

Review

Tumor Innervation: Cancer Has Some Nerve

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Over the past decade, several landmark reports have demonstrated that the nervous system plays an active role in cancer initiation and progression. These studies demonstrate that ablation of specific nerve types (parasympathetic, sympathetic, or sensory) abrogates tumor growth in a tissue-specific manner. Further, many tumor types are more densely innervated than their normal tissues of origin. These striking results raise fundamental questions regarding tumor innervation, how it is initiated, and how it molecularly contributes to disease. In this review, we aim to address what is currently known about the origin of tumor-infiltrating nerves, how they may be recruited to tumors, and how their presence may give rise to aggressive disease.

Nerve Dependence across Cancer Types

Neural circuitry provides a means of communication between the brain and the rest of the human body. Canonical somatosensory nerves relay signals to the central nervous system for processing stimuli, while efferent motor and autonomic nerves relay signals from the central nervous system to target tissues throughout the body in order to respond to stimuli (Figure 1). However, it has recently become appreciated that normal neuron development and communication cues may be altered in the context of disease, including, but not limited to tumorigenesis.

While preliminary reports of nerves infiltrating tumors surfaced in the early 20th century, it was long thought that these components of the tumor microenvironment (TME) were merely passive bystanders [1,2]. However, the tumor innervation field has recently gained attention following the observation that cancer cells intimately interact with nerves infiltrating the tumor and that they do so with a purpose [3,4]. In 2001, cocultures of mouse neurons with prostate cancer cells revealed that neural processes actively extend towards cancer cells and ultimately stimulate their growth [5]. This study inspired the idea that nerves may not simply be bystanders within the TME, but instead may be functionally promoting tumorigenesis; cancer cells preferentially recruit these nerves to exploit nerve-mediated effects that fuel their expansion. What began as an observation has since transformed into an entire research focus dedicated to understanding when, how, and why nerve signals influence cancer progression.

Initial *in vitro* coculture experiments that rationalized studying tumor innervation led to the next logical question: What happens to tumors in the absence of nerves? A 2013 study set out to answer this, finding that prostate tumors are innervated by both adrenergic sympathetic and cholinergic parasympathetic nerves and that ablation of these nerves impairs *in vivo* tumorigenesis [6]. This paper was succeeded by other important nerve ablation studies in breast and gastric cancers that demonstrated a similar dependence on autonomic innervation [7,8]. However, it is important to note that there appears to be a tropic relationship between the tumor type and the innervation source. For example, stimulation of sympathetic nerves is associated with breast and pancreatic cancer progression, while stimulation of parasympathetic nerves is associated with a reduction in tumor growth [7,9,10] (Table 1). Further, models of head and neck squamous cell carcinoma, pancreatic ductal adenocarcinoma, cervical carcinoma, basal cell carcinoma, melanoma, and high-grade serous ovarian carcinoma demonstrate dependence on sensory

Highlights

Tumors may promote their innervation by recruiting locoregional nerves, neural progenitor cells (NPCs), and/or reprogramming other cell types.

Nerves may be attracted to the tumor microenvironment (TME) by both soluble secreted factors as well as functional cargo transported within tumor-derived small extracellular vesicles (sEVs).

Nerves contribute to disease progression, in both tumor cell-dependent and tumor cell-independent manners.

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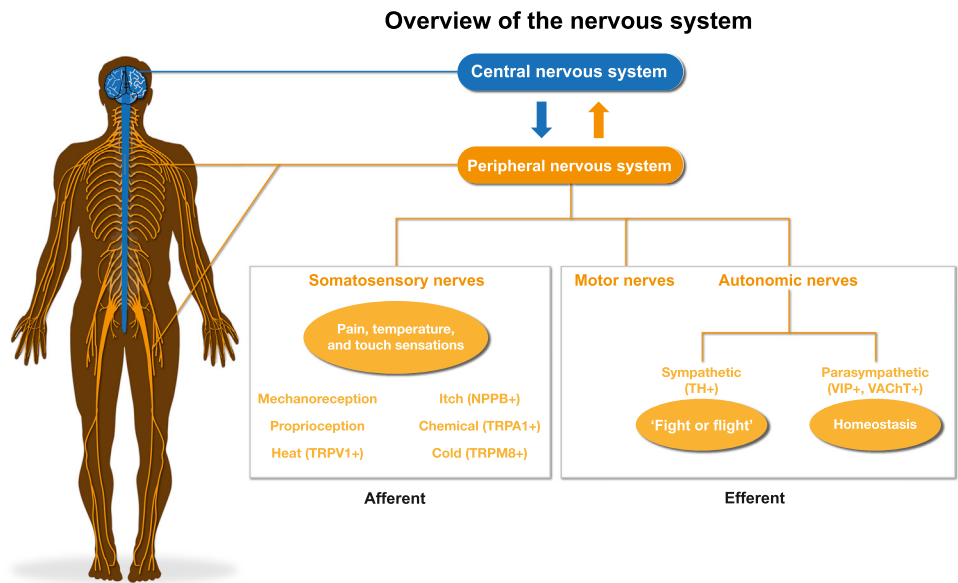
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Figure 1. Overview of the Nervous System. The central nervous system is comprised of the brain and the spinal cord (blue), that integrates afferent signals from the peripheral nervous system (orange) and consequently triggers a response by means of efferent signals to the body. Somatosensory nerves in the peripheral nervous system may be stimulated by activation of mechanoreceptors, proprioceptors, natriuretic peptide B (NPPB+) itch, transient receptor potential cation channel, subfamily A member 1 (TRPA1+) chemical, transient receptor potential vanilloid (TRPV1+) heat, or transient receptor potential cation channel subfamily M member 8 (TRPM8+) cold receptors. Efferent signals consist of both motor and autonomic nerves. Autonomic nerves may either be tyrosine hydroxylase (TH+) sympathetic nerves that control 'fight or flight' responses, or vasoactive intestinal peptide/vesicular acetylcholine transporter (VACHT+/VIP+) parasympathetic nerves that maintain homeostasis of the body.

Table 1. Summary of Innervation Studies across Different Cancer Types^a

	General innervation	Sympathetic	Parasympathetic	Sensory	Relevant guidance molecules	Refs
Basal cell	Undefined	Undefined	Undefined	+	Undefined	[15]
Breast	+	+	–	+	NGF, VEGFA ^b	[7,33,48,49]
Cervical	Undefined	Undefined	Undefined	+	sEVs	[13]
Colon	+	Undefined	–	Undefined	Undefined	[50–52]
Gastric	Undefined	–	+	Undefined	NGF	[8,53]
Glioma	+	Undefined	Undefined	Undefined	Undefined	[41,42,54,55]
Head and neck	+	Undefined	Undefined	+	EphrinB1 (sEV), miR-34a (sEV)	[12,14]
Melanoma	Undefined	Undefined	Undefined	+	Undefined	(S. Talbot, unpublished)
Ovary	Undefined	+	Undefined	+	BDNF	[16,56]
Pancreatic	+	+	–	+	SEMA3D	[9–11,35,37]
Prostate	+	+	+	Undefined	SEMA4F, proNGF, GCSF	[6,31,32,34,47]
Thyroid	+	Undefined	Undefined	Undefined	proNGF	[57]

^aInnervation associated with protumorigenic processes are annotated '+', and antitumorigenic processes are annotated '-'. Relevant neurotrophic factors regulating tumor innervation are listed when applicable. sEV indicates that small extracellular vesicle cargo is associated with the observed phenotype.
^bAbbreviations: BDNF, brain-derived neurotrophic factor; SEMA4F, semaphorin 4F; VEGFA, vascular endothelial growth factor A.

nerves [11–16] (S. Talbot, unpublished). Altogether, these findings highlight the importance of studying tumor innervation in a tissue-specific manner; it is not simply true that all innervation types promote disease progression in all cancer types.

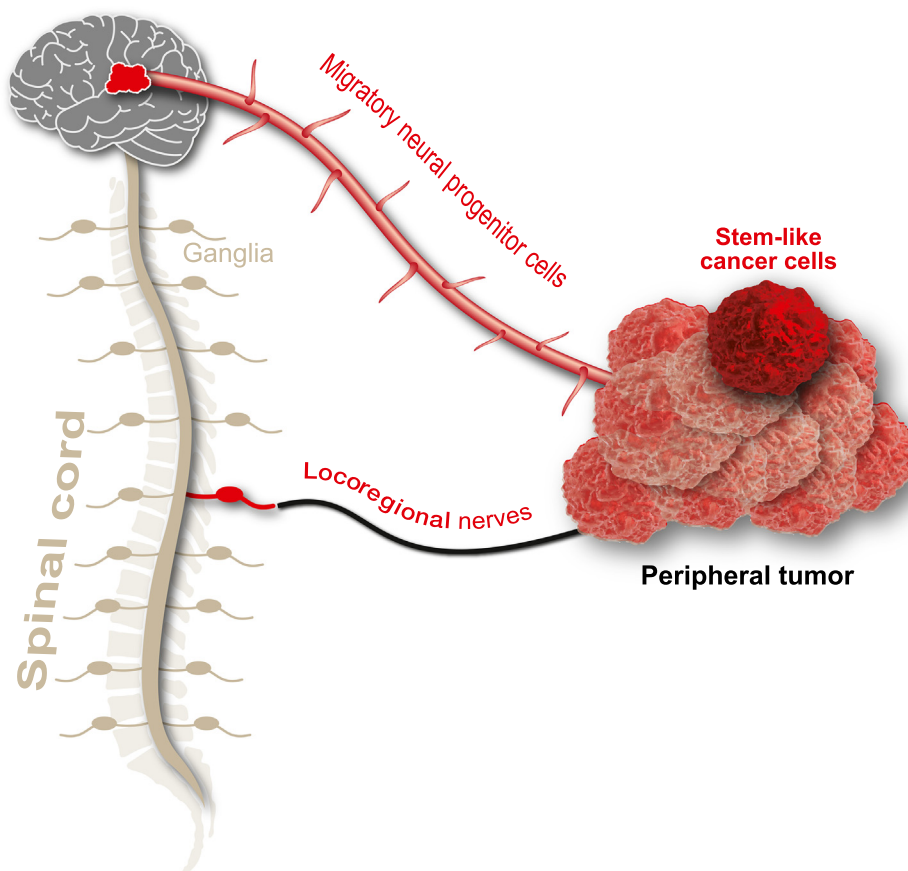
Tumor Nerve Origins

Locoregional Nerves

It seems logical that cancers recruit local peripheral nerves that normally innervate their tissue of origin. This hypothesis has recently been tested using uniquely engineered adeno-associated viral (AAV) vectors. Specifically, this study took advantage of retroAAVs that are retrogradely transported from nerve terminals to the nerve soma. By incorporating a neuron-specific promoter driving expression of diphtheria toxin (DTA) into the retroAAV, the authors were able to ablate specific subpopulations of tumor-infiltrating nerves. For instance, intratumoral injection of a retroAAV-TH-DTA results in retrograde transport of DTA, where expression is driven by the tyrosine hydroxylase promoter (TH, sympathetic); such expression specifically ablates sympathetic tumor-infiltrating nerves. Selective ablation of these TH+ nerves led to drastic reductions in metastasis and primary tumor volumes, highlighting the fact that tumor growth is regulated by nerves that directly interact with the tumor, as opposed to an artifact of systemic genetic or pharmacological nerve ablation [7]. Alternatively, other groups have employed mechanical severing of locoregional nerves to study breast cancer progression [8]. However, whether or not these nerve terminals extend from the ganglia in the spinal cord that typically provide innervation to normal tissue remains unknown (Figure 2). Another recent study suggests that locoregional nerves may be reprogrammed in the TME upon cancer cell transformation. Evidence suggests that *TP53* mutant oral cavity squamous cell carcinoma cells reprogram trigeminal sensory nerves to restore TH+ adrenergic nerves that are ablated by pharmacological sympathectomy with 6-OHDA [14]. Furthermore, severing of the trigeminal nerve decreased tumor growth in *TP53* mutant xenograft models. Therefore, it is indeed plausible that tumors may be exploiting locoregional neural plasticity to fuel their own growth.

Remote Neural Progenitor Cells

Curiously, the subtypes of nerves that are found in tumors are not always identical to the subtypes of resident nerves that infiltrate the tissue of origin. For example, transient receptor potential vanilloid (TRPV)1+ nociceptive pain sensory nerve ‘twigs’ are abundant in high grade serous ovarian carcinomas, yet absent from the normal fallopian tube and ovaries [16]. While it is possible that these *de novo* ‘twigs’ could be derived from existing sensory nerves in neighboring tissues, their origins may also be explained by an alternative, more provocative origin of tumor innervation. A recent prostate cancer study utilized lineage-tracing of doublecortin-positive (DCX+) neural progenitor cells (NPCs) that originate in the subventricular zone (SVZ) of the brain to examine whether tumor innervation can originate from the central nervous system. Importantly, the authors showed that SVZ-derived NPCs can escape the blood–brain barrier and travel through the circulation to colonize the prostate tumor. Upon arrival in the TME, these NPCs mature to form TH+ sympathetic nerves, which were previously shown to promote prostate tumorigenesis [6]. In addition, they show that depletion of these DCX+ NPCs abrogates prostate tumor initiation and progression and that transplantation of DCX+ cells from the SVZ enhances tumor xenograft growth and metastasis *in vivo* [17]. This model provides important insight into how cancer may be taking advantage of normal systemic developmental processes like neurogenesis and further indicates that complex signals exist between peripheral tumors and the central nervous system. NPCs maintain their quiescent, undifferentiated state at least in part by cell–cell interactions with vascular endothelial cells [18]. Therefore, cancer-derived factors in the circulation could be recruiting these cells to the TME. In support of this idea, a 1998 study showed that NPCs in the SVZ of adult human cancer patients actively incorporate the thymidine-analog BrDU into their DNA, indicating that



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Figure 2. Schematic of Potential Sources of Tumor Innervation. Densely innervated tumors may be supported by neurons derived from many different origins. In addition to canonical locoregional innervation extending from spinal cord ganglia, neural progenitor cells from the subventricular zone may be able to migrate through the vasculature to peripheral tumor sites. Alternatively, plastic or stem-like cancer cells may transdifferentiate within the tumor microenvironment and support disease progression.

under pathological conditions, these cells are indeed capable of escaping their quiescent state and re-entering the cell cycle [19]. Given that NPCs from the SVZ are thought to be tumor progenitor cells in glioblastoma, this concept of atypical NPC activation is further strengthened [20]. However, the scale of SVZ neurogenesis in the adult human is highly controversial. While several groups have established the proliferative potential of NPCs in the SVZ, it is still heavily debated whether or not these NPCs are capable of noncanonical migration and/or persist into adulthood [21–23]. Further studies into the migratory capacity of adult NPCs will be necessary to determine the potential of these cell populations to contribute to tumor infiltrating nerves.

Cancer Stem Cells and Plastic Cancer Cells

Stem cells are classically considered to be cells that can both self-renew and differentiate to generate progeny of different lineages [24]. In the context of cancer stem cells, this means that tumor heterogeneity may be informed by a single cell with the capacity for ‘stemness.’ By this notion, it is not unreasonable to assume that fate determination during the expansion of cancer stem cell progeny could, in fact, give rise to neural populations within the tumor that are capable of differentiating into fully functional nerves in the TME. Lineage tracing of *ex vivo*-tagged human gastric and colorectal carcinoma stem cells revealed that these cells

not only produce tumors in xenograft mouse models, but also that the tagged human cancer stem cells differentiate and express neural proteins upon engraftment. Moreover, inhibition of this differentiation capacity abrogated xenograft tumor growth [25]. Taken together, these data strongly support the idea that cancer stem cells can expand and mature into heterogeneous cell populations, including neuron-like cells, that are found in the TME. Identification of stem-like progenitors in prostate cancer revealed that a subset of these cells exhibit neurogenic gene expression profiles, consistent with the cancer stem cell nerve origin hypothesis [26]. While it is appreciated that both cancer stemness and nerve density are independently associated with aggressive disease, further investigation is required to determine the relative contribution of cancer stem cells to tumor innervation.

Cancer cell plasticity has also been implicated in the generation of neuron-like cells in the TME. By contrast to cancer stem cells, 'plastic' cancer cells are not necessarily multipotent, self-renewing cells, but rather cancer cells whose identity is capable of being reversibly reprogrammed [27]. For example, *in vitro* studies demonstrate that a cocktail of epigenetic inhibitors (HDACi + EZH2i + LSD1i + DNMTi) can induce expression of neural gene programs in a variety of cancer cell lines [28]. With the notion that cancer cells are plastic and capable of gaining neural identity, these data raise the question as to whether a neural reprogramming phenomenon may be occurring naturally *in vivo* during tumorigenesis. 3D cocultures of rat SVZ neural stem cells with human pancreatic cancer cells have revealed that cancer cells aggregate on the surface of neurospheres and begin to mimic the morphology of differentiated neurons. Further, implantation of these hybrid cancer cells into nude mice accelerates tumor progression, indicated by decreased survival of mice harboring hybrid xenografts when compared with the parental pancreatic cancer cell line [29]. These data not only underscore the correlation between neural cells and aggressive disease, but also suggest that these populations may arise from reprogrammed cancer cells themselves.

Mechanisms of Nerve Recruitment

Growth Factors and Axon Guidance Molecules

Under normal physiological conditions, nerve growth is dictated by changes in intracellular neuronal signaling in response to local biochemical signals, including neuropeptides, neurotransmitters, growth factors, and morphogens, that recruit axons to target tissues. As cancer is known to exploit normal developmental processes to fuel tumorigenesis, evidence suggests that secretion of these proneural signals, particularly growth factors, in the TME may facilitate nerve recruitment [30] (Table 1). For example, prostate cancer cells secrete a precursor to neural growth factor (proNGF) that induces axonogenesis *in vitro* [31]. Granulocyte-colony stimulating factor (GCSF) has also been implicated in prostate tumor innervation, indicating that this process is multifaceted and may be mediated by multiple secreted factors [32]. Further, inhibition of NGF in breast cancer cells partially abrogates neurite outgrowth, bolstering the idea that these factors secreted by the tumor can alter the behavior of nerves and highlighting the tissue-dependent nature of tumor innervation [33].

In addition to circulating growth factors, axon guidance molecules may either be secreted or present on the surface of tumor cells in the TME to promote directional cues that ensure tumor infiltration. Functional testing reveals that overexpression of semaphorin 4F (S4F) increases both the frequency of neurite outgrowth as well as neurite length in *in vitro* cocultures with mouse neuroblastoma cells [34]. Another recent study has further implicated semaphorin overexpression in the enhancement of nerve-dependent tumorigenesis, reporting on another member of the semaphorin family, SEMA3D. In this study, the authors found that SEMA3D secreted by pancreatic cancer cells can engage and activate plexin D1 (PLXND1) on the neural

surface. The resulting innervation observed in PLXND1 active pancreatic cancer models was abrogated upon SEMA3D knockdown and/or inhibition of this paracrine signal with a PLXND1 neutralizing antibody [35]. Taken together, these results suggest a functional role of semaphorin-mediated axon guidance in recruitment of nerves to the TME. However, it is important to note that while these neurotrophic factors have been implicated in axonogenesis, whether they are directly secreted into the extracellular space or released as extracellular vesicle cargo remains under investigation.

Small Extracellular Vesicles

While it has been long appreciated that small extracellular vesicles (sEVs) released from cancer cells increase tumor growth [36], recent evidence suggests that these sEVs also have axonogenic properties. In *in vitro* coculture experiments with rat pheochromocytoma (PC12) cells and sEVs isolated from cervical carcinoma cell cultures, robust neurite outgrowth is observed [13]. In accordance with these data, sEVs isolated from high-grade serous carcinomas (HGSCs) and oncogene-transformed fallopian tube cell lines similarly induce PC12 neurite outgrowth *in vitro*, whereas sEVs isolated from normal fallopian tube cell lines do not [16]. Not only does this imply that sEVs play an important role in signaling with neural cells in the TME, but also that newly transformed cancer cells may be initiating this conversation early on in tumorigenesis. These data are further supported by similar reports in head and neck squamous cell carcinoma, where genetic and pharmacological inhibition of sEV release not only abrogate tumor growth, but also decrease tumor innervation [12]. Further analysis of sEV cargo revealed that this innervation phenotype is enhanced by the membrane protein EphrinB1. Another group recently employed miRNA profiling of sEV cargo to find that miR-34a is transported within sEVs from TP53-WT (wild type) head and neck cancer cells to suppress neuritogenesis of local sensory nerves by disrupting the expression of genes involved in sensory nerve differentiation [14]. However, aside from these two studies, the functional components of the sEV cargo that mediate tumor innervation remain largely unknown.

Effects of Tumor Innervation on Disease Progression

Direct Effects on Cancer Cell Proliferation and Migration

Cancer-mediated recruitment of nerves to the TME triggers reciprocal signaling whereby interactions with nerves support the progression of disease. For example, cocultures of pancreatic cancer cells with dorsal root ganglia (DRG) exhibit increased proliferation, as indicated by strong Ki-67 staining, as well as decreased apoptosis in the presence of these sensory neurons [37]. Similarly, in prostate cancer, cells that are closer to the nerve exhibit increased Ki-67 staining and decreased apoptosis when compared with cancer cells that are further away from the site of the nerve, which is believed to be mediated by increasing NF- κ B signaling in the cancer cells [38]. From a paracrine perspective, several cancer types exhibit neuropeptide receptors on their cell surface, providing a plausible mechanism for nerve-to-tumor communication. In support of this idea, inhibition of neuropeptide receptors on esophageal squamous cell carcinoma cells decreases cell proliferation and viability in a PI3K/AKT/NF- κ B-dependent mechanism [39]. When evaluating cancer cell surface receptors that may be responsive to nerve-derived factors, it is also important to mention that several tumor types express neurotrophic growth factor receptors, indicating that signaling molecules may also act on the cancer cells in an autocrine manner [40]. Alternatively, two pivotal studies in glioma have recently revealed that cancer cells may be capable of forming direct, functional synapses with neurons [41,42]. Not only are postsynaptic signals detectable in glioma cells, but direct activation of the presynaptic neurons stimulates glioma proliferation. These studies are the first of their kind, showing that electrical stimulation of neurons mediates direct communication with cancer cells in a synaptic manner. Altogether, these data raise questions as to whether direct interactions of neurons and cancer

cells are primarily mediated by electrical and/or biochemical signaling processes and will need to be further investigated in peripheral tumor types.

In addition to the nerve-mediated pro-growth signaling, *de novo* innervation may also stimulate intracellular migratory programs. *In vitro* pancreatic cancer/DRG cocultures [37] support this, where increased proliferation and decreased cell death were accompanied by retrograde extension and migration of cancer cells towards the sensory nerves. It is important to distinguish that this process is distinct from the clinical process of perineural invasion (PNI); PNI is a process whereby tumor cells migrate along existing nerve bundles that serve as a 'highway' for tumor dissemination. Conversely, *de novo* tumor innervation refers to the infiltration of nerve 'twigs' that lack the same structure and organization seen in resident tissue nerves. Additional investigation will be required to determine the mechanisms of *de novo* tumor innervation on cancer cell migration.

Indirect Effects on Non-cancer Cells within the TME

Several recent reports suggest a role for activated nerves in modulating other non-tumor cell populations within the TME. Importantly, immune responses are susceptible to modulation by the nervous system [43,44]. For example, stimulation of the vagus nerve induces the release of acetylcholine, which inhibits tumor necrosis factor (TNF)-mediated macrophage activation in the presence of endotoxins [45]. With this in mind, it is reasonable to hypothesize that prolonged exposure to secreted neural factors may sculpt the immune landscape to allow a tumor to evade immunosurveillance (Figure 3). In the context of the innervated TME, recent reports suggest that the adaptive immune landscape changes quite significantly upon sympathectomy. In analyzing the T cell landscape, ablation of sympathetic nerves decreases expression of the immune checkpoint molecule programmed cell death-1 (PD-1) on T cells, indicating that these lymphocyte populations should be more effective at mounting an antitumor immune response. Moreover, they report that sympathetic innervation increases immunosuppressive FOXP3+ regulatory T cell populations [7]. Gene expression analysis within

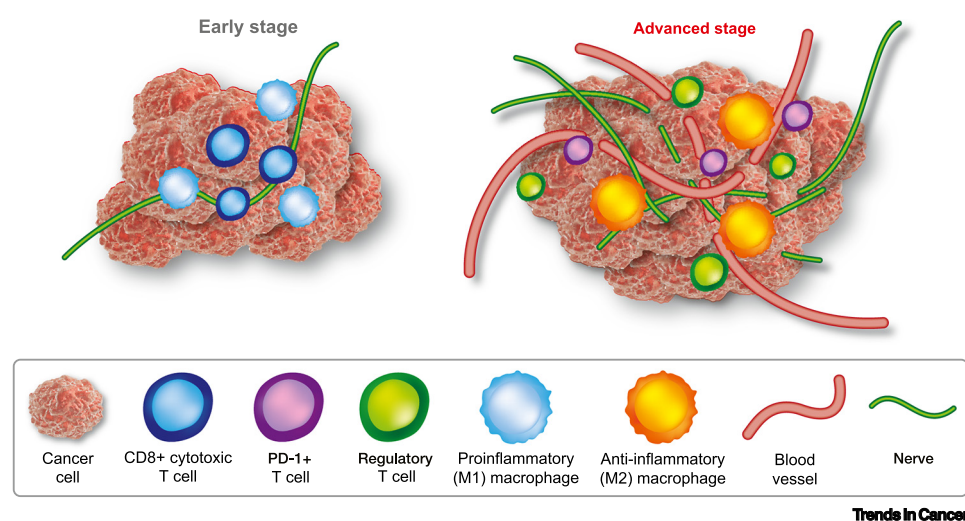


Figure 3. Dense Innervation Influences Immune Cell Populations and Vasculature within the Tumor Microenvironment. During early tumorigenesis, tumors are likely sparsely innervated and vascularized, with active immunosurveillance managed by proinflammatory macrophages and active cytotoxic T cells. As the cancer progresses, dense innervation yields increased angiogenesis and preferentially supports the survival of immunosuppressive cell populations [anti-inflammatory M2 macrophages, exhausted/PD-1 (programmed cell death-1) expressing T cells, regulatory T cells].

triple-negative breast cancer patients revealed that patients with a high neurogenic signature also exhibit a more immunosuppressive profile when compared with other subtypes, marked particularly by M2-macrophage polarization [46]. In a more recent melanoma study, TRPV1+ sensory nerve ablation restores cytotoxic CD8+ T cell activity, as indicated by decreases in the number of exhausted cytotoxic T cells present in the TME. This phenotype is mediated by neuropeptides released from TRPV1+ sensory nerves that are enriched in the melanoma TME, suggesting a direct role for innervation in tumorigenesis by suppressing the immune response (S. Talbot, unpublished).

During development, nerves extend alongside endothelial cells that construct the vasculature. Importantly, recent reports show that early angiogenesis, another hallmark of cancer, is regulated by sympathetic adrenergic nerves during tumor development [47]. While this study has broadened the implications of tumor innervation on remodeling the vasculature of the TME (Figure 3), it is one of the first of its kind and will need to be investigated in other contexts.

Concluding Remarks

While it has become increasingly clear that nerves play a functional role in cancer, there is still much to be uncovered. With several investigations into tumor innervation in different tumor types, we can appreciate that 'one size' does not fit all. Further studies into the role of each nerve type in different cancer contexts will help to elucidate potential therapeutic approaches. Understanding the balance between stemness and natural innervation will provide further biological insight into how cancer hijacks normal developmental processes (see Outstanding Questions). Identifying the relevant players that participate in attracting these nerves could identify targetable growth factors and/or sEV-enclosed neurotrophic factors that could be successful in staving off nerve-mediated tumor onset. Lastly, understanding the interplay between these tumor infiltrating nerves, tumor cells, and non-tumor cells may inform potential combination therapeutics to further weaken the TME barrier that protects these malignancies. Altogether, the identification of nerve dependence in cancer has illuminated the field and should prove to be a very exciting new therapeutic opportunity.

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Outstanding Questions

Do tumors hijack locoregional nerves?

How abundant are neural progenitor-derived tumor infiltrating nerves?

Are plastic/cancer stem cells capable of transdifferentiating and forming mature nerves?

What are the neurotrophic contents of cancer-derived sEVs?

Do nerves form functional synapses with cancer cells in non-glioma cancers?

How do nerves primarily change cancer intrinsic and extrinsic programs to support tumorigenesis?

Can nerve origins, neuroattractants, tumor-neural synapses, and effects of innervation be mitigated therapeutically?

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