accompanying metabolite secretion in creating a supportive TME is also increasingly recognized.[13] Advances in single-cell metabolomics and spatial multi-omics,[14] combined with *in vivo* experimentation, are expected to improve our understanding of metabolite crosstalk and competition in the TME.

The ECM facilitates intercellular communication by acting as a reservoir for the sequestration of secreted molecules and as a substrate for cell adhesion and migration. ECM remodeling by proteases liberates the tethered molecules, thus generating localized high concentrations of released mediators. Moreover, cancer and TME cells directly contact the surrounding ECM via receptors, including integrins and CD44, contributing to the complex signaling networks functioning in cancer[15] (Figure 2).

Collectively, dialogue between neoplastic and non-neoplastic cells can occur at multiple levels and via diverse mechanisms. Throughout this review, representative examples illustrating intercellular TME crosstalk mechanisms along the tumorigenesis trajectory will be discussed.

Context matters

The composition and functional state of the TME can vary considerably between patients, even within the same cancer type.[16–18] Patient-specific factors including age, gender, lifestyle, body mass index, and the microbiome can impact the TME, as can the organ in which the tumor arises. Different organs have unique tissue-resident immune and stromal cell types, and the type of tissue can dictate the functional state of these cells. Illustrative examples include the functionally distinct macrophage populations found in different organs.[19] For instance, resident macrophages in the liver, termed Kupffer cells, differ in their transcriptome and physiological functions from the alveolar macrophages in the

Table 1. Major cellular and non-cellular components of the TME

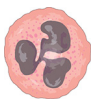
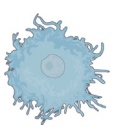
Cell type	Function in the TME	References
Immune cells		
Adaptive immune cells		
$CD8^+$ T cells 	$CD8^+$ T cells are powerful effector cells in the anti-tumor immune response. $CD8^+$ T cells can specifically recognize cancer cells by binding with their T cell receptor (TCR) to MHC-peptide complexes expressed by cancer cells. Upon TCR engagement, $CD8^+$ T cells destroy target cells through granzyme and perforin-mediated apoptosis or via FASL-FAS-mediated cell death. In tumors, many different $CD8^+$ T cell states can be found. Often, intratumoral $CD8^+$ T cells have a dysfunctional or exhausted phenotype. Immune checkpoint blockade aims to unleash $CD8^+$ T cell responses against cancer.	Philip and Schietinger,[61] van der Leun et al.[63]
$CD4^+$ T cells 	$CD4^+$ helper T cells influence a variety of other immune cells; in particular, they contribute to effective $CD8^+$ T cell responses. In cancer, $CD4^+$ T cells play a dual role. In particular, the Th1 subtype of $CD4^+$ T cells exerts anti-tumorigenic functions by providing help to anti-tumor cytotoxic $CD8^+$ cells and B cells and by direct killing of cancer cells via the production of interferon γ (IFNγ) and TNF-α. On the other hand, the Th2 subtype secretes anti-inflammatory mediators that exert pro-tumoral functions. There is growing evidence that $CD4^+$ T cells may play important roles in efficacy of immune checkpoint blockade (ICB).	DeNardo et al.,[49] Borst et al.[328]
Tregs 	Regulatory T cells (Tregs) are a highly immunosuppressive subset of $CD4^+$ T cells and function as gatekeepers of immune homeostasis. Tregs can be subdivided into thymic-derived and peripherally induced Tregs. In cancer, Tregs suppress effective anti-tumor immunity through different mechanisms. Their exact effector program is dependent on context-dependent cues. Treg-targeted cancer therapies are under investigation but are challenging given the key role of Tregs in preventing autoimmunity.	Togashi et al.[329]
B cells 	B lymphocytes are key mediators of humoral immunity. In cancer, B cells can exert anti-tumor effects through antibody-dependent cell cytotoxicity and complement activation. B cells can reside in intratumoral tertiary lymphoid structures (TLSs), where they contribute to T cell activation via antigen presentation. B cells can also support tumor growth by promoting inflammation and immunosuppression via secretion of anti-inflammatory and pro-angiogenic mediators, via immune-complexes, and via complement activation. A subpopulation of immunosuppressive B cells, Bregs, are involved in immunological tolerance.	Yuen et al.,[330] Laumont et al.[331]
Myeloid immune cells		
Macrophages 	Tumor-associated macrophages (TAMs) represent a highly plastic immune cell population with both pro- and anti-tumorigenic functions. TAMs comprise multiple subsets that arise from different origins (yolk sac-derived tissue-resident macrophages or bone marrow-derived infiltrating macrophages). Moreover, multiple TAM subsets co-exist in tumors. Pro-tumorigenic functions of TAMs include promoting angiogenesis, immunosuppression, metastasis formation, and therapy resistance, while TAMs can also counteract cancer progression by direct phagocytosis of cancer cells or activation of anti-tumor immune responses.	Guc and Pollard,[68] DeNardo and Ruffell[87]
Neutrophils 	Neutrophils are the most abundant immune cells in blood. Besides their recruitment to primary tumors, neutrophils frequently accumulate in blood and distant organs of tumor-bearing hosts. Depending on cues from the TME and their maturation status, neutrophils can exert anti- or pro-tumorigenic functions. Their systemic accumulation contributes to immunosuppression and extracellular matrix (ECM) remodeling in distant organs, which promote (pre)metastatic niche formation. Neutrophil diversity and plasticity in cancer is a topic of intense investigation.	Guc and Pollard,[68] Jaillon et al.[332]
Monocytes 	Monocytes circulate in the bloodstream and migrate into tissues where they differentiate into macrophages and dendritic cells (DCs). Several subtypes of monocytes exist, including classical, non-classical, and intermediate monocytes. Recent single-cell RNA sequencing studies demonstrated additional monocyte subpopulations. In cancer, monocytes exert pro- and anti-tumoral functions. Monocytes can produce tumoricidal mediators and stimulate natural killer (NK) cells. However, in the TME, they contribute to immunosuppression, ECM remodeling, angiogenesis, and cancer cell intravasation. Moreover, they differentiate into tumor-supporting TAMs.	Olingy et al.[333]
DCs 	DCs are a diverse group of antigen-presenting cells critical for initiating and regulating adaptive immune responses. By integrating information from the TME and relaying it to other immune cells, most notably T cells, DCs have the potential to shape anti-tumor immunity. However, tumors, in turn, employ a variety of strategies to limit and manipulate DC activity to evade immune control. Harnessing the power of DCs to improve immunotherapy response and the development of DC-based vaccines is an active field of cancer research.	Gerhard et al.,[334] Wculek et al.[335]

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Table 1. ***Continued***

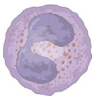
Cell type	Function in the TME	References
Mast cells 	Mast cells are granulocytes that mediate host defense and maintenance of homeostasis by swiftly degranulating histamines, cytokines, and chemokines. They are well known for their role in allergies and autoimmunity, but they can also infiltrate tumors. Mast cells exert both pro- and anti-tumorigenic activities depending on the microenvironmental stimuli. They can directly target tumor cells, but they mainly regulate the recruitment and activity of other immune populations and the endothelium.	Majorini et al.[336]
Eosinophils 	Eosinophils are known for their role in allergic diseases and parasite infections. More recently, their function in the TME is becoming apparent. Eosinophils have the capacity to directly kill tumor cells via the release of cytotoxic molecules, but eosinophils can also modulate the tumor vasculature and regulate the immune composition of the TME, and as such, they can have both pro- and anti-tumorigenic functions depending on the activation signals they receive. In addition, there is a growing interest in the role of eosinophils in promoting immunotherapy response.	Grisaru-Tal et al.,[337] Blomberg et al.[338]
Myeloid-derived suppressor cells 	Myeloid-derived suppressor cells (MDSCs) are a heterogeneous population of myeloid cells, consisting of (immature) monocytic and neutrophilic cells with potent immunosuppressive capacities. These cells expand in patients with cancer and mouse cancer tumor models, and their presence in the TME is associated with poor clinical outcome. MDSCs suppress T cells, NK cells, B cells, and DCs via paracrine and cell-cell contact mechanisms.	Veglia et al.[105]
Platelets 	Platelets, also named thrombocytes, are fragments of cytoplasm derived from megakaryocytes in the bone marrow. Platelets lack a nucleus, are abundant in blood, and are essential for blood clotting. Platelets promote tumor progression and metastasis through a range of different mechanisms. They bind to circulating tumor cells (CTCs), promoting CTC survival by shielding them from physical stress and immune attack. Platelets also release pro- and anti-angiogenic mediators, and they bind to endothelial cells, through which they modulate angiogenesis and vascular integrity. Platelets contribute to tumor-associated inflammation and immune evasion by activating myeloid cells.	Braun et al.[339]
Immune cells at the interface of adaptive and innate immunity		
NK cells 	NK cells are cytotoxic innate lymphoid cells. They recognize and kill stressed cells that lack MHC class I expression. Circulating and intratumoral NK cell levels are predictive for improved survival in patients with cancer. NK cells have potent anti-cancer abilities; however, progressing tumors evade elimination by NK cells via several mechanisms, such as the upregulation of inhibitory receptors that diminish NK cell cytotoxicity and the mobilization of immunosuppressive myeloid cells and Tregs. There is a growing interest in utilizing NK cells in the next generation of immunotherapeutic modalities either by engaging endogenous NK cells or by NK cell-based cellular therapies.	Chan and Ewald[340]
Invariant NK T cells 	Invariant NK T (iNKT) cells are CD1d-restricted lipid-specific T lymphocytes that bridge innate and adaptive immunity and can mediate a plethora of immune functions depending on tissue distribution. In several experimental models, iNKT cells exert cancer immunosurveillance through direct tumor cell killing or by orchestrating the activity of both pro- or anti-tumorigenic immune cells. Cancer-associated immunosuppression can skew iNKT cell activity toward more regulatory functions.	Fujii and Shimizu[341]
Gamma delta T cells 	Gamma delta ($\gamma\delta$) T cells form an unconventional T cell population expressing $\gamma\delta$ TCRs, but not $\alpha\beta$ TCRs, that recognize target antigens in an MHC-independent manner. Depending on the subset, $\gamma\delta$ T cells exert effector or regulatory functions. In cancer, $\gamma\delta$ T cells may promote disease progression by suppressing anti-tumor immune responses via the production of cytokines, including IL-17. Anti-tumor immunity can also be induced by $\gamma\delta$ T cells via direct cytotoxicity mediated by TCR- or NK-receptor interactions or production of effector molecules.	Silva-Santos et al.[342]
Innate-like lymphocytes 	Innate-like lymphocytes (ILCs) are a highly diverse group of immune cells that reside in tissues and that function at the intersection of adaptive and innate immunity. Besides NK cells, ILCs include ILC1s, ILC2s, and ILC3s. ILCs lack antigen-specific receptors and exert their immunoregulatory functions through secretion of a diverse array of cytokines and other inflammatory mediators. In cancer, ILCs play opposing roles. Depending on the tumor types and on cues from the TME, a different composition and activation phenotype of ILC subsets can be found in human tumors. Our understanding of the roles of the different ILCs subtypes in cancer is still very limited.	Bruchard and Ghiringhelli[343]

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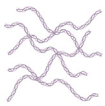
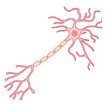
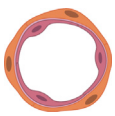
Table 1. *Continued*

Cell type	Function in the TME	References
Stromal cells and matrix		
Cancer-associated fibroblasts 	Cancer-associated fibroblasts (CAFs) are a key component of the tumor stroma. CAFs are composed of multiple functionally distinct subtypes that display an enormous plasticity. CAFs exert pleiotropic and opposing functions within the TME. CAFs synthesize and remodel the ECM, which changes the mechanical properties of the ECM and alters the behavior of cancer cells and immune cells. CAFs impact angiogenesis, and they have a strong immunomodulatory capacity and contribute to immune evasion of cancer.	Sahai et al.,[115] Kalluri[146]
ECM 	The ECM is a non-cellular structural component of the TME and comprises a network of fibrous proteins, such as collagens, glycoproteins, and proteoglycans. The ECM is a dynamic structure that is continuously remodeled by proteases produced by a variety of cells in the TME. The composition of matrisomal proteins in the ECM varies between tumor types and stages. The ECM facilitates intercellular communication in the TME by acting as a reservoir for the sequestration of secreted molecules and as a substrate for cell adhesion and migration. ECM remodeling by proteases liberates tethered molecules, thus generating localized high concentrations of released mediators. Cancer and TME cells directly contact the surrounding ECM via receptors including integrins and CD44, which form part of the diverse signaling networks that are activated in cancer.	Timaner et al.[344]
Adipocytes 	Adipocytes are present in numerous tissues, and they are specialized in storing energy as fat. Obesity is a key risk factor for multiple cancer types. Cancer-associated adipocytes are emerging key contributors to cancer types. They release free fatty acids, hormones, cytokines, adipokines, and growth factors that impact cancer cells as well as host cells in the TME. There is active interchange of metabolites and amino acids between adipocytes and cancer cells. Cancer-associated adipocytes have strong immunoregulatory capacity. They contribute to pro-tumorigenic low-grade chronic inflammation by producing chemoattractants for myeloid cells.	Quail and Dannenberg,[345] Pallegar and Christian[346]
Neurons and nerves 	Neurons and nerve fibers are present in the TME. Accumulating evidence demonstrates that neurons contribute to tumorigenesis. Perineural invasion (PNI) is a process by which cancer cells locally extend along nerves, which is observed in several solid cancer types and is associated with poor outcomes. Moreover, there is active crosstalk between neurons and cancer cells in the TME via reciprocal paracrine signaling. Neurons release neurotransmitters, neurotrophins, and chemokines, which stimulate cancer stemness, resistance to apoptosis, and enhanced proliferation. Moreover, nerves regulate inflammation and immune response in the TME, in the central nervous system, and in extracranial organs and is an active field of cancer research.	Wang et al.[347]
Vascular cells		
Blood vascular endothelial cells 	Endothelial cells (ECs) form a single cell layer that lines all blood vessels. Tumor ECs display a remarkable heterogeneity and plasticity, and they control the passage of proteins, cells, oxygen, and fluid into the surrounding tissue. ECs that line tumor blood vessels differ from normal ECs. Tumor ECs express lower levels of adhesion molecules, which causes an impaired barrier function, and they express increased levels of inhibitory immune checkpoint molecules, which contributes to immunosuppression.	De Palma et al.,[180] Amersfoort et al.[199]
Lymphatic ECs 	Lymphatic ECs (LECs) form the walls of lymphatic vessels. In the TME, lymphatic vessels provide a dissemination route for cancer cells in addition to blood vessels. LECs have recently also been recognized as direct regulators of anti-tumor immunity and immunotherapy response. LECs can present tumor antigens but also immune checkpoint molecules.	Ma et al.[348]
Pericytes 	Pericytes, also known as mural cells, surround blood vessels and are embedded in the basement membrane of vessels and adjacent to ECs. They support the maturation and permeability of the vasculature. In tumors, an impaired interaction between pericytes and ECs contributes to a leaky and dysfunctional tumor vasculature. Pericytes also interact with other stromal cells and cancer cells via paracrine mechanisms, resulting in modulation of the TME. In particular, there is growing interest in the immunomodulatory activity of pericytes.	Sun et al.[349]

lungs or the microglia in the brain. Technological advances have also revealed adaptations of cellular phenotype, activation state, and fate to the tissue context for other immune and stromal cell types, including neutrophils, fibroblasts, T cells, ECs, and adipocytes.[20–25] The organ-specific transcriptional programs of these cells may be instigated by local cues upon their arrival in tissues or may already be epigenetically imprinted during tissue development, as in the case of long-lived fibroblasts.[26]

There is increasing recognition that organ-specific imprinting of cells under homeostatic conditions can partially explain the diverse phenotypes and functions of these cells in different tumor types.[27,28] For example, in human and mouse non-small cell lung cancer (NSCLC), tissue-resident macrophages have a distinct temporal and spatial distribution and function versus monocyte-derived macrophages.[29] Along with differences in cellular programming among organs, the composition of matrisomal proteins in the ECM also varies.[30] Given the importance of the ECM in regulating cell phenotype and behavior, such tissue-dependent ECM properties contribute to generating organ-specific TMEs.

In addition to anatomical site-dependent mechanisms, arguably the most important regulator of the TME is the cancer cell itself. This is supported by the finding that gliomas, which arise in the brain, have different immune landscapes than brain metastases that originate from extracranial tumors.[18,31] It is becoming clear that cancer cell-intrinsic features, including altered (epi)genetics, metabolic reprogramming, and deregulated signaling, are key determinants of how tumors shape their microenvironment. Preclinical studies have shown that manipulating cancer cell-intrinsic wiring changes the secretome, alters cell surface receptors or ligands, impacts the cargo and abundance of EVs, and modifies nutrient usage, resulting in extensive changes in the tumor immune contexture and impaired ICB response.[32,33] For example, Myc activation in *KRasG12*-driven lung adenomas resulted in CCL9 and interleukin-23 (IL-23) secretion by epithelial cells. This created an inflammatory, angiogenic, and immunosuppressed TME, which enhanced tumorigenesis.[34] In melanoma, tumor-intrinsic β-catenin signaling induced expression of the transcriptional repressor ATF3, which inhibited CCL4 secretion. This impaired $CD103^+$ dendritic cell (DC) recruitment, leading to T cell exclusion and ICB resistance.[35] Finally, mutation or loss of *Trp53* in cancer cells led to a myeloid-rich immunosuppressed environment, which promoted tumor progression,[36,37] metastasis,[38] and ICB resistance.[39] With rapidly advancing high-resolution profiling technologies, many more associations between cancer-intrinsic features and the TME will be revealed, which—upon experimental proof of causality—may set the stage for the rational design of TME-targeted strategies tailored to individual tumors.

TUMOR INITIATION: DISRUPTION OF TISSUE HOMEOSTASIS

There are multiple bottlenecks that malignant cells must overcome to successfully form a tumor, many of which depend on subverting normalizing cues from the surrounding tissue, followed by hijacking microenvironmental processes to support the developing tumor. In this section, we will take a closer look at how nascent tumors evade immune attack, how they transform the surrounding stroma into a tumor-supportive TME, and how they acquire a sufficient supply of oxygen and nutrients to meet their high metabolic demands.

Tumor initiation: Tipping the balance from immune attack to immune evasion

Our immune system is essential for protection against life-threatening pathogens, for wound healing, and for eradication of damaged cells. To execute these functions, the immune system is incredibly diverse and adaptable, with tightly controlled mechanisms to limit tissue damage and restore homeostasis. However, despite the ability of adaptive immune cells to recognize and eliminate pathogens and cells expressing non-self-antigens, cancer cells can evade destruction and develop into full-blown tumors (Figure 1). The finding that T cells specific for (neo)antigens expressed by cancer cells can be detected in established tumors,[40] and that high T cell density and T cell-activation signatures correlate with improved survival across cancer types,[41,42] indicates that the adaptive immune system has the potential to recognize cancer cells. Indeed, preclinical studies using highly immunogenic tumor models provided early experimental proof for the cancer immunosurveillance theory, postulating that the adaptive immune system may constrain and sculpt tumors.[43,44] Nonetheless, many developing cancers successfully prevent or counteract immune attack, already early during tumor initiation. For example, while patients with suppressed adaptive immune systems, such as those with AIDS or who have undergone organ transplants, are at elevated risk of developing viral-associated malignancies, the incidence of many non-viral-associated epithelial cancers is not increased.[45] Similarly, in various transgenic mouse models, tumor incidence is not always increased upon genetic elimination of adaptive immune cell components but sometimes is even reduced.[46–49] These findings underscore the need to better understand the mechanisms driving immune evasion in cancer.

Recent studies exploiting single-cell technologies and multiplexed spatial analyses have shed light on the earliest steps of premalignant progression and the co-evolving spatial, molecular, structural, and functional changes in the immune milieu.[50] Analysis of lung cancer evolution revealed that initial, low-grade lesions were characterized by an influx of naive T cells, indicating that the immune system was sensing the transformation at its earliest stages. However, as the lesions progressed, a transition toward an accumulation of activated T cells and myeloid cells, and upregulation of genes involved in immunosuppression, was observed.[51] Similarly, the comparison of immune cell composition in breast ductal carcinoma *in situ* (DCIS) versus normal breast tissue revealed more total leukocytes, more neutrophils, and a decreased CD8/CD4 ratio in DCIS. The progression of DCIS to invasive ductal carcinomas was accompanied by transitioning to a suppressed immune milieu characterized by fewer activated $CD8^+$ T cells, increased PD-L1 and CTLA4 expression, more regulatory T cells (Tregs), and less diverse T cell receptor (TCR) clonotypes.[52] In patients with head and neck cancer, early-stage disease was associated with immuno-stimulatory neutrophils in draining lymph nodes, which switched to immunosuppressive neutrophils at later tumor stages.[53] Together, these analyses of patient samples indicate that early neoplastic lesions are sensed by the adaptive and innate immune system and that as these lesions progress, a transition toward an immunosuppressed TME ensues.

Studies in preclinical tumor models further support the early onset of immune evasion during neoplastic progression. Time-resolved single-cell profiling of premalignant stages of mammary tumorigenesis, driven by loss of function of *Brca1* and *p53* in a transgenic breast cancer model, revealed the early establishment of a potentially immunosuppressive environment, characterized by the accumulation of Tregs and tissue-resident

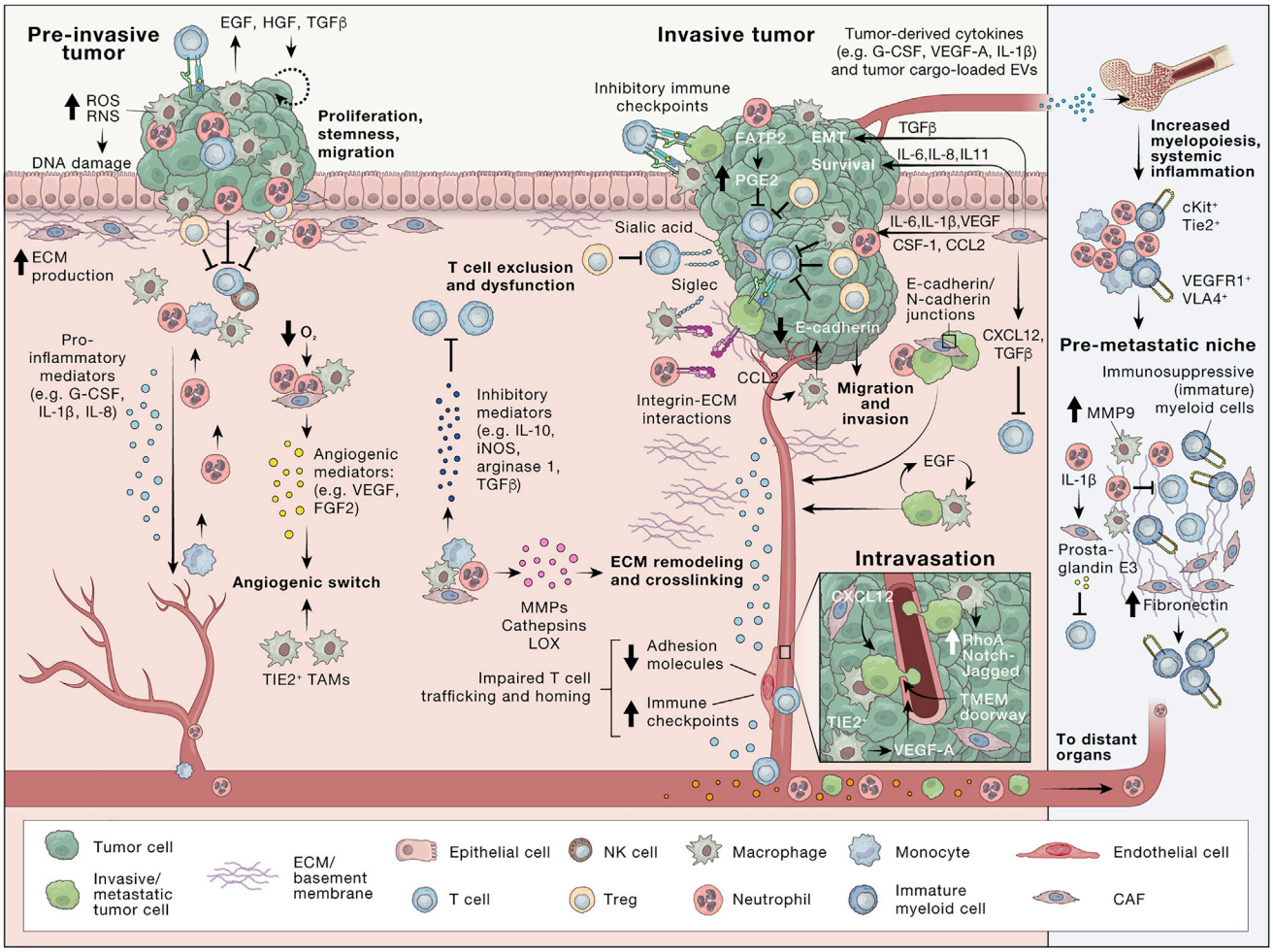


Figure 2. Primary tumor progression and the complex interplay within the TME

The primary tumor niche is supported by various stromal and immune cells. At the earliest stages of tumor initiation, cancer cells may be targeted for destruction by the immune system. Fibroblasts and macrophages can also help suppress tumor growth initially, but they may eventually be influenced by the developing cancer to gain pro-tumorigenic functions. For example, tumor-associated macrophages (TAMs) can support angiogenesis and invasion by secreting growth factors, cytokines, and proteases. CAFs can become activated to secrete ECM proteins and angiogenic factors including VEGF-A, thus further contributing to the complex intertwined primary TME. During intravasation, macrophages localize to perivascular niches, where they can help cancer cells traverse vessel barriers through TME of metastasis (TMEM) doorways.

macrophages.[54] Similarly, early neoplastic progression in mice with mutant *Kras* in pancreatic cells was accelerated by concomitant tissue damage via the alarmin cytokine IL-33.[55] Administration of recombinant IL-33 was sufficient to cause chromatin dysregulation and to accelerate the development of pancreatic intraepithelial neoplasia (Pan-IN) in *Kras*-mutant pancreata, illustrating how gene-environment interactions can trigger gene-regulatory programs that underlie cancer.[55] The timing and mechanisms of immune evasion during tumor initiation will likely depend on the tissue context, tumor-initiating genetic alterations, and host features.

Several cancers arise following chronic inflammation and thereby take advantage of an already-subverted myeloid-adaptive immune cell crosstalk that favors immunosuppression. Chronically inflamed tissues are often characterized by Th2-type immune responses and an accumulation of myeloid cells polarized toward an immunosuppressed functional state, secreting reactive oxygen species (ROS), pro-inflammatory cytokines, chemokines, growth factors, and pro-angiogenic mediators. Together, these may cause tissue injury, epithelial mutagenesis, endothelial dysfunction and angiogenesis, immunosuppression, and matrix remodeling, culminating in tumor initiation and progression.[56,57] Well-known examples of inflammatory conditions that drive tumor initiation include chronic inflammatory bowel disease, which predisposes to colorectal cancer; chronic hepatitis and non-alcohol fatty liver disease, which underlie liver cancer; and asbestos-induced inflammation, which can lead to mesothelioma.[57] Obesity-induced chronic inflammation has also been linked to increased risk of many different cancer types, including breast and uterus cancer.[58–60]

Regardless of whether cancer develops as a consequence of long-standing chronic inflammation or an initiating tumor orchestrates a tumor-supportive inflammatory environment during the early stages of tumorigenesis, almost all progressing tumors

induce varying levels of T cell, natural killer (NK) cell, and DC exclusion or trigger programs of dysfunction in $CD8^+$ T cells.[61–65] This occurs while tumors simultaneously stimulate the recruitment and activation of myeloid cells, particularly macrophages and neutrophils, which collectively form a tumor-supportive inflammatory milieu.

Inflammation: A catalyzer of tumor progression

Cancer-induced inflammation has been compared with the inflammatory response observed in wounds.[66] However, while wound healing is characterized by a well-orchestrated dynamic interplay between adaptive and innate immune cells resulting in inflammation resolution and restored tissue homeostasis, inflammation in the context of a developing tumor is characterized by a subverted adaptive-innate immune cell crosstalk that does not resolve. Under the influence of prolonged inflammatory signaling, hypoxia, low pH, and altered metabolite levels, this inflammation becomes chronic and damaging. Hence, Dvorak postulated that tumors are like wounds that do not heal.[67] In this section, we discuss several key features of cancer-associated inflammation. Since the mechanisms governing the composition, spatial organization, and activation state of tumor-associated immune cells are diverse and vary considerably between tumors, it is important to consider that the type of inflammation and its effect on cancer progression will differ by tumor type and by patient.

As tumors grow, the co-evolving immune milieu undergoes profound changes as a consequence of progressive decreases in cytotoxic $CD8^+$ T cells and NK cells, increased dysfunctional $CD8^+$ T cells, immunosuppressive $CD4^+FoxP3^+$ Tregs, and regulatory B cells, while $CD4^+$ T cells are skewed toward a pro-inflammatory Th2 phenotype, and DCs display defective maturation and functionality (Table 1). In parallel, myeloid cells are increasingly mobilized to the TME, where they adapt their phenotype to local inflammatory cues. Tumor-associated macrophages (TAMs) and neutrophils (TANs) are often the most abundant myeloid cells in different TMEs and have been extensively studied.[68–70] Key tumor-derived mediators that drive the mobilization and activation of these cells include CSF-1, CCL2, VEGF-A, tumor necrosis factor α (TNF-α), and semaphorin 3A for macrophages and G-CSF, GM-CSF, IL-6, CXCL1, CXCL2, IL-1β, and IL-8 for neutrophils.[68] Their presence in human tumors is generally associated with worse prognosis and poor therapy response, although in some cases, their abundance correlates with a favorable outcome.[42,68–70] Recent studies have revealed the immense diversity and plasticity of tumor-associated myeloid cells. From initial classifications into simple binary states of classical versus alternative activation, i.e., M1 and M2 macrophages[71] or N1 and N2 neutrophils,[72] (single-cell) transcriptomic profiling studies and functional analyses have provided important new insights.[73,74] For example, single-cell RNA sequencing (scRNA-seq) analyses demonstrated the co-existence of multiple macrophage and neutrophil subsets within individual tumors, revealing that TAMs simultaneously co-express canonical M1 and M2 marker genes.[74,75] It remains unclear whether these subsets represent distinct populations or different states of the same population. However, an increasing number of studies support the view that these different myeloid cell clusters display distinct, and sometimes opposing, functionalities.[73] For example, TANs frequently exert immunosuppressive functions, but a unique TAN subset was shown to have antigen-presentation capabilities in early-stage human lung tumors.[76] Within TAM populations, subsets with immunosuppressive or pro-angiogenic features have also been identified.[70] A recent pan-cancer analysis revealed that macrophage subsets exhibit distinct transcriptomic patterns between tumor types,[74] supporting the concept of organ- and cancer-type-specific imprinting of tumor-associated myeloid cells. Understanding the full spectrum of myeloid cell subsets in cancer, both intratumorally and systemically, is essential for designing strategies to therapeutically harness myeloid immune subsets with anti-cancer properties while inhibiting or depleting those with tumor-supportive roles.

An additional layer of complexity regarding macrophage diversity in evolving tumors is that tissue-resident macrophages, which are originally seeded by embryonic-derived macrophages in many organs, are functionally distinct from recruited monocyte-derived macrophages.[19,68] While similar differences in ontogeny have not yet been described for neutrophils, preclinical studies revealed that inflammatory mediators derived from developing tumors reprogram BM hematopoiesis, skewing it toward the myeloid lineage and altering neutrophil output from the BM.[77] This is followed by additional transcriptional and epigenetic adaptation of their fate and behavior in a tissue- and tumor-specific manner.[21] Most likely, other myeloid cells, including eosinophils, mast cells, basophils, and DCs, undergo a similar multi-layered process of tumor-induced education, the extent of which may be shaped by the varying lifespan and turnover of the different myeloid cell subsets.

Consistent with the significant association between the abundance of TAMs and TANs and poor patient outcome,[42,68–70] depletion, inhibition, or reprogramming of these cells in mouse models impairs the development or progression of many cancer types[1,58,59,78–83] and also improves the efficacy of chemo-, radio-, and immunotherapy.[1,84–89] However, in some preclinical settings, the net effect of macrophages or neutrophils is anti-tumoral.[69,90–94] Whether these tumor-restraining properties are instigated by certain cancer (sub)types, stages, or other tumor- or host-related characteristics requires detailed investigation. Tumor-associated myeloid cells show high functional plasticity and can influence many tumorigenic processes including (1) regulating the fate and behavior of cancer cells directly via their proliferation, survival, and invasive capacity, (2) creating an immunosuppressed TME, (3) activating tumor angiogenesis, and (4) remodeling the ECM.

Our understanding of the mechanisms at play in different tumors is still limited, and distinct mechanisms may co-exist in the same tumor, may be confined to specific spatial regions within tumors, or may be sequentially activated as cancers progress. We highlight here several examples illustrating the rich diversity of myeloid cell effector mechanisms in the TME and refer the reader to recent reviews for detailed discussions.[68,69,87,95] One important mechanism for how chronically activated macrophages and neutrophils contribute directly to malignant conversion of epithelial cells is via the production of reactive oxygen and nitrogen species that can directly induce DNA damage in epithelial cells.[56,57] Tumor-associated myeloid cells also secrete abundant growth factors and cytokines that modify the fate and behavior of cancer cells, including epidermal growth factor

(EGF), which sustains cancer cell proliferation and migration[96]; hepatocyte growth factor (HGF), which increases the metastatic potential of cancer cells[97]; and transforming growth factor β (TGF-β), IL-6 and IL10, and GPNMB, which support cancer cell stemness.[98–101] However, under certain conditions, such as in some early-stage tumors or during antibody-based therapies, myeloid cells can also kill or phagocytose cancer cells, or contribute to antibody-dependent cellular cytotoxicity,[76,92,95] highlighting the opposing roles of these cells in the TME.

In addition to directly impacting cancer cells, TAMs, their monocytic progenitors, neutrophils, and the less-studied mast cells can indirectly contribute to tumorigenesis by orchestrating tumor-supportive processes in the TME. Proof for a potent immunosuppressive role of myeloid cells comes from preclinical studies showing that depletion or functional reprogramming of TAMs or TANs reduces immune exhaustion programs in tumor-infiltrating T cells, restores anti-tumor immune responses, and synergizes with ICB therapies.[81,87,89,102] Moreover, TAM and TAN abundance frequently correlate with poor ICB response in patients.[103]

Myeloid cells employ a variety of mechanisms to support immune evasion by tumors. They can secrete inhibitory mediators of T cells and NK cells, including IL-10, ROS, iNOS, arginase 1, and TGF-β, express immune checkpoint molecules such as PD-L1, and produce the inflammatory mediators IL-1β, TNF-α, and IL-6 to amplify the inflammatory response.[68,87,104,105] For example, in early-stage human lung cancers, a subset of infiltrating macrophages with high levels of PPARγ, reduced CD86, and increased PD-L1 was associated with diminished T and NK cell presence compared with macrophages in healthy lungs.[65] Pan-cancer scRNA-seq analyses identified an IL-4I1$^+$ PD-L1$^+$ IDO1$^+$ TAM subset linked with T cell exhaustion, tryptophan degradation, and Treg accumulation.[106] Consistently, preclinical studies showed that macrophages contribute to the intratumoral pool of suppressive Tregs by promoting their expansion[29] and the intratumoral conversion of conventional CD4$^+$ T cells into Tregs.[107] Due to their low RNA content, TANs are often underrepresented in scRNA-seq datasets. However, there is ample clinical and experimental evidence for their potent immunosuppressive capacity intratumorally and systemically.[108] In fact, due to this function, these neutrophils and monocytic cells are often grouped under the unified term myeloid-derived suppressor cells (MDSCs).[105,109] Since the immunosuppressive capacity of these cells is only one facet of the many different tumor-supportive functions they can exert, we will adhere to the traditional nomenclature herein.

An emerging mechanism by which myeloid cells promote immune evasion and angiogenesis in cancer is via their metabolic adaptation in the TME and depletion of nutrients and essential amino acids. Tumor-associated myeloid cells often have altered glycolytic activity and increased consumption of glutamine and fatty acids, which can support tumor growth via nutritional and immunological mechanisms.[110,111] For example, tumor-induced upregulation of the fatty acid transport protein 2 (FATP2) in neutrophils enables arachidonic acid processing into prostaglandin E2, thereby enhancing their pro-tumoral, immunosuppressive properties.[112] Myeloid cells can also inhibit T cell activation by depleting cystine and cysteine in the TME.[113] For a detailed overview of the metabolic plasticity of myeloid cells in the TME, we refer the reader to recent reviews.[110,111,114]

In addition to modulating immunosuppression, myeloid cells orchestrate ECM remodeling and activation of angiogenesis during tumor initiation and progression. These processes, and how they are promoted by tumor-associated immune cells, are discussed below.

Multi-faceted roles of CAFs and ECM remodeling in the evolving TME

Along with immune cells, CAFs form a dominant component of many tumors.[115,116] Some tumors, such as hepatocellular carcinoma, develop as a consequence of aberrantly activated fibroblasts, particularly in fibrotic or cirrhotic livers.[117] Other types of cancer can also induce fibrosis, often referred to as desmoplasia, during their initiation and progression.[118] Recent advances in single-cell technologies have uncovered the previously unappreciated phenotypic and functional diversity of CAFs.[116] For example, scRNA-seq of precursor lesions of human pancreatic adenocarcinoma (PDAC) revealed dynamic changes in the composition and transcriptome of CAF subsets during tumor initiation.[119] The progression of premalignant Barrett's esophagus to esophageal adenocarcinoma is characterized by increased inflammation-related gene expression by stroma and fibroblasts.[120] Likewise, in multiple mouse models, alterations in CAF composition and transcriptome are early events during neoplastic development, and these changes evolve as tumors progress.[121–123]

The origin of CAFs in tumors remains controversial and may vary depending on the tumor stage and cancer type. Expansion of local tissue-resident fibroblasts can represent a source of CAFs in early-stage tumors.[115,124] Other studies have revealed that some tissues harbor distinct fibroblast lineages,[125,126] which can contribute to different cellular states or functionally diverse CAF subsets.[126] CAFs may also arise from the conversion of other cell types, including myofibroblasts, BM-derived mesenchymal stem cells (MSCs), stellate cells, and adipocyte-derived CAFs.[127–130] These different origins of CAFs contribute to their phenotypic and functional heterogeneity.

CAFs also show plasticity in response to dynamically changing cues from the TME. The extent of this plasticity is not fully understood, but recent studies indicate that CAFs consist of multiple subtypes that change during tumor progression and that are spatially regulated.[115,131] In pancreatic cancer, three different CAF subtypes co-exist: myofibroblasts (myCAFs), inflammatory CAFs (iCAFs), and antigen-presenting CAFs (apCAFs), with functionally distinct properties and transcriptomic plasticity.[131–133] In other cancer types, similar, but also additional CAF subsets have been identified.[131,134–136] Interestingly, CAF cluster distribution can change with mechanotransduction disruption or following immunotherapy,[137] suggesting strategies to modulate CAF subset composition.

CAFs are activated by various mechanisms in the TME, including exposure to inflammatory mediators, changes in ECM stiffness and composition, and altered metabolites.[115] Key soluble activators include TGFβ, IL-1, IL-6, and TNFα,[115] which also drive chronic inflammation in developing tumors, underscoring the connection between inflammation and CAFs during tumor onset and progression, as discussed further below. Matrix stiffness contributes to transcriptional rewiring by stimulating the YAP and MRTF-SRF regulatory networks in CAFs,

which drives a pro-fibrotic response, production of ECM proteins, angiogenesis, and cancer cell invasion.[138,139] Tumor-derived signals can also regulate complex signaling networks in CAFs. For example, in PDAC, cancer cells activate Hedgehog signaling in CAFs in a paracrine manner.[140] Early preclinical studies demonstrated that targeting the Hedgehog pathway sensitized PDAC tumors to gemcitabine.[141] However, clinical trials of Hedgehog pathway inhibitors in combination with chemotherapy did not show any therapeutic benefit, and in some cases even accelerated tumor progression.[142–144] It is now recognized that Hedgehog signaling is activated differentially in myCAFs versus iCAFs. Consequently, Hedgehog pathway inhibition reduces myCAFs and increases iCAFs, leading to a more immunosuppressed TME.[143]

Mirroring tumor-associated immune cells, CAFs similarly exert pleiotropic and functionally opposing functions within the TME.[145] Early evidence for tumor-promoting functions of CAFs came from experiments in which cancer cells were co-injected with CAFs in mice.[123] Further preclinical studies, in which endogenous CAFs were genetically or therapeutically targeted, additionally revealed potent pro-tumorigenic roles for CAFs.[131,146–148] However, depletion or targeting of specific CAF subsets, including myofibroblasts, accelerated tumor growth in certain mouse models,[149,150] implicating functionally opposing CAF subsets in different TMEs.

CAFs are primarily responsible for ECM deposition and remodeling within the TME.[151] For example, fibrosis in the TME causes tissue stiffness, which is significantly associated with poor survival of patients with pancreatic cancer and breast cancer.[152] The mechanical properties of the ECM directly influence the signaling and behavior of cancer cells, additionally impact immune cell recruitment and activation, and reduce drug access to tumors. Additionally, in this context, CAFs and immune cells work together. Fibrotic tumors have an inflamed phenotype, and inflammation promotes fibrosis.[153–155] Myeloid cells are an important source of ECM remodeling enzymes, matrix metalloproteinases (MMPs) and cathepsins, and collagen-crosslinking enzymes, including lysyl oxidase (LOX). In multiple preclinical models, these have been shown to promote tumorigenesis, invasion, and therapy resistance.[153,156–159]

Recent research has revealed that CAFs can help tumors evade immune control via several mechanisms. In human cancers, CAFs are associated with T cell dysfunction and exclusion, and preclinical studies have shown that CAFs directly prevent T cell recruitment or activation via secretion of CXCL12 and TGF-β or by creating a physical barrier via ECM deposition.[134,160–162] In patients, CAF-induced T cell exclusion may be an early event during tumorigenesis, as $MYH11^+$ αSMA^+ CAFs form a single layer around tumor nests in some early-stage NSCLC lesions, which correlated with decreased T cell density inside those nests.[134] Interestingly, in PDAC, a subset of major histocompatibility complex (MHC) class II-expressing CAFs display antigen-presentation capacities similar to $CD4^+$ T cells but lack costimulatory molecules, which may cause defective T cell activation,[131,132] thus conferring another layer of immunomodulatory functions on CAFs.

CAFs also interfere indirectly with anti-tumor immunity by mobilizing and programming immunosuppressive myeloid cells via secretion of mediators including IL-6, IL-1β, VEGF, CSF-1, CCL2, and Chitinase 3-like1 and by promoting the accumulation and immunosuppressive activity of Tregs.[163–167] These immunoregulatory properties of CAFs provide interesting opportunities to reverse immunosuppression and improve ICB treatment. Indeed, multiple preclinical studies have demonstrated enhanced T cell influx and ICB efficacy following CAF modulation.[160,168,169] Further mechanistic understanding of the reciprocal interactions between CAFs and immune cells, and the heterogeneity across patients and cancer types, could inspire novel combination therapies aimed at reversing CAF-induced immunosuppression while stimulating T cell function.

CAFs additionally influence cancer cells directly. In human breast and lung tumor samples, a $CD10^+GPR77^+$ CAF subset provides a survival niche for cancer stem cells via IL-6 and IL-8 secretion, thereby contributing to tumor formation and chemoresistance.[170] In colorectal cancer, TGF-β-driven secretion of IL-11 by CAFs promotes a GP130/STAT3-dependent survival program in disseminating cancer cells.[171] A pancreatic tumorigenesis-induced lipid metabolic shift in pancreatic stellate cells leads to lysophosphatidylcholine secretion, which supports PDAC cell proliferation and migration and AKT activation.[172] These representative examples illustrate that the mechanisms by which CAFs alter cancer cell signaling and behavior are both varied and tissue dependent.

In summary, the constellation of CAF functions is highly diverse and context dependent. Recent advances in single-cell sequencing and multi-omics approaches, along with sophisticated lineage-tracing models and improved understanding of the contextual functional properties of defined CAF subsets, will facilitate the development of refined targeting strategies directed against tumor-promoting CAF subsets.

Angiogenesis enables cancer progression

Angiogenesis, the process of developing new blood vessels, is essential for tumorigenesis (Figure 1). Once a tumor grows beyond 1–2 mm, it must establish its own vascular supply of oxygen and nutrients.[173] In autopsy studies of seemingly healthy individuals, microscopic quiescent tumors were detected in several organs, including breast, prostate, and thyroid, at a much higher prevalence than expected based on the reported cancer incidence in these tissues.[174–177] The lack of angiogenesis is thought to be why some microscopic lesions do not develop into invasive cancer but remain in a dormant state.[177]

In healthy tissues, the vasculature is stable, and ECs, the main building blocks of vessels, are not actively dividing. By contrast, the onset of angiogenesis during tumor initiation, also termed the angiogenic switch,[178] is a complex process involving extensive crosstalk between ECs, pericytes, mural cells, cancer cells, tumor-associated immune cells, and CAFs.[179,180] The physical changes to the vasculature during the sprouting of new capillaries from existing vessels, and the remarkable heterogeneity and plasticity of ECs have been described in detail.[25,181–183] Tumor vessels are constantly exposed to pro-angiogenic cues, leading to a disorganized, leaky, and tortuous vasculature with defective pericyte coverage and discontinuous lining by ECs. This affects the oxygenation of tumors, alters immune cell dynamics, and reduces drug penetration into tumors.[179,180] An alternative vascularization process involves vessel co-option,

in which tumors expand preexisting blood vessels, without the need to stimulate new angiogenesis.[184] Cancer cells can migrate along the abluminal surface of host vessels, and these vessels may be incorporated into developing tumors.[185] Vascular mimicry represents yet another strategy for progressing tumors to gain access to the circulation, which has been reported in melanoma and glioblastoma. This involves the formation of cancer cell-lined channels, with or without matrix protein deposition, that connect to the existing vasculature.[186] The mechanisms behind these processes are not fully understood, but they may allow tumors to resist anti-angiogenic therapies.[184]

Hypoxia, the lack of oxygen in tissue, is a major trigger for angiogenesis. Many molecules that respond to hypoxia can promote angiogenic switching, of which vascular endothelial growth factor (VEGF) and its downstream signaling pathway are the predominant drivers. In patients, high intratumoral and systemic VEGF levels correlate with poor disease outcomes across cancer types.[187,188] Inhibiting VEGF signaling can prevent angiogenesis and tumor growth in mice,[189,190] indicating that angiogenesis is a critical step in tumorigenesis. Other molecules that promote angiogenesis, such as basic fibroblast growth factor (FGF2) and placental growth factor (PIGF), are also found in tumors, as well as inflammatory mediators including TNF, BV8, and G-CSF.[179] The formation and continuous adaptation of the vascular network during tumor evolution are regulated by cancer cells and host cells in a context-dependent manner.[180] In this section, we highlight myeloid cells and CAFs as archetypal drivers of tumor angiogenesis. For further discussion of how other tumor-associated host cells regulate angiogenesis, we refer the reader to comprehensive reviews.[179,180]

Tumor-associated myeloid cells promote tumor angiogenesis and increased vascular permeability via pro-angiogenic mediators, including VEGF-A, FGF2, PIGF, TNF, and BV8. These cells also produce proteases, such as MMPs and cathepsins, which break down the ECM and release sequestered pro-angiogenic molecules, rendering them bioavailable.[180,191] A growing body of evidence indicates that specific subsets of myeloid cells have pro-angiogenic functions.[73] For instance, neutrophils that produce MMP-9 and BV8 drive angiogenesis in a pancreatic islet carcinogenesis model.[192,193] Macrophages expressing TIE-2, which reside in the perivascular niche (PVN) of tumors, drive angiogenesis in different mouse tumor models.[194–196] Hypoxic TAMs that have undergone metabolic changes also contribute to the formation of disorganized, unstable tumor vessels by competing with ECs for glucose.[197] Single-cell profiling of tumor-associated myeloid cells in multiple human cancers has identified subsets with prominent angiogenic gene signatures, although a unifying molecular annotation of these cells has not yet been established.[73,74]

Importantly, the interactions between the vasculature and immune cells are reciprocal. There is growing evidence that tumor-induced angiogenesis contributes to immunosuppression and immune evasion.[198,199] For example, vascular adhesion molecules, which regulate the homing and trafficking of immune cells, can be downregulated. Tumor-associated ECs express lower levels of ICAM-1, VCAM-1, E-selectin, and P-selectin, which results in a barrier against immune cell infiltration into tumors.[198,199] Conversely, inhibitory immune checkpoint molecules including IDO, TIM3, and PD-L1 can be upregulated on tumor vessels.[198] Moreover, tumor-induced FasL expression by ECs was reported to selectively kill effector $CD8^+$ T cells, resulting in immune evasion.[200] Single-cell studies have revealed new insights into immunoregulatory phenotypes of different EC subsets in health and disease.[199] Pro-angiogenic mediators can also directly impact immune cells. For example, VEGF-A suppresses the maturation of DCs, increases Tregs, and enhances the immunosuppressive state of tumor-associated myeloid cells.[198,201–203] Lastly, the altered physical properties of tumor vessels, the ECM, and hypoxic niches also impact immune cell infiltration and function.[204]

Similarly, tumor lymphatics also have important immunoregulatory properties.[205,206] Like blood ECs, lymphatic ECs can suppress T cell responses through various mechanisms, including expression of immune checkpoint molecules and antigen presentation in the absence of co-stimulatory molecules.[205,206] High levels of VEGF-C, the predominant driver of lymphangiogenesis, are associated with increased metastasis and reduced survival.[207] However, paradoxically, in melanoma, immunotherapy is more effective in tumors with high VEGF-C levels and pronounced lymphangiogenesis.[208] Understanding the tumor-supportive reciprocal feedback between angiogenesis and immunosuppression may help to exploit anti-angiogenic therapies as a means to reverse immunosuppression and reinstate anti-tumor immunity. Indeed, in preclinical studies, disrupting angiogenesis improves the effectiveness of various immunotherapies.[209]

Underscoring the complex interconnectedness of the TME, CAFs are also key orchestrators of tumor angiogenesis. Like immune cells, CAFs produce several pro-angiogenic mediators, including VEGF-A, FGF2, and CXCL12, among others.[180,210] Additionally, by recruiting and activating EC progenitors and myeloid cells with pro-angiogenic capacities in the TME, CAFs indirectly contribute to tumor angiogenesis. Moreover, the CAF-mediated desmoplastic response impacts the vascularization of developing tumors.[211] CAFs produce collagen crosslinking enzymes, including LOXs and hydroxylases, and ECM-degrading proteases, which alter the mechanical properties of tumors and impact angiogenesis.[151] However, several of the collagen fragments released after ECM proteolysis, such as endostatin and tumstatin, can inhibit angiogenesis,[211] indicating that both CAF-dependent pro- and anti-angiogenic processes are at play in the TME.

SETTING THE STAGE FOR METASTATIC SPREAD

Cancer cell invasion and migration

Once tumors have successfully established the mutually reinforcing connections between angiogenesis, inflammation, and fibrosis, they can enter the next phase of disease progression: local invasion. Invasive growth is one of the key hallmarks of cancer and sets the stage for metastatic dissemination (Figures 1 and 2). Invasion is a complex, multi-step process that involves cancer cells detaching from each other, migrating away from the primary tumor mass, and invading the surrounding stroma.[212] Cancer cells can invade as single cells or collectively in strands or clusters.[213] During invasion, cancer cells are exposed to changing cellular and molecular components of the TME and must switch phenotypes to complete this process.

To detach from their neighboring cancer cells, epithelial cell-cell adhesion must be disrupted. Loss of the intercellular adhesion protein E-cadherin is central to this process and often accompanied by an epithelial-to-mesenchymal (EMT)-like transitional state. Cancer cells lose epithelial features and gain mesenchymal traits that facilitate stem-like properties and migration.[214,215] Cues from the TME promote the phenotype switching of cancer cells, enabling local invasion[216,217] (Figure 2). For instance, in a mouse model of HER2$^+$ mammary tumorigenesis, CCL2 produced by epithelial and myeloid cells in premalignant lesions recruits CD206$^+$Tie2$^+$ macrophages that downregulate E-cadherin junctions and stimulate Wnt signaling. This leads to an EMT-like response that facilitates early dissemination.[218] Macrophage depletion via CSF1R inhibition reversed this process, resulting in increased E-cadherin expression in hyperplastic ducts and reduced cancer cell dissemination.[218] In early stages of NSCLC, cancer cells localize proximally to tissue-resident alveolar macrophages. Transcriptomic analysis of alveolar macrophages isolated from early mouse NSCLC lesions revealed increased expression of antigen presentation and tissue-remodeling genes, including proteases. Mechanistic *ex vivo* and *in vivo* studies showed that tissue-resident macrophages instigate an EMT and invasion phenotype in adjacent cancer cells.[29] A powerful inducer of this phenotypic plasticity in cancer cells is TGF-β, which can be secreted by cancer cells themselves or host cells in the TME.[219] For instance, CAF-associated TGF-β signaling enhances cancer cell invasion under *in vitro* and *in vivo* conditions.[220,221] Additionally, the catalytic activity of MMPs and cathepsin proteases alters the biophysical properties of cancer cells, for instance through cleavage of E-cadherin from epithelial cells and via the modulation of integrins, which enables mechanoadaptation of cancer cells to matrices of different stiffness.[156,159,222,223]

While E-cadherin loss and phenotypic plasticity facilitate cancer invasion, not all tumors undergo EMT-like switching during metastatic dissemination.[224,225] The exact mechanisms underlying the invasion of cancer cells that retain epithelial characteristics are poorly understood, but there is increasing evidence showing that CAFs enable collective cancer cell invasion by physically generating tracks in the ECM through their remodeling properties and by exerting physical pulling forces.[226,227] Heterotypic adhesion between CAFs and cancer cells via E-cadherin/N-cadherin junctions triggers a mechanotransduction response in cancer cells, enabling collective invasion.[228] Other TME cells can also promote cancer cell invasion (Figure 2). For instance, perineural invasion (PNI) is a process by which cancer cells locally extend along nerves that is observed in several solid cancer types and is associated with poor outcome.[229] In a mouse model for PDAC-associated PNI, Schwann cells at the site of PNI released CCL2, which attracted inflammatory monocytes. These subsequently differentiated into cathepsin B-producing macrophages that potentiated nerve invasion.[230] Intravital microscopy (IVM) studies in mouse breast cancer models have revealed that invasion and migration of EGFR$^+$ cancer cells were dependent on the co-migration of EGF-producing TAMs.[96,231] Together, these findings demonstrate that co-option of CAFs, immune cells, and tissue-resident cells foster the invasive behavior of cancer cells.

In healthy tissues, the basement membrane forms a physical barrier between the epithelium and underlying stroma.[232] This barrier must be breached to enable the invasion of cancer cells into the surrounding tissue (Figure 1). The ability of cancer cells to do this depends on a combination of factors, including their internal programming, the architecture of the ECM, and signaling cues from the TME.[212,233] CAFs are key players in remodeling the basement membrane and ECM network through secretion of proteases but also by exerting contractile forces that generate gaps in the basement membrane, which can then be utilized by cancer cells to cross through.[234,235] Cancer cells are subsequently influenced by the composition and mechanical properties of the ECM and by the interstitial fluid pressure, which affects their ability to migrate and invade.[217,236] They can sense the remodeled and crosslinked ECM molecules through integrins and other transmembrane receptors, which impacts cancer cell-intrinsic signaling and enhances invasion and migration.[217,237] For instance, a stiff ECM triggers integrin clustering on cancer cells, which stimulates FAK/Src complex assembly and downstream activation of PI3K/Akt and ERK signaling, promoting cancer cell invasion, migration, and survival.[217] In various mouse tumor models, pharmacological or genetic inhibition of ECM remodeling and ECM crosslinking, inhibition of FAK, and other strategies to reduce stromal stiffening or the cancer cell's response to a stiff ECM attenuated tumorigenesis.[152,217,223,238]

Intravasation

The next rate-limiting step in the metastatic cascade is the intravasation of cancer cells into the blood or lymphatic circulation (Figure 1). The mechanisms by which cancer cells cross endothelial layers to enter the circulation are complex, context dependent, and influenced by cancer cell-intrinsic features, the physical properties of the ECM and type of vasculature, microenvironmental cues, and the extent of hypoxia[239] (Figure 2). As discussed above, the integrity of the blood vasculature in tumors is often impaired. The vascular basement membrane and the endothelial barrier may be disrupted, which increases vascular leakiness and facilitates cancer cell intravasation.[180] Mouse IVM studies have provided key insights into the intravasation process.[240] Often, TAMs associate with intravasating cancer cells.[196,240–244] IVM and mechanistic experiments in mice with implanted PyMT mammary tumors revealed that CXCL12-secreting CAFs located proximal to blood vessels can attract TAMs and accompanying cancer cells toward perivascular regions, where intravasation takes place.[245] VEGF-A signaling induced by TIE2$^+$ perivascular TAMs caused focalized loss of vascular junctions, resulting in a transient increase in vascular permeability that facilitated cancer cell intravasation.[196] Consequently, macrophage depletion can reduce vascular permeability and the number of circulating tumor cells (CTCs).[196] Besides creating gateways to access the vasculature, TAMs directly reprogram cancer cells to undergo the intravasation process. Macrophages activate RhoA signaling in cancer cells, which induces cancer cell invadopodium formation and subsequent intravasation *in vitro*.[244] Moreover, TAMs promote cancer stemness programming in cancer cells via Notch-Jagged signaling, resulting in a slow-migratory, invadopod-rich cancer cell phenotype that enhanced their intravasation.[242,243] The

tripartite structures containing VEGF-expressing TIE2$^+$ macrophages, cancer cells, and ECs, also termed TME of metastasis (TMEM) "doorways," promote intravasation in a contact-dependent manner (Figure 2). TMEMs have been observed in human breast tumors, and their density predicts elevated risk of distant metastasis.[246,247] Neoadjuvant chemotherapy in patients with breast cancer and mouse breast cancer models increased the density and activity of TMEM sites by promoting the mobilization of Tie2$^+$/VEGF^{hi} macrophages to tumors, which was associated with increased CTCs and metastatic foci in chemotherapy-treated experimental models.[248] TIE2 inhibition reversed the chemotherapy-mediated pro-metastatic effects.[248] The relevance of TMEM doorways in intravasation stems mostly from studies on breast cancer. Whether a similar mechanism underlies intravasation in other cancer types remains to be established. In addition, besides macrophages and ECs, neutrophils, pericytes, CAFs, adipocytes, and mechanical features of the TME, including ECM structure and interstitial fluid pressure, also influence cancer cell intravasation via direct or indirect mechanisms.[239]

Lymphatic intravasation is another route that cancer cells may take to disseminate, although the underlying mechanisms are incompletely understood. Intratumoral lymphatic vessels are often compressed, and the structure of lymph vessels differs from that of blood vessels and may thus require a different mode of intravasation.[206,249] The importance of the lymphatic route for the formation of distant metastases is debated and may be organ dependent.[249]

THE LONG-DISTANCE REACH OF PRIMARY TUMORS: FORMATION OF THE PREMETASTATIC NICHE

Importantly, the impact of a developing tumor on the host is not limited to the local TME (Figure 1). Through paracrine effects, primary tumors trigger a cascade of events by which they generate cancer cell-conducive microenvironments in distant organs before metastatic spread occurs.[250] The realization that primary tumors reach far beyond their boundaries by preparing distant sites for the future arrival of disseminated cancer cells, termed premetastatic niches, led to a paradigm shift in our understanding of metastasis. The existence of the premetastatic niche was initially reported in studies using the LLC lung and B16 melanoma tumor models.[250] It was shown that these primary tumors triggered VEGF- and PlGF-mediated induction of MMP9 in distant lung ECs and macrophages, which promoted lung metastasis formation. MMP9 was also upregulated in lung ECs of patients with cancer with primary tumors in other organs than the lungs, which was not observed in patients without cancer.[251] Another study, using the same mouse tumor models, reported that fibronectin, a VLA-4 ligand, was induced in fibroblasts in premetastatic distant organs, which directed the accumulation of pro-metastatic VEGFR1$^+$VLA-4$^+$ BM-derived hematopoietic progenitor cells. Importantly, VEFGR1$^+$ cellular clusters were also observed in common sites of metastasis in patients with cancer but not in patients without cancer.[252] Since this pioneering research, substantial progress had been made in our knowledge regarding the molecular and cellular mechanisms underpinning the premetastatic niches that form a fertile soil for disseminated cancer cells.[250,253]

The initiating signals that trigger the series of systemic changes leading to premetastatic niche generation include tumor-derived soluble mediators, most notably G-CSF, VEGF-A, PLGF, TGFβ, S100 proteins, and TNF, and EVs loaded with tumor cargo that can be transferred to BM cells and resident cells in distant organs.[250] Some of these mediators influence the BM niche, where they activate and program immune cells and their progenitors to mobilize to future metastatic sites.[10] Other tumor-secreted mediators directly modify distant organs. For example, LOX secretion by hypoxic 4T1 breast cancer cells disrupted normal bone homeostasis by inducing osteoclastogenesis, which facilitated the homing and colonization of CTCs.[254] In LLC and B16 tumor-bearing mice, tumor-derived EVs loaded with small nuclear RNAs activated Toll-like receptor 3 (TLR3) in lung epithelial cells, stimulating the release of neutrophil chemoattractive mediators, culminating in lung premetastatic niche formation via neutrophil recruitment.[255] These and other recent studies demonstrate that primary tumors subvert the crosstalk between different tissue-resident cells and newly mobilized BM-derived immune cells in distant organs, thus contributing to premetastatic niche formation.[250,253,256] The critical involvement of tissue-resident cells underlies organ-specific differences in premetastatic niche formation, explaining in part the organotropism of metastasis. Another element contributing to the organ specificity of metastatic spread is dictated by the expression of adhesion molecules on tumor-secreted EVs. In mouse models, it was observed that depending on the integrin expression profiles, tumor-derived EVs homed to different distant organs, and depending on the organ, different resident cells demonstrated uptake of the tumor EVs. Consequently, inoculation of tumor-secreted EVs could redirect the organotropic behavior of cancer cells.[257] Moreover, in patients with cancer, EVs with specific integrin expression patterns were identified, which correlated with the location of metastases.[257]

Another mechanism by which primary tumors prepare the host for metastatic disease is via the induction of tumor-induced systemic inflammation and immunosuppression, favoring immune escape of disseminated cancer cells.[258] For instance, IL-1β-secreting TAMs in primary *Trp53*-deficient mouse mammary tumors induced IL-17- and G-CSF-dependent mobilization of immunosuppressive neutrophils from the BM to distant organs, facilitating the metastatic spread to lungs and lymph nodes by suppressing CD8$^+$ T cells.[11,12,81,259] Primary tumor-induced systemic immunosuppression does not exclusively affect the future sites of metastasis, but does impact the entire host, and therefore formally does not fall under the concept of the premetastatic niche formation. However, systemically mobilized immunosuppressive myeloid cells may trigger tissue-context-specific programs to enable organ-specific metastasis. For instance, in breast cancer mouse models, systemic mobilization of IL-1β-secreting neutrophils enhanced prostaglandin E3 secretion from lung-resident adventitial fibroblasts, resulting in reduced anti-tumor immunity and enhanced lung metastasis.[260]

Collectively, while the exact paracrine mediators, cellular players, and cascade of events underlying the formation of the premetastatic niche may differ by tumor type, key features of the resulting permissive niches in distant organs include increased vascular permeability, ECM remodeling, alterations in resident cells including fibroblasts and epithelial cells,

mobilization of BM-derived cells, and immunosuppression. In the following sections, the impact of different organ microenvironments on the fate of metastatic cells will be further discussed.

CTCs AND THE BATTLE FOR SURVIVAL IN THE CIRCULATION

Following an often lengthy process of evolution and adaptation, which progressively sculpts the TME at the primary site, once tumor cells intravasate into the circulation (blood or lymphatic), they are immediately subjected to an array of different insults and challenges in this foreign microenvironment (Figure 3). In the more common situation of hematogenous dissemination, these include anoikis, resulting from cellular detachment; high shear forces in the blood circulation; and immune-mediated attack, collectively resulting in the death of most CTCs. From clinical and preclinical analyses, it has been estimated that between 20,000 and 700,000 CTCs are shed from solid tumors per gram of tissue per day, depending on the tumor type analyzed. Detailed blood-exchange analyses, using multiple genetically engineered mouse models (GEMMs), have estimated the half-life of endogenous, naturally shed CTCs in the circulation to be several minutes.[261] Interestingly, a recent study also found that there is a circadian rhythmicity to CTC release.[262,263] The vast majority of CTCs will die, however, underscoring the highly inefficient nature of the dissemination process, which is one of the key rate-limiting steps in the invasion-metastasis cascade (Figure 1).[264,265]

Nonetheless, for the small proportion of CTCs that survive passage through the circulation, they can evade destruction through a variety of mechanisms. These include CTC clustering, which promotes stemness via induction of NANOG, SOX2, and OCT4[266]; association with specific immune cells such as neutrophils or platelets; and, conversely, evasion of the effects of other types of cytotoxic immune cells including NK cells (Figure 3). Indeed, clustering of CTCs with neutrophils, in a VCAM-1-dependent manner, results in increased CTC proliferation while in the circulation, thereby promoting more efficient metastatic colonization.[267] Consistent with these mechanistic insights, the enrichment of CTC clusters versus single CTCs is generally associated with worse patient prognosis,[266] and a high neutrophil-to-lymphocyte ratio in the circulation correlates with poor outcome across multiple cancers.[268]

Another highly abundant cell type in the blood, platelets, have long been recognized as key promoters of CTC survival through several mechanisms, including the enhancement of CTC adhesion and clustering, resulting in a "platelet cloak" around the CTCs that can shield them from both physical stress and surveillance by the immune system[269] (Figure 3). One intriguing mechanism for evading immune attack involves a type of molecular mimicry where the transfer of MHC class I-containing vesicles from platelets to the tumor cell surface protects CTCs from recognition by NK cells.[270] In turn, CTCs can activate platelets, for example via the G protein-coupled receptor (GPCR) CD97.[271] This leads to both enhanced tumor cell invasion via CD97-LPAR signaling and ATP release that promotes vasodilation and, consequently, CTC extravasation from the circulation, as discussed in the following section.

Counteracting the CTC-protective effects of neutrophils and platelets is the destructive power of immune surveillance by NK cells, cytotoxic T cells, DCs, and others. Given that these diverse and rapid cellular interactions are taking place in the fast-moving circulation, it remains an open and intriguing question as to whether these simply occur in a stochastic manner—based on which type of immune cell first interacts with the CTCs or whether there is a dynamic immune "battle" that ultimately determines CTC fate. Interrogation of patient CTCs via liquid biopsies can be used as a minimally invasive means to follow disease evolution, including therapeutic response and the emergence of adaptive resistance, and may help answer this question. Such analyses have revealed a plethora of factors in addition to CTCs and various immune cells, including EVs and non-coding RNAs, which may also impact CTC viability and thus have prognostic relevance.[272] However, it has proven very challenging to detect and isolate CTCs from peripheral blood samples at early stages of cancer, which is when these types of analyses would of course be most beneficial from an interventional perspective.

ORGAN TROPISM AND EXTRAVASATION

For the small proportion of CTCs that survive passage through the circulation, the next rate-limiting step in their metastatic journey is to extravasate into a secondary organ. This is determined in part by the underlying organ tropism for each primary cancer, classically known as the "seed and soil" hypothesis as first coined by Paget in the 1880s.[273] Metastatic tropism can be highly stereotypical; for example, breast cancer predominantly spreads to lungs, liver, bone, and brain, and prostate cancer shows a high propensity for dissemination to bone.[274] This organotropism is influenced by multiple mechanisms including signaling by factors such as chemokines, metabolites, and EVs that contribute to the directed migration of CTCs to specific organs. In addition, the specific circulatory routes that CTCs take and the range of different vascular barriers that cancer cells must cross in order to gain entry into a given organ further influence their ultimate destination (reviewed in Massagué and Ganesh[265]). This vascular diversity is exemplified by comparing the relative ease of CTC entry into the BM across largely fenestrated capillaries versus the formidable challenge of traversing the multiple, tightly integrated cell layers of the blood-brain barrier.[275,276]

For the process of extravasation itself, tumor cells must first arrest and attach to the lumen of the endothelium while continuously being subjected to high shear forces from the rapidly flowing bloodstream around them (Figure 3). This step is facilitated by cell adhesion molecules and their ligands, integrins, and ECM components expressed by both tumor cells and ECs[277] and shares some similarities with the molecular mechanisms involved in blood leukocyte rolling, adhesion, and extravasation.[278] Platelets and neutrophils, which may still be traveling with the CTCs, can further enhance tumor cell adhesion to the vasculature via selectins or GPCRs or via the production of neutrophil extracellular traps (NETs), respectively.[271,279]

Following adhesion, the CTCs next traverse EC junctions, and possibly also additional vascular cell layers (e.g., pericytes, smooth muscle cells) and the ECM, to gain entry into the new

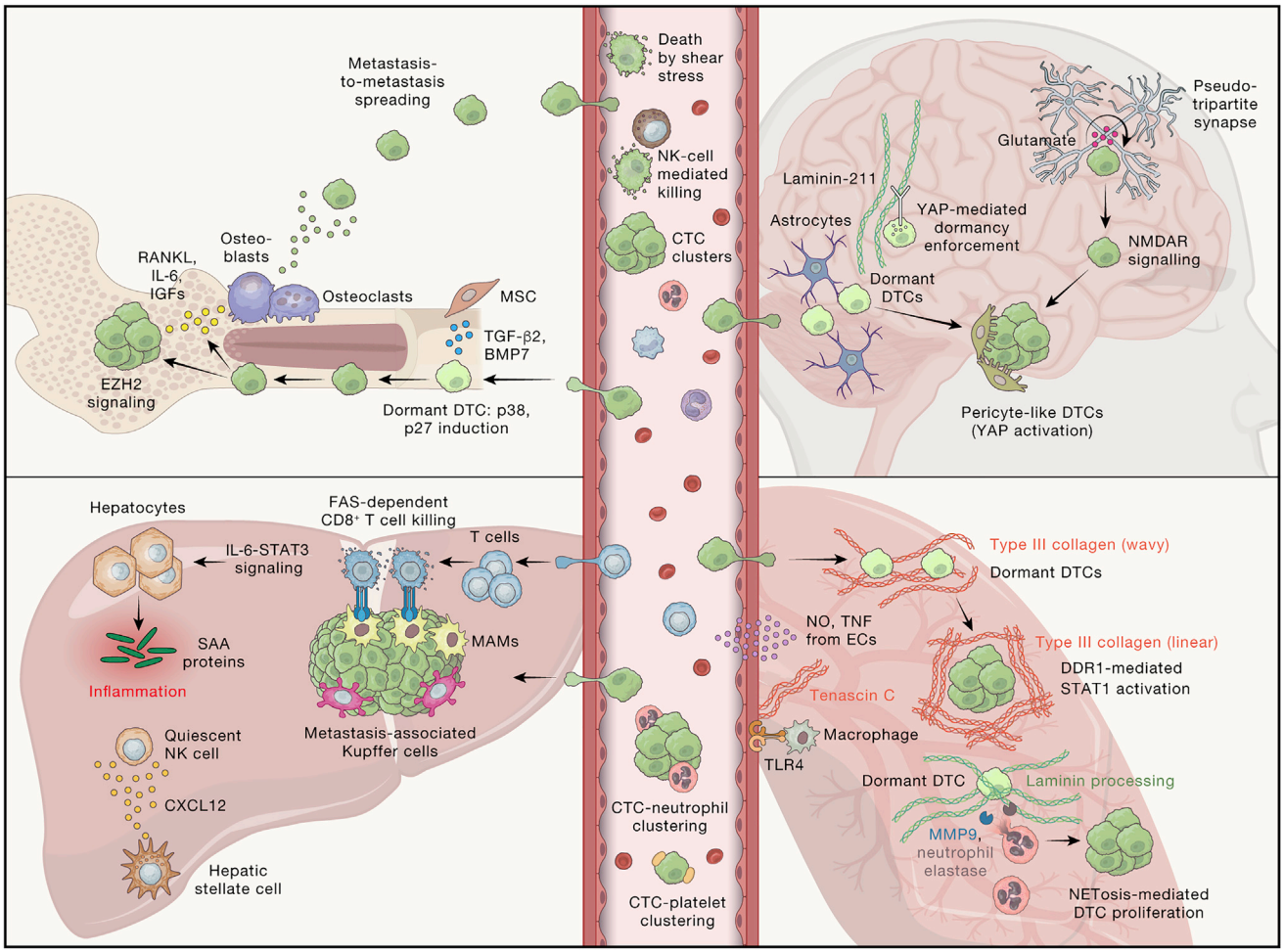


Figure 3. Regulation of metastatic cell fate in different tissue environments

The fate of metastatic cells in the circulation can be considered a "battle" between different types of immune cells, as depicted in the center of this figure, with neutrophils and platelets promoting circulating tumor cell (CTC) survival, while natural killer (NK) cells and other adaptive immune cells can eliminate CTCs. Once cancer cells extravasate into secondary tissues, the microenvironment must be permissive to their colonization and expansion for overt disease to develop. TME cell types involved in regulating this process include mesenchymal stem cells (MSCs) and metastasis-associated macrophages (MAMs). Four major secondary organs are shown here, bone, brain, liver, and lungs, which have shared as well as tissue-specific mechanisms for controlling the fate of disseminated tumor cells (DTCs), as depicted for each metastatic site.

organ parenchyma (Figure 3). This typically requires active proteolysis and/or degradation of cell adhesion molecules, including junctional adhesion molecules, cadherins, and others, particularly for multi-cellular CTC clusters to cross the vasculature. Cancer cells not only rely on the proteases and degradative enzymes that they produce for this step,[280,281] but they can additionally trigger the release of these enzymes from non-cancerous cells, including platelets, monocytes, and also neutrophils via the production of NETs. Given that immune cells are adept at transiting through the different organs of the body to execute their physiological functions, it is perhaps not surprising that cancer cells may undergo a type of "immune mimicry" by which they produce factors typically enriched in immune cells, including chemokines, proteases, and cell adhesion molecules.[280,281] Moreover, resident immune cells in the sites of future metastasis can further promote CTC extravasation through doorways as shown by IVM in the lung,[282] much as for intravasation at the primary site, as discussed above. A similar enhancement of metastatic cell extravasation by resident microglia was also revealed in the brain[283] (Figure 3).

Tumor cell plasticity at both the phenotypic and the physical levels is thus a key trait for conferring the adaptability necessary for survival.[284] Indeed, CTCs can additionally extravasate via non-proteolytic mechanisms, such as diapedesis, which involves mechanical deformation to squeeze through the EC junctions one cell at a time.[285] This mode of extravasation is also more typical for CTCs exiting from lymphatic vessels into the lymph nodes. While lymphatic dissemination, perineural migration, and growth in the pleural cavity or other body spaces have been reported for several cancers,[265] these other routes for CTC trafficking are considerably less frequent than hematogenous spread. Intriguingly, a recent study found that metastatic colonization of the lymph nodes did not serve as a hub for subsequent evolution of the metastatic clones, per se, but rather led

to systemic tumor-specific immune tolerance mediated by immunosuppressive Tregs.[286] This rendered distant tissues more hospitable to metastatic seeding via the blood circulation. Following extravasation, CTCs often remain near blood vessels, and this is critical for determining their fate, as discussed in the next section.

METASTATIC SEEDING AT SECONDARY SITES, AND THE COMPLEX INTERPLAY BETWEEN TUMOR DORMANCY AND OUTGROWTH

After extravasation into the secondary site, disseminated tumor cells (DTCs) face a new series of challenges from the foreign tissue environment, and once again, the vast majority of tumor cells are killed by host defense mechanisms, including immune surveillance.[287] The minority of DTCs that survive to seed a new organ often remain near the vasculature, indicating that they receive regulatory cues from blood vessels. Indeed, molecular signals from the PVN, as well as tissue-specific niches such as the endosteal niche in the bone, initially appear to hold the DTCs in a dormant state, which may protect them from recognition and killing by the immune system (Figure 3). Dormancy represents the least-well-understood stage in the metastatic cascade, in part because of the inherent challenges in studying these rare cells, which stop proliferating and can survive in a quiescent state, sometimes for years to decades. However, recent studies, including those harnessing the power of intravital imaging,[240,282,288] are beginning to reveal important insights into the mechanisms controlling dormancy initiation, its maintenance during the latency phase, and its reemergence from dormancy—and, notably, the critical interplay with the microenvironment for this exquisite regulation of organ-specific metastasis.

Bone metastasis

The bone is one of the most studied organs in relation to DTC biology, in part due to the large proportion of patients found to have micrometastases in this site, particularly for breast and prostate cancers.[289] The bone microenvironment is home to a plethora of different cell types including tissue-resident osteoblasts, osteoclasts, and osteocytes, along with adipocytes, abundant vasculature, and immune cells, and a rich marrow and ECM[289] (Figure 3). Together, this results in a dynamic interplay that regulates hematopoietic stem cell (HSC) development under homeostasis but which may ultimately be co-opted in the "vicious cycle" of bone metastasis accompanied by bone destruction and fractures.

In the earliest stages of DTC colonization, these cells can occupy different niches in the bone, with the majority of DTCs located in the BM, often long before the diagnosis of overt metastasis.[289,290] For example, in the HSC niche, $NG2^{+}$/$Nestin^{+}$ MSCs produce TGF-β2 and BMP7, which activates a quiescence pathway in DTCs via p38-kinase signaling and p27 induction.[291] In patients with estrogen receptor $(ER)^{+}$ breast cancer, those without evidence of systemic recurrence showed higher levels of TGF-β2 and BMP7 in BM plasma. Accordingly, either MSC depletion or MSC-specific deletion of TGF-β2 resulted in metastatic outgrowth of dormant bone DTCs in mice.[291] The endosteal niche also facilitates bidirectional interactions between DTCs and bone-resident cells, which may ultimately foster disease progression. Osteoblasts and osteoclasts can be induced by the cancer cells to secrete factors including RANKL, IL-6, IGFs, and matrix-degrading enzymes that collectively promote metastatic outgrowth, osteolysis, and the skeletal changes that underly many of the clinical manifestations of late-stage bone metastasis.[290]

Interestingly, preclinical experiments incorporating phylogenetic barcode tracing *in vivo* have revealed that osteoblast-derived cytokines in the bone microenvironment can promote the stemness of breast or prostate DTCs via epigenetic regulation in an EZH2-dependent manner.[292,293] This enhances their further dissemination to other organs, termed multi-organ metastasis-to-metastasis spreading, which can be substantially reduced by EZH2 inhibition in mice.[292] These results are also consistent with clinical observations that the bone is typically the site where breast and prostate cancer metastases are first detected.[289,290] Moreover, the bone microenvironment can epigenetically modulate ER expression in DTCs, thereby rendering the cells resistant to endocrine therapy,[293] again modeling a major challenge in the clinic. Similarly, DTC resistance to systemic chemotherapy is often encountered in patients. This has been attributed to quiescent DTCs, which are not proliferating and thus will not be targeted by such therapies and may later emerge from dormancy. However, recent preclinical studies have found that the bone PVN can protect DTCs from chemotherapy, independent of their cell-cycle status, via vascular adhesion molecules including integrins β1, αvβ3, and VCAM-1.[294] Consequently, blocking integrin-mediated interactions between the PVN and DTCs sensitized these cells to chemotherapy and significantly enhanced survival.[294]

Lung metastasis

DTC seeding, dormancy, and outgrowth have also been investigated in the lungs, which is a major site for metastatic dissemination. Indeed, given the extensive vascularization of the lung and large surface area, which are critical for normal lung functions, there are numerous opportunities for CTCs to arrest, extravasate, and colonize this organ (Figure 3). Key insights into this process have been revealed through IVM, showing that already at the primary site, tumor cells acquire a pro-dissemination and dormancy phenotype controlled by the transcriptional regulator NR2F1 and which is further enriched in proximity to macrophages.[282] This state is initially maintained in DTCs after their arrival in the lungs and subsequently lost during outgrowth.[282] Similar to other organs, cues from the PVN are critical in determining DTC fate, with ECM molecules including type III collagen and tenascin C playing important roles.[295,296] Imaging analyses, incorporating second-harmonic generation to assess collagen fiber orientation, revealed that solitary dormant DTCs are surrounded by type III collagen in a non-linear wavy orientation. By contrast, in concert with induction of proliferation in micrometastases, there is a shift toward more aligned collagen organization and associated ECM remodeling.[295] Manipulation of these different ECM niches, via targeting of the identified COL3A1-DDR1-STAT1 pathway, was thus proposed as a strategy to maintain DTC dormancy.[295] Another secreted ECM molecule that regulates DTC fate in the lung is tenascin C, which sequentially activates neighboring

interstitial macrophages via TLR4 signaling, inducing ECs to secrete nitric oxide (NO) and TNF, thereby generating a pro-metastatic PVN niche.[296]

Interestingly, the importance of stromal regulation of lung DTC dormancy is also evident from preclinical studies in the context of aging.[297] While dermal fibroblasts in the aged skin suppress the growth of melanoma cells, they can drive melanoma phenotype switching and dissemination via the soluble WNT antagonist sFRP2.[298] When these DTCs arrive in the lung, they encounter the related sFRP1 antagonist, which is secreted at higher levels by aged lung fibroblasts. This results in the inhibition of WNT5A in melanoma cells, in a PROS1-AXL-dependent manner, which overcomes dormancy and ultimately leads to subsequent melanoma outgrowth.[297] Consequently, genetic manipulation of the different components of this paracrine signaling pathway resulted in altered lung metastasis,[297] which may have important implications for therapeutic targeting of cancer specifically in the aged population.[299] Another instigator that can awaken dormant cancer cells is inflammation. Indeed, sustained inflammation, for example from tobacco smoke exposure, induces the formation of NETs.[83] NETs are filled with proteases, including MMP9 and neutrophil elastase. Release of these proteases remodeled laminin in the lung, resulting in the proliferation of dormant cancer cells in an integrin $\alpha 3\beta 1$-dependent manner[83] (Figure 3).

Liver metastasis

The liver represents a very frequent organ for metastasis, in part because it is extensively vascularized with a dual blood supply from the hepatic portal vein and hepatic arteries and also because the hepatic vasculature is highly fenestrated, thereby facilitating CTC extravasation[300] (Figure 3). Alterations in the stroma and ECM are also important in regulating metastatic outgrowth in the liver as for the organs discussed above.[30] For example, in a large study of patients with colorectal cancer, those with a high liver fibrosis score had significantly worse outcomes regarding hepatic metastasis and relapse.[301] Tissue-resident hepatocytes have additionally been shown to facilitate liver metastasis in preclinical models through the formation of a pro-metastatic niche.[302] Early in pancreatic tumorigenesis, hepatocytes in the liver orchestrate an inflammatory response via activation of IL-6-STAT3 signaling and subsequent increased production of serum amyloid A (SAA) proteins. Analysis of patients with liver metastasis revealed similar alterations.[302] This results in an altered fibrotic and immune microenvironment in the liver that is associated with enhanced metastatic seeding in mice. Interestingly, removing either SAA proteins or Stat3 through hepatocyte-specific deletion, blocked the pro-metastatic niche.[302]

Another liver-resident population, hepatic stellate cells, also play important roles in regulating the fate of breast cancer cells in this organ.[303] Stellate cells can drive fibrotic injury via secretion of the immune-inhibitory CXCL12 chemokine, which renders NK cells quiescent. This suppresses the key immune surveillance functions of NK cells, resulting in the reemergence of DTCs from dormancy.[303] A similarly intriguing study found that the presence of liver metastases can negatively impact the efficacy of immunotherapy by siphoning activated $CD8^+$ T cells from the systemic circulation.[304] Interactions with macrophages in liver metastases were found to drive T cell killing in a Fas-dependent manner. Interestingly, this effect was overcome in preclinical models via liver-directed radiotherapy—which eliminated these immunosuppressive macrophages and consequently reduced T cell siphoning. Given that patients with liver metastasis were also found to have reduced T cell numbers, diversity, and function, these results may have important implications for strategies to improve immunotherapy efficacy for this patient cohort.[304]

Brain metastasis

In the brain, tissue-resident astrocytes constitute a key component of the blood-brain barrier (BBB), and quiescent DTCs have been found to reside in a PVN surrounded by astrocytic endfeet[288] (Figure 3). The astrocyte-derived basement membrane protein laminin-211 can enforce this dormancy via induction of dystroglycan receptor engagement with the YAP in DTCs. This prevents YAP trafficking to the nucleus and thereby blocks its pro-metastatic functions. Conversely, proliferating DTCs were only found in association with vascular structures that were stripped of astrocytes and their endfeet. Consequently, *in vivo* modulation of these various molecular components resulted in brain metastatic outgrowth.[288] Interestingly, YAP activation has also been implicated in the acquisition of pericyte-like features by the DTCs themselves, which promotes their elongation and metastatic outgrowth via the cell adhesion molecule L1CAM, both in the brain and other metastatic sites.[305] In another intriguing example of the plasticity of DTCs, these cells can even integrate themselves into the neural networks of the brain.[306] In this case, DTCs position themselves proximal to an existing synapse between two glutamatergic neurons to form a "pseudo-tripartite" synapse. This results in the release of glutamate by the neurons which leads to N-methyl-D-aspartate receptor (NMDAR) signaling and subsequent metastatic outgrowth.[306] Interestingly, the NMDAR pathway can also promote pancreatic neuroendocrine tumor invasion,[307] and the burgeoning field of cancer neuroscience[308] represents a particularly insidious co-option of the host microenvironment by tumors.

Collectively, these illustrative examples underscore the importance of different organ microenvironments, populated by unique tissue-resident cell types as well as recruited immune cells, in regulating DTC fate. Given that DTCs can remain dormant for years to decades, while their activation represents a major clinical challenge, it will be imperative to fully understand the underlying mechanisms, as this critical stage still represents a "black box" in the cancer field. Similarly, efforts to therapeutically target dormant DTCs are inherently challenging, compared with the primary tumor for example, and manipulation of the TME is likely to be essential for this to be successful.

CONCLUDING REMARKS: CHALLENGES AND OPPORTUNITIES

From the very beginning of the TME research field, therapeutic targeting of cells, processes, and signaling pathways in the TME was viewed as a promising strategy that could, in principle, be generalizable across all cancer types. The plethora of co-opted immune and stromal cells found in the TME are genetically

stable and are thus more straightforward to target compared with genomically unstable cancer cells. Moreover, the development of acquired resistance, at least via mutation-based selection mechanisms as observed for cancer cell-directed therapies, was thought to be less likely for similar reasons. Finally, there was also the hope that TME therapies might even represent a universal approach that could be applied to any tumor type, regardless of the organ in which it develops.

However, as the field has evolved in scope and understanding in recent years, we have come to realize that these early predictions were overly simplistic. As discussed in this review, we now appreciate the immense complexity and interconnectedness of the TME, as well as its diversity among different organs and patients. We also recognize that adaptive and intrinsic resistance can be an obstacle for TME-directed therapies. Moreover, it is evident that standard-of-care treatments, including chemotherapy and radiotherapy, elicit changes to the TME that modulate their therapeutic efficacy in a cancer cell-extrinsic manner, either augmenting or interfering with the response.[309] For example, radiotherapy and certain chemotherapies can elicit immunogenic cell death, which enhances their efficacy by engaging the adaptive immune system.[310] However, in other contexts, many of these same treatments can provoke an inflammatory reaction, including via TAMs, which then interfere with therapeutic response[85] and may even drive metastatic dissemination.[86]

Despite these challenges, there is also significant promise in terms of the expanding array of strategies for therapeutically targeting the TME, as recently reviewed.[1] These include therapies that either deplete or "reprogram" cancer-promoting host cells in the TME; interventions that modify the ECM, matrisome, and EVs; cell-based therapies and vaccines; and immune checkpoint inhibitors.[1] The key question now is how to combine these various approaches in a rational and optimal manner. Indeed, in the clinic, there are many more open combination trials than there are eligible patients to enroll in them.[311] This represents a formidable obstacle. The use of reliable preclinical models offers a means to first evaluate all logical combinations systematically. Indeed, the need to incorporate accurate preclinical avatars in the evaluation of new therapies is underscored by a recent meta-analysis of immunotherapy clinical trials, which found that >70% of such therapies were in trials without prior significant preclinical evidence supporting the combination being evaluated.[312] Additionally, a majority of trials were conducted in populations without any biomarker selection.[312] This highlights another challenge: how to optimally stratify patients for TME therapies, particularly for cancers that are not straightforward to biopsy for this purpose. It will be important to determine whether analysis of the relative abundance and/or phenotypes of circulating immune cells or EVs could represent a surrogate approach for analysis of the tumor.[313,314] Another critical question is how to target cancer cells that have disseminated and entered into a dormant state, as the regulation of their later emergence from dormancy undoubtedly involves modulation by the TME (Figure 3).

We will conclude by briefly highlighting some recent examples that can offer a "roadmap" for how to therapeutically target the TME, including efforts to extend the efficacy of immunotherapies beyond the current subset of patients with cancer to a much broader patient population. Indeed, there is increasing evidence that the TME also plays a crucial role in regulating immunotherapy efficacy (as for chemotherapy and radiotherapy), as highlighted throughout this review. This can vary from the physical exclusion of cytotoxic T cells, leading to immune-excluded TMEs, to the generation of an immunosuppressed TME in which T cells are present but dysfunctional due to interactions with immunosuppressive cells.[40] As such, strategies to combine ICB or cell-based immunotherapies with modulation of the TME are actively being explored in preclinical models or are already under clinical evaluation.[315,316] These include the administration of immuno-stimulatory cytokines such as IL-12 and IL-15, which activate NK cells[317]; local delivery of IL-2 (a key cytokine for T cells) into the TME via engineered MSCs[318]; and the development of variants of IL-2, engineered to boost T cell abundance and cytotoxic functions while avoiding similar effects on immunosuppressive Tregs.[316]

Additional combinatorial strategies take advantage of anti-angiogenic inhibitors, which were among the first TME-targeted treatments and subsequently found to have potent vascular modulation effects.[319] For example, anti-VEGF therapies can promote infiltration of immuno-stimulatory cells, block immunosuppressive effects in the TME, and improve drug delivery.[320] The complex interplay between the vasculature and immune system can additionally be exploited via interventions to enhance the formation of high endothelial venules (HEVs) given the association between tumor-associated HEVs, increased lymphocyte infiltration, and favorable prognosis for certain cancers.[321] These and other vascular-directed therapies are now being widely incorporated into combination strategies with ICB- and cell-based therapies to enhance immune cell infiltration and cytotoxicity in tumors.[1,320,321] Similarly, repurposing clinically approved treatments in novel combinations or via different dosing regimens that convert "cold" to "hot" TMEs represents another strategy to rapidly translate findings from preclinical models to the clinic, as revealed in recent representative studies.[1,322,323]

Looking forward, we are optimistic that key advances will be made to fully realize the promise of targeting the TME in the coming years. Instead of studying the TME one cell type at a time, we expect the field to adopt a comprehensive systems-level approach that analyzes and integrates the TME in all its complexity to identify and therapeutically target critical nodes. Integration of multi-modal data and advanced computational analyses, including the use of artificial intelligence,[324,325] will be crucial in achieving this goal. We also anticipate significant advances in bioengineering that will enable platforms for large-scale testing, such as *ex vivo* organoids and tissue slices that accurately recapitulate organ-specific TMEs.[326,327] It will also be essential for the field to understand the additional layers of complexity beyond the tumor itself, including systemic influences and the external environment (Figure 4). For example, how do the microbiome, diet, exercise, and metabolism impact the TME and therapeutic response? Additionally, what are the contributions from the underlying physiology of individual patients, such as obesity, cachexia, circadian cycle, inflammation, and aging? Finally, we can expect new insights into the impact of external environments, such as pollution and carcinogen exposure, on inflammation and the TME, which should motivate

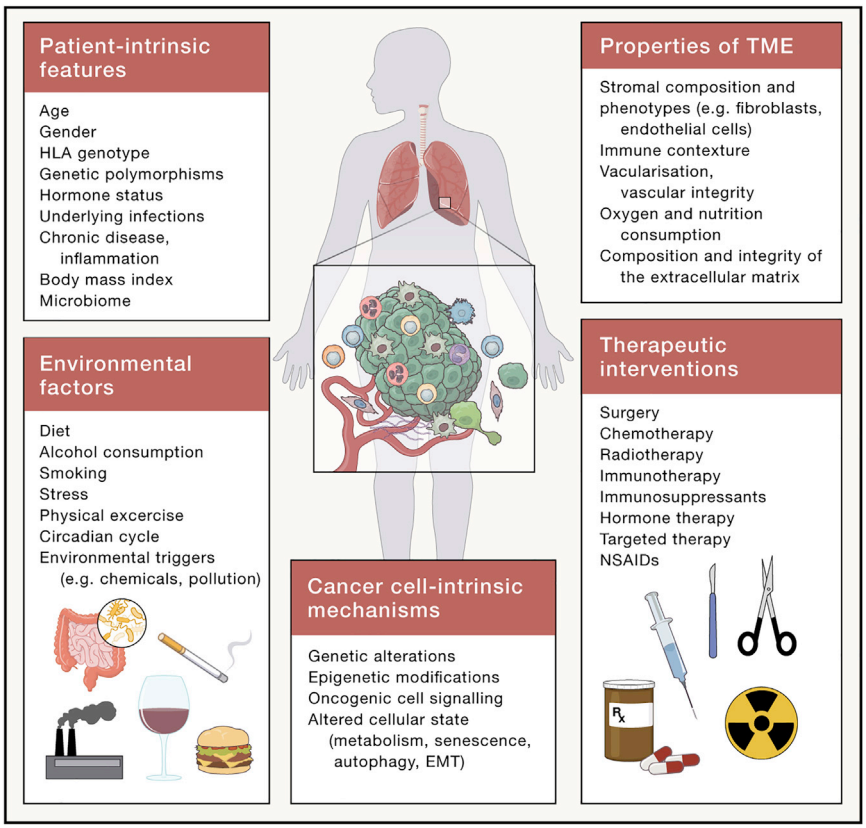


Figure 4. A multitude of cell-intrinsic and systemic factors impact the patient and the TME

This figure depicts the multiple layers of complexity that we must consider when endeavoring to holistically understand, and therapeutically target, cancer in patients. This includes tumor cell-intrinsic, and -extrinsic factors, along with systemic mediators of disease progression, such as underlying infection, inflammation, and chronic pathologies. Environmental factors are also emerging as important modulators of cancer progression, including pollution and other carcinogens. Finally, therapeutic interventions, including surgery and chemo-, radio-, and immunotherapy, can impact the TME and, conversely, be influenced by the TME and systemic changes in the patient. Therefore, rational combinations to effectively target this multitude of factors will be critical for developing effective, long-term control of the disease.

urgently needed public health responses. Collectively, by integrating and leveraging these key perspectives, we are optimistic that we will be able to therapeutically target the TME for the benefit of many more patients in the near future.

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