Salting the soil: Targeting the microenvironment of brain metastases

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Abstract

Paget's 'seed and soil' hypothesis of metastatic spread has acted as a foundation of the field for over a century, with continued evolution as mechanisms of the process have been elucidated. The CNS presents a unique soil through this lens, relatively isolated from peripheral circulation and immune surveillance with distinct cellular and structural composition. Research in primary and metastatic brain tumors has demonstrated that this tumor microenvironment (TME) plays an essential role in the growth of CNS tumors. In each case, the cancerous cells develop complex and bi-directional relationships that reorganize the local TME and reprogram the CNS cells, including endothelial cells, pericytes, astrocytes, microglia, infiltrating monocytes, and lymphocytes. These interactions create a structurally and immunologically permissive TME with malignant processes promoting positive feedback loops and systemic consequences. Strategies to interrupt interactions with the native CNS components, on 'salting the soil,' to create an inhospitable environment are promising in the preclinical setting. This review aims to examine the general and specific pathways thus far investigated in BrM and related work in glioma to identify targetable mechanisms that may have general application across the spectrum of intracranial tumors.

Introduction

Brain metastases (BrMs) represent the most common intracranial tumor and affect an estimated 10-20% of all cancer patients.[1–3] The incidence of BrM continues to rise, likely due to increased detection with magnetic resonance imaging (MRI) and improved survival from continued progress in cancer management. Lung, breast, and melanoma are the most common primary tumors to metastasize to the brain. However, renal cell and colorectal BrMs remain significant.[4] There have been many recent advances in the multimodal management of BrMs across surgery, radiotherapy, and systemic therapies; in particular, stereotactic radiosurgery and targeted therapies with greater intracranial penetration have altered the treatment paradigm in many cancers.[5] Despite this, the presence of BrMs continues to portend a poor prognosis as long-term survival rates remain unacceptably low.[6,7] Furthermore, neurologic symptoms such as headache, seizures, focal deficits, and cognitive impairment, as well as toxicity from treatment, can impair a patient's quality of life and contribute to morbidity.[8]

With this context, the origins of the classical ‘seed and soil’ view of metastatic spread reach back to Paget’s work in 1889.[9] In the ensuing 130 years, a significant evolution in our understanding of these processes has, of course, taken place. However, some fundamental ideas remain true to this day. A greater biological understanding of BrM pathophysiology and the metastatic cascade is crucial to developing novel and improved therapeutic strategies. This review will focus on the soil itself, the central nervous system (CNS) tumor microenvironment (TME), and discuss the current state of knowledge regarding how brain-metastatic cells manipulate and restructure the native components and architecture to create an actively pro-tumorigenic setting. Characterizing the changes within this ‘soil’ and understanding the existing literature on preventing or reversing these processes will allow for the identification of common pathways shared across a range of primary tumor sources in order to pursue therapeutic strategies aimed towards creating an inhospitable CNS TME both before and after the establishment of macrometastatic lesions.

Physiologic Brain Microenvironment

It is necessary to appreciate the unique CNS microenvironment in non-pathologic conditions. The brain contains a dense microvasculature network that circulates roughly 15-20% of the total cardiac output, with outflow filtered into the dural sinuses, and eventually returned to the venous system.[10] The CNS is isolated from peripheral circulation at the boundary of this vasculature by the blood-brain barrier (BBB). This highly selective filter regulates the passage of solutes into the extracellular fluid of the CNS.[11] Beyond the BBB, the CNS's cellular elements predominantly consist of neurons and supportive glial cells, including astrocytes, microglia, pericytes, and oligodendrocytes. The BBB itself comprises endothelial cells connected by tight junctions and supported through astrocyte projections with pericytes, similar to vascular smooth muscle cells, embedded in the basement membrane.[12] The BBB permits the diffusion of hydrophobic molecules and small polar molecules in the physiologic state while restricting that of larger or hydrophilic solutes, relevantly including pathogens, antibodies, and many chemotherapeutic drugs.

Astrocytes within the CNS act as the primary support cell for neurons, with a range of functions that include regulation of nutrient and solute availability, neurotransmitter reuptake, blood flow, and the response to areas of inflammation or injury.[13] Microglia are the primary effector cells of the innate immune

system within the brain, the CNS equivalent of peripheral monocytes, while oligodendrocytes supply the myelin sheaths surrounding the axons of neurons in a manner analogous to peripheral Schwann cells.[14,15] Lastly, the extracellular matrix of the CNS plays essential roles in physical and homeostatic support, from the pericyte-containing basement membrane of the BBB to the perineuronal and intraparenchymal matrices.[16] Throughout the metastatic process, tumor cells manipulate and reorganize these cellular and extracellular components of the CNS through targetable mechanisms to create a pro-tumorigenic, therapy-resistant environment, as will be discussed in the sections below.

Metastatic Cascade

The metastatic spread, described as the 'metastatic cascade[17,18], begins with local invasion at the primary tumor site, migration into blood vessels, extravasation at a distant site, the initial proliferation of micrometastases, and the eventual establishment of a macrometastatic lesion.[19] The CNS setting is unique relative to other sites of metastasis for several reasons. First, circulating tumor cells must pass through the BBB at extravasation (depicted in Figure 1 [A-C]). Notably, alternative pathways that bypass the BBB have also been suggested, including traversal across the laminin-rich basement membrane of bridging vessels into the subarachnoid space in the case of leptomeningeal metastases of acute lymphoblastic leukemia or via functional lymphatic vessels lining the dural sinuses[20,21]. Regardless, the would-be metastatic cells then encounter a set of native cellular components and non-cellular architecture with distinct immune parameters once within the CNS. In this setting, and before encountering it, a complex and bi-directional interplay occurs in which the metastatic cells manipulate the CNS TME to their advantage. Understanding the factors that set the stage for extravasation at the CNS rather than other locations and the subsequent changes within the microenvironment is critical to generating therapeutic strategies based on preventing or mitigating those factors.

The premetastatic niche

Even before the arrival of circulating tumor cells at the distant site, the scene's initial setting has occurred with creating the premetastatic niche. This phase of the cascade encompasses preparation of the colloquial 'soil' in advance of colonization by circulating tumor cells. The process is mediated through the secretion of cytokines, chemokines, and angiogenic factors from the primary tumor site. Such mechanisms have been demonstrated in several primary and metastatic sites, with less direct investigation in BrMs. In the general case of systemic metastases, implicated actors include vascular endothelial growth factor A (VEGFA), lysyl oxidase-like protein (LOXL2), C-C motif ligand 2 (CCL2), C-X-C motif chemokine ligand 17 (CXCL17), tumor necrosis factor-α (TNF-α), transforming growth factor-β (TGF-β), vascular adhesion molecule 1 (VCAM-1), and C-X-C motif receptor 4 (CXCR4), among others.[22,23,23–28]

Regarding BrM, several secreted factors have been shown to influence the permeability of the BBB including VEGFR, angiopoietin-2, matrix metalloproteinases (MMPs, specifically MMP-2 and MMP-9), and placental growth factor (PLGF).[29–32] Feng *et al.* and Li *et al.* demonstrated that these changes are mediated through disruption of tight junction proteins, including ZO-1, claudin 5, and occludin.[30,32] Soto *et al.* highlighted the importance of premetastatic conditioning in the CNS with their finding that brain vascular endothelial cells upregulate cell adhesion molecules (CAMs) soon after the injection of metastatic cells into

the peripheral circulation, including VCAM-1, ALCAM, ICAM-1, VLA-4, E-selectin, and β_4-integrin, at the same time corresponding ligands are upregulated on circulating tumor cells. In consideration of potential therapeutic application, this group also demonstrated that neutralization of these CAMs through targeted monoclonal antibodies significantly reduced tumor seeding within the brain[33]. Another study by Liu *et al.* in mice found that before the development of BrM, the brains of mice bearing orthotopic breast tumors showed significant accumulation of bone marrow-derived $CD11b^{+}Gr1^{+}$-myeloid cells expressing inflammatory chemokines S100A8 and S110A9. These inflammatory mediators attracted both the tumor cells and myeloid cells through Toll-Like Receptor-4 (TLR4), and treatment with both anti-Gr1 and cyclooxygenase-2 inhibitors (as well as analogous knockout mouse models) reduced the infiltration of myeloid cells and subsequent formation of BrM.[34]

Tumor-derived exosomes are another factor in conditioning the eventual metastatic site. These exosomes are extracellular vesicles containing tumor-produced factors, including proteins, lipids, and nucleic acids, released into circulation from the primary site. The exosomes then interact with resident cells at distant locations through extracellular signaling or fusion with subsequent intracellular cascades.[35,36] Studies in extracranial metastases of various primary tumors have demonstrated the role of tumor-derived exosomes in the induction of a pro-tumorigenic premetastatic niche by modifying the inflammatory, immunologic, and angiogenic parameters of the eventual metastatic location. Some of the factors involved include PD-L1, miRNAs, intracellular signaling mediators, inflammatory cytokines, and various chemokines.[37–41] Importantly, these exosomes have been shown to have site-specificity dependent on their integrin (ITG) profile, with $ITG\beta_3$ specific to the brain.[42] These exosomes subsequently promote a site-specific local premetastatic niche in part through S100 gene regulation, and both knockdown and drug inhibition models aimed at target integrins have successfully blocked organ-specific tropism *in vitro* and *in vivo.*[42] Another fascinating study made use of engineered nanoparticles to capture circulating breast cancer tumor-derived exosomes *in vivo* with significantly reduced rates of systemic metastases.[43]

In applying these concepts to the CNS premetastatic niche, a recent study by Morad *et al.* demonstrated that such tumor-derived exosomes are capable of migrating through the BBB *in vivo* via transcytosis.[44] Exosomes have been shown to contain miRNA that suppresses glucose uptake in astrocytes *in vitro*, through miR-122, which creates an environment favoring the proliferation of metastatic cells. The same study verified that the miR-122-containing tumor-derived exosomes increased BrMs *in vivo* and that anti-miR-122 treatment reduced metastasis to both the brain and lungs.[45] Exosomes containing the miRNA miR-181c in another brain-seeking breast cancer metastatic model were shown to promote the breakdown of the BBB *in vitro* and increase BrMs *in vivo*. The group corroborated these findings with increased miR-181c in patient serum samples from those with BrM compared to those without.[46] A study by Rodrigues *et al*. identified a particular protein, cell migration-inducing, and hyaluronan-binding protein (CEMIP), enriched in brain-tropic breast and lung cancer tumor-derived exosomes. The group showed that CEMIP induces upregulated cytokine and chemokine production and angiogenesis in the brain, promoting metastatic colonization of the CNS. Furthermore, knocking out CEMIP reduced BrMs by 70% *in vivo*, indicating that CEMIP is required for the early stages of metastatic colonization. These results were corroborated in clinical samples with the correlation of CEMIP expression to BrMs and survival.[47] Extending these results and those identified in other distant sites to the CNS presents an opportunity to target tumor-

derived exosomes and their associated pathways, with supportive preclinical data, for the prevention of BrMs long before they become clinically relevant.

Therapeutic applications of targeting the premetastatic niche will need preclinical research strategies aimed at multiple sites. Reduced incidence of BrM was demonstrated with monoclonal antibodies targeted towards upregulated CAMs and brain-specific ITGs, and treatment with anti-Gr1 and cyclooxygenase-2 inhibitors, as well as with genetic knockdown and knockout studies of additional targets in the pathways. Such a strategy could prove immensely beneficial in actively preventing BrMs rather than responding after the fact.

Extravasation through the BBB and seeding of the CNS

The first step distinguishing BrMs from other sites is the BBB transversal by circulating tumor cells, a process that is, as of yet, incompletely understood. The BBB is the basis of the 'immune-privilege' designation of the CNS, though its immutability has been disproven with the identification of the CNS lymphatic network and mechanisms of infiltration by circulating immune cells, particularly in states of injury and inflammation.[21,48]

Various groups have suggested both paracellular and transcellular routes through the BBB, particularly with co-opting existing pathways for leukocyte extravasation.[49] Several surface molecules and soluble factors have been identified as essential factors in the process of BBB transmigration, including selectin ligands, integrins, cadherins, proteases, and various chemokines and cytokines. The range of these molecules is broad across primary tumor histologies, indicating multiple mechanisms with common factors. On the circulating tumor cells, specifically identified mediators include the expression of the adhesive membrane proteins ST6GALNAC5 and CD44, upregulation of COX2, CXCR4, HBEGF, EREG, and ITG$\alpha_v\beta_3$, increased secretion of VEGF, angiopoietin-2, PLGF, and S100A4 from tumor cells and brain endothelial cells, secretion of proteases including cathepsin S, matrix metalloproteinase (MMP) MMP-1, MMP-2, MMP-3, MMP-9, and ADAM-8, surface melanotransferrin expression on melanoma cells, rho kinase signaling in small cell lung cancer (SCLC), and various other upregulated CAMs.[29,30,38,50–56] These factors' shared effect is to increase the permeability of and adherence to the BBB, permitting transmigration by the circulating tumor cells. Thus far, inhibition of a number of these factors, including MMP1, COX2, HBEGF, EREG, ST6GALNAC5, VEGF, and endothelial Rho-kinase, has been shown to significantly reduced incidence of BrM in preclinical studies.[29,50,53,57(p1),58]

Many of these studies utilized RNA knockdown or transgenic knockout experimental strategies. The existence of small molecule inhibitors and monoclonal antibodies against several targets presents an opportunity to disrupt essential pathways and protect the CNS from metastatic reprogramming, with supportive data for the COX-2 inhibitor celecoxib, rho kinase inhibitor Fasudil, anti-EREG monoclonal antibody, and HBEGF inhibitors.[34,53,59,60] These results provide proof of concept for a strategy to prevent BrM by targeting the factors that mediate initial access to the CNS.

Initial tumor proliferation and colonization of the brain parenchyma

Single-cell *in vivo* studies have demonstrated that the vast majority of metastatic tumor cells fail to proliferate beyond the micrometastatic phase after initial transmigration through the BBB.[61] For cells that

progress, the development of a complex and evolving TME begins as the metastatic cells interact with the resident CNS components. Initially, the metastatic cells remain near the extravasation site at the blood vessel's abluminal surface, where the developing tumor is supplied with essential nutrients to facilitate its accelerating growth.[61] As the metastatic cells proliferate, these needs multiply, and the tumor manipulates the local vasculature through co-option of existing vessels and induced angiogenesis.[62] These vascular remodeling processes are thought to be directed through VEGF, integrins, and cell adhesion molecules (particularly ITG$\alpha_v\beta_3$, ITGβ_1, and L1CAM) from both metastatic and CNS cells.[63] Following perivascular migration, colonization of the brain parenchyma by metastatic tumors is dependent on the activation of diverse signaling networks that promote crosstalk within the TME and the metastatic cell's acquisition of neuronal phenotypes.[5,64,65] Examples include co-option of γ-Aminobutyric acid (GABA) as an oncometabolite and the activation of an AXL-ABL2-TAZ signaling axis to promote the expression of neuronal-related factors in brain-metastasizing lung adenocarcinoma cells.[65,66] Among these factors is the neuronal CAM L1CAM, a target of TAZ-dependent transcription, which regulates vascular co-option and migration and tumor outgrowth.[67,68] Whereas there are no effective therapies to target L1CAM, pharmacological inhibition of either ABL or AXL tyrosine kinases downregulates TAZ-driven L1CAM gene expression and decreases BrMs in lung adenocarcinoma models.[66]

Bohn *et al.* demonstrated in their preclinical model that bevacizumab reduces BrMs when administered 10 days after circulating tumor cell injection; however, whether this disrupted seeding or subsequent vascular remodeling is unclear from their design. Ilhan-Mutlu *et al.* showed a potential preventative role for the therapy with the finding that administration of bevacizumab 24 hours after circulating tumor cell injection reduced single-cell, micro-, and macrometastases in the CNS at subclinical doses, along with prolonged overall survival and correlated clinical data from the AVAiL trial.[69,70] Furthermore, inhibition of PLGF has also shown success in slowing the growth of VEGF-resistant tumors as well as reducing the rate of metastasis and TAM M2 polarization, with promising Phase 1 trial evidence supporting its safety.[71–75]

Another implicated group throughout the initial phase of BrM establishment are the MMPs. The strategy of interrupting MMP activity has been validated with RNA interference studies in CNS metastatic models of leukemia and breast cancer, and with MMP pharmacologic inhibitors in *in vivo* preclinical models.[32,58] The history of MMP inhibitors in clinical trials has been unfortunately unsuccessful; while significant preclinical data supported their use, trials throughout the early 2000s showed few successes and significant musculoskeletal side effects. However, with the development of novel, specific MMP inhibitors, re-visiting this strategy as a method to prevent tumor-driven reorganization of the CNS is a new opportunity for the defense of the CNS microenvironment. Specific targeting of MMP-9 in colorectal cancer has shown successes without the characteristic musculoskeletal side effects, and a similar strategy may be useful in preventing BrMs.[76] Notably, the earlier previous clinical trials were conducted on patients at all stages of progression, and a focus instead on preventing BrMs may be the most promising avenue forward. Relevantly, *in vivo* administration of an MMP inhibitor (targeted at MMP-2, MMP-9, and MMP-13) two days after orthotopic breast cancer inoculation showed a significant reduction in tumor size and lung metastases. However, similar studies have not yet been performed in BrMs.[77] Interestingly, doxycycline is a multi-specific MMP inhibitor with activity against MMP-9, and similar tetracyclines have been shown to prevent

lung metastases from renal adenocarcinoma and bone metastases of breast cancer in combination with a COX-2 inhibitor, as well as inhibiting glioma growth.[78–82]

Targeting this initial phase of metastatic propagation could significantly improve the effectiveness of the early anti-tumor response. The studies above showed success in reducing BrMs by blocking the influence of key tumor-initiated signaling pathways in the early phases of vascular remodeling and parenchymal invasion. The existence of current targeted drugs for these purposes presents an opportunity to further explore BrM treatment before the development of clinically significant lesions.

Cellular interactions

While the metastatic lesion grows, its interactions with the surrounding TME form an evolving relationship with distinct temporal profiles. The initial response is a frequently effective anti-tumor program initiated by activated astrocytes termed reactive astrocytes (R.A), which successful metastatic cells evade through the plasminogen-activator inhibiting protein neuroserpin.[61,68] From there, the metastatic cells quickly begin to influence the native CNS components towards a supportive and accelerative growth milieu. The key cellular actors in the CNS include the R.A., endothelium, pericytes, neurons, microglia, and bone marrow-derived macrophages (together called tumor-associated macrophages [TAMs]), and tumor-infiltrating lymphocytes (TILs). While more significant work has been done in glioma, the current literature suggests several potentially targetable interactions in the BrM TME. Thus far, most investigation has focused on communication between metastatic cells, R.A.s, and TAMs, and a summary of these interactions is depicted in Figure 1 (D).

Endothelium and the perivascular niche

The initial perivascular niche remains an important tumor development site and interaction with the vascular architecture, endothelial cells, and pericytes. However, most work in the CNS has been completed in gliomas, and characterization of these interactions in BrM growth should be considered extrapolation. Notably, in glioma, the perivascular niche is an essential location for cancer stem cells, a population within the tumor defined by its ability to sustain growth and angiogenesis with particular resistance to radio- and chemotherapy, in part mediated through Akt signaling pathways.[83,84] These cancer stem cells have even been shown in glioma to transform into vascular endothelial cells, pericytes, and mural cells, directly driving the essential vascular reorganization of the CNS.[85–87] While similar processes have not yet been explored in BrMs, interactions with endothelial cells are essential to the initial extravasation of the metastatic cells. This interaction continues within the perivascular niche as the tumor co-opts and manipulates the local vasculature[88,89]. This signal has also been proven to be bidirectional in glioma, as brain endothelial cells drive glioma growth through direct interactions with the cancer stem cells, potentially through nitric oxide (NO) signaling .[90,91]

Notably, cerebral microvessels have also been found to have a 10-30x greater pericyte:endothelial cell ratio compared to other tissues, highlighting their importance in the early TME.[92] Valdor *et al.* demonstrated that pericytes promote glioblastoma (GBM), and contribute to local immunosuppression through a GBM cell-induced secretion of anti-inflammatory cytokines (IL-10 and TGF-β) with corresponding decreases in pro-inflammatory cytokines.[93] Within the perivascular niche, even the extracellular components of the vascular basement membrane, largely collagen type IV and laminins, are central to the

initial metastatic cell proliferation through interactions with the surface ITGβ_1 subunit.[94] In total, the perivascular niche is an early and essential site of interactions between metastatic cells and the native CNS components.

Currently, there is little data on strategies aimed to disrupt the perivascular niche of BrMs. However, with the importance of cancer stem cells in driving tumor growth and recurrence, it may prove a particularly relevant context in promoting long-term survivorship. Extending glioma models to BrMs will elucidate whether these interactions are conserved and identify targets that could be specific or general in intracranial tumors.

Reactive astrocytes

The relationship between the metastatic cells and R.A.s may be the most intimate connection within the TME. Release of inflammatory mediators by R.A.s, including tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6), and IL-1β, are induced by lung cancer BrMs.[95] Furthermore, the development of connexin-43 (Cx43)-based gap junctions between metastatic cells and R.A.s has been identified in preclinical models of breast and lung cancer BrMs. Through this mechanism, the metastatic cell initiates a cGAMP-mediated paracrine signaling loop that promotes R.A. release of inflammatory mediators, including interferon-α (IFN-α) and TNF-a.[96] Consequently these inflammatory cytokines drive cell survival and chemoresistance mechanisms within the tumor cells via upregulation of STAT1, NF-κB, GSTA5, BCL2L1, and TWIST1[97]. This interaction can be directly disrupted via BBB-penetrant gap-junction targeting drugs, including meclofenamate and tonabersat, which were both shown to inhibit BrMs *in vivo*.[96] Additionally, R.A.s release miR-19a-containing exosomes that inhibit the expression of tumor suppressor PTEN in metastatic cells, consequently increasing tumor chemokine secretion as well as recruiting pro-tumorigenic brain-derived myeloid cells into the TME.[98]

Priego *et al.* identified STAT3 as the essential driver within these pro-tumorigenic R.A.s, further promoting pro-tumorigenic TAMs, and showed that inhibition of STAT3 activation through the BBB-penetrant drug silibinin disrupted astrocyte activation, reduced BrMs, and showed efficacy against established BrMs. The same group administered the STAT3 inhibitor to 18 patients with treatment-failed lung cancer BrMs and found significantly improved overall survival to a matched historical control, regardless of driver mutation status.[99] Furthermore, a multi-specific tyrosine kinase inhibitor, pazopanib, that targets several mediators of angiogenesis has been demonstrated to reduce the population of metastasis-associated R.A.s in a metastatic breast cancer model and significantly inhibit BrMs.[100,101]

Several other tumor-promoting paracrine loops have been identified between R.A.s and metastatic cells. Estrogen-dependent signaling in breast BrMs has been identified to stimulate ER+ R.A.s toward pro-tumorigenic chemokine secretion through a pathway mediated by S100A4, BDNF, and tropomyosin kinase receptor B (TrkB).[102,103] Sartorius *et al.* demonstrated that disruption of S100A4 activity through shRNA knockdown prevented the pro-tumorigenic R.A. activity and identified a potential role for anti-estrogen therapies and aromatase inhibitors in BrMs.[102] Contreras-Zarate *et al.* supported the efficacy of letrozole, an aromatase inhibitor, in preventing BrMs of triple-negative brain-seeking breast cancer cells injected intravascularly with improved overall survival, mediated through a pathway involving ER+ R.A.s.[103] TrkB knockdown and inhibition also reduced the incidence of BrMs, and together these results provide a

rationale for implementing anti-estrogenic therapies even in the setting of triple-negative breast cancer. Furthermore, Xing *et al.* showed that breast BrMs could create a positive feedback loop in which upregulation of c-Met increases HGF-dependent tumor cell secretion of pro-tumorigenic IL-1β, IL-8, and CXCL-2, which subsequently increase HGF secretion by local R.A.s.[104] The same group demonstrated that inhibition of the c-Met pathway by BBB-penetrant pterostilbene significantly blocked BrM development *in vivo* and extended survival.[104]

IL-1β has also been demonstrated to drive RA-mediated activation of pro-tumorigenic Notch signaling in cancer stem-like cells of breast BrMs[105]. Jandial *et al*. demonstrated that R.A.s upregulate pro-tumorigenic Reelin signaling in HER2+ breast BrMs, while Choy *et al.* found that R.A. produced bone-derived neurotrophic factor (BDNF) is essential to the proliferation of HER2+ breast BrMs via TrKB signaling, with targeted inhibition of BDNF and HER2 by cyclotraxin B and lapatinib, respectively, significantly inhibiting tumor cell growth *in vivo*.[106,107] Kim *et al.* showed that IL-6 and IL-8 production by breast BrMs increased endothelin production by R.A.s and upregulated E.T. receptors on metastatic cells, subsequently promoting a range of tumor proliferative and chemo-resistance signaling pathways.[108] R.A.s can also be induced by metastatic melanoma cells to express IL-23 via MMP2, which increases parenchymal invasion that can be blocked by IL-23 inhibition.[109] Another study highlighted the role of TGF-β2 from R.A.s in upregulating ANGPTL4 in triple negative breast cancer BrMs, a gene involved in tumor progression through an unknown mechanism.[110] This interaction is stimulated through metastatic cell release of IL-1β and TNF-α. R.A.s have also been shown to contribute to local immunosuppression via the induced STAT3-dependent expression profile that inhibits CD8+ T cell activation and polarizes TAMs to the anti-inflammatory M2 profile.[111]

While a comprehensive model of the interactions between metastatic cells and R.A.s has yet to be developed, the findings above highlight several common mediators and their roles and relationships in BrM progression that can potentially be interrupted pharmacologically. Researchers above have validated methods of disrupting specific intercellular signaling pathways and intracellular pathways within the R.A.s themselves, with promising preclinical results. Given existing safety data for some candidate therapies, translation into clinical use may be closer than typically feasible.

Tumor associated macrophages (TAMs)

Less thoroughly investigated is the interaction between metastatic cells and TAMs, a group consisting of microglia and infiltrating bone marrow-derived macrophages. Bone marrow-derived macrophages are infiltrating monocytes from the peripheral circulation. These two cell populations are indistinguishable by current experimental techniques. However, murine models and clinical samples show that up to 30% of the total tumor mass consists of TAMs.[51] Classically, two polarizations have been described with M1 considered a pro-inflammatory profile and M2 anti-inflammatory. However, the validity of this distinction has been debated.[14,112] The pro-tumorigenic M2 TAMs demonstrate inhibited cytotoxic activity and secrete factors involved in local immunosuppression, tumor growth, and ECM remodeling.[113] Andreou *et al.* showed that selective depletion of M2 TAMs significantly reduced BrM in a metastatic breast cancer model.[114] Induction of this TAM profile is regulated by WNT, CXCR4, and PI3K pathway signaling, with targeted inhibition of each leading to reduced TAM-associated parenchymal infiltration.[115–117] Breast

BrMs have been shown to secrete neurotrophin-3 to reduce TAM cytotoxicity and drive a broad shift towards the M2 polarization profile.[118] Xing *et al.* demonstrated that downregulation of X-inactive specific transcript (XIST) in breast BrMs promotes metastatic growth through increased secretion of miR-503 from metastatic cells, which suppresses microglial cytokine progression and subsequently T cell proliferation.[119] The group found that a drug targeting XIST-low breast metastatic cells blocked BrMs *in vivo* and correlated these findings with XIST quantification in patient tumor samples.[119] In glioma, TAMs have been shown to additionally produce VEGF, driving angiogenesis, and express IL-10 and TGF-β, which stimulate Tregs and perpetuate the immunosuppressive environment.[120]

Notably, the polarization of TAMs is known to exist on a reversible spectrum, dependent on dynamic extracellular or intracellular cues.[121] While tumors manipulate this fluidity to their advantage, targeting the opposite is another potential therapeutic approach as treatment with the PI3K inhibitor buparlisib in a breast cancer model inhibited metastatic tumor growth and specifically drove TAMs towards the more classically activated phenotype.[115] Significant work remains to be done in characterizing the role of TAMs in the progression of BrM and identifying potentially additional targetable interactions between the metastatic cells and TAMs.

Infiltrating immune cells

After the initial seeding and development of the metastatic niche, an additional element of the CNS TME arrives in the form of TILs. $CD4^+$ T cells, $CD8^+$ T cells, and Tregs infiltrate significantly in both preclinical models, and clinical specimens of NSCLC and melanoma BrMs.[122] Similar findings have been reported in glioma with more extensive research into mechanisms and implications.[123] In non-pathologic states, Tregs function to resolve inflammation; however, in the TME, this action encourages further proliferation of the metastatic tumor.[124] As such, these cells present an attempted immune response and another route of local immune suppression. Glioma research demonstrated chemokines' role, including C-C motif ligand 2 (CCL2), and local induction as the cause for the enriched Treg population within Glioma.[125(p2)] Similar experiments have not yet been conducted in metastatic models to confirm an analogous pathway. However, direct extrapolation from glioma should be viewed with some skepticism, as recent work highlighted significant differences between the TME of the two. Study of multiple tumor subtypes demonstrated that, in general, BrMs contain significantly greater populations of T cells and neutrophils compared to the immunologically cold glioma, with relevant differences in their genomic and proteomic profiles. These findings highlight a contrast that could be particularly relevant to the future of immunotherapeutics in the CNS.[126,127]

In consideration of differential treatment responsiveness, studies across BrMs from various primary tumors have also noted differences in the profile of TILs in the metastatic lesions, with lung cancer metastases showing more significantly upregulated immune-checkpoint expression, including programmed death-ligand 1 (PD-L1), PD-L2, and iodothyronine deiodinase 1 (IOD1), compared to breast and colorectal cancer.[128] In comparison to the primary tumor site, the NSCLC BrMs show fewer TILs in total with more anti-inflammatory TAMs, presenting a uniquely immunosuppressed local environment that supports tumor proliferation.[129] Notably, Berghoff *et al.* examined the density and distribution of infiltrating immune cells in clinical BrM samples and found no correlation with overall TIL or Treg density and survival.[62] Recent work

investigating the mechanisms of local and systemic immunosuppression associated with intracranial tumors, including the sequestration of functional T cells in the bone marrow, is also relevant to understanding the presence and function of these TILs[130–132]. Thus far, significant effort has been invested in attempts to reverse the immunosuppressive environment and permit infiltrating immune cells to actively engage with metastatic lesions, with the most relevant clinical studies involving immunotherapies and targeted strategies summarized in Table 1.

Leptomeningeal metastases

Metastatic spread to leptomeninges, either focally or diffusely, and with or without BrMs, is seen in 8% of cancer patients in autopsy studies and also seems to be increasing as patients with cancer live longer.[133] Haematologic, melanoma, lung, and breast cancer are common causes of such spread. Leptomeningeal tumors usually elicit an inflammatory response, even without malignant cells in the cerebrospinal fluid (CSF), often called carcinomatous meningitis.[133] The preclinical study by Boire *et al.* suggests that C3 expression in primary tumors is predictive of leptomeningeal relapse. Pharmacologic manipulation with C3 signaling was shown to suppress leptomeningeal metastasis in preclinical models.[134] Considering strategies to intervene in these processes may also potentially prevent access to the CNS and present another avenue for further research.

Therapeutic challenges and opportunities

Until recently, surgical resection followed by radiotherapy was the main therapy strategy for patients with BrMs, with laser interstitial thermal therapy (LITT) use rising for patients with recurrent disease.[135] Tailoring of radiotherapeutic doses, schedule, and techniques has advanced significantly to improve efficacy and limit toxicities. These include stereotactic radiosurgery (SRS) alone instead of whole-brain radiotherapy (WBRT) and the use of hippocampal sparing strategies with memantine administration in patients requiring WBRT.[136,137] Traditionally, BrMs have been notably resistant to both radio- and chemotherapy. In particular, melanoma and renal cell carcinoma metastases are known to be radioresistant, though SRS does extend survival in these patients as well.[138] Choi *et al*. demonstrated that TopBP1 and Claspin genes are increased in such radioresistant cells and their targeted depletion enhances sensitivity, as does interruption of DNA damage checkpoint pathways.[139,140] Several studies have also investigated various radiation protocols for optimization against such radioresistant tumors, with success in fractionated and hypofractionated SRS plans.[138,141,142] Many conventional chemotherapy and targeted drugs lack effective penetration of the BBB and are actively extruded from the brain, and encounter several further resistance mechanisms once within the CNS parenchyma.[143–145] Some of this hurdle is directly linked to the interplay between metastatic cells and the native CNS components, as demonstrated with the findings that R.A.s actively enhance chemoresistance through calcium sequestration and the upregulation of survival genes in tumor cells.[97,146] Niessner *et al*. similarly found that interactions between melanoma metastases and brain-derived factors lead to therapy resistance mediated by AKT hyperactivation and PTEN loss.[147] Specific signals from the brain microenvironment upregulating the PI3K-AKT-mTOR pathway has also been implicated in treatment resistant breast cancer BrMs.[148,149] This protective role of R.A.s in treatment resistance has been shown to be conducted through direct cellular

contact and supported across a number of primary tumor sources.[97,146,150] The structural and functional heterogeneity within the brain microenvironment and the tumor must also be considered, both within lesions and across tumor subtypes.[151] The development of improved biomarkers of drug penetrability and delivery will allow for greater evaluation of the efficacy of novel therapeutic strategies for BrM.

Recent advances in targeted and immunotherapies have stimulated the development of clinical trials specific to patients with BrMs (Table 1). Small molecule inhibitors and targeted antibodies have demonstrated varying efficacy in the treatment of BrMs in patients with oncogene driven cancers such as HER-2, ALK, EGFR, AXL, ABL, and BRAF driven tumors.[152–158] Immune checkpoint inhibitors, having transformed the landscape in melanoma, lung cancer, and many other solid tumors, have also shown encouraging efficacy in patients with BrMs.[159–161] Overall prognosis of patients with BrMs remains poor, as therapy responses are often short-lived. Many BrM tumor types are neither driven by targetable oncogenes nor responsive to immune checkpoint blockade. The local immunosuppressive environment-induced, as described above, presents an additional challenge to the broad application of immunotherapeutics in the CNS.[162] BrMs, therefore, remains an active area of unmet clinical need, and further research is needed in order to exploit their molecular and immunologic vulnerabilities. Harnessing our growing understanding of the metastatic cascade and pursuing a strategy that targets the surrounding TME is one path forward that may have a role in future clinical practice.

Targeting the microenvironment

The review above characterized and highlighted the range of complex interactions that occur between BrMs and the native CNS components. Considering the therapeutic value of these investigations requires a broad view of the shared and specific implicated pathways and an understanding of analogous mechanisms in more thoroughly studied primary and systemically metastatic cancers. Even before metastatic cells have gained a foothold in the CNS, there are opportunities to disrupt and evade their influence, as with the destruction of circulating tumor-derived exosomes through novel nanoparticles, monoclonal antibody-directed blockade of essential endothelial adhesion mediators, inhibitors of essential chemotactic mediators, and targeted disruption of the BBB-transversal pathway.[33,34,42,43,53,59,60] Once within the CNS, various groups have shown the efficacy of interrupting specific signaling pathways between the metastatic cells and surrounding cellular components, such as blocking the formation of gap junctions or estrogen-dependent signaling in all subtypes of breast cancer, or BDNF in HER2+ breast cancer. Other groups have shown the potential for disrupting the intracellular cascades within R.A.s or TAMs, as with pharmacologic STAT3, cMET, and PI3K inhibition.

Furthermore, common factors appear at various stages throughout the metastatic process, such as VEGF and MMPs. Potential avenues for their inhibition and the existing preclinical data are discussed above, with promising directions for future therapeutic opportunities. Continued research into halting CNS invasion mechanisms and the reprogramming of native CNS components through pre-emptive or reactive pharmacologic intervention presents a new strategy to reduce and treat BrMs. The findings discussed throughout this review emphasize the numerous potential targets therein.

Conclusion

BrMs present a clinical problem with limited therapeutic answers thus far. The CNS is a unique environment for metastatic spread due to its relative isolation from the rest of the body and distinct immune and cellular milieu. The development of the metastatic TME begins likely long before circulating tumor cells cross the BBB, with the initial setting of the premetastatic niche by secreted factors from the primary site. As the TME evolves with selective pressures from the metastatic cells, the growth of the lesion becomes dependent on the local cellular and non-cellular components of the CNS. Understanding the range of these interactions presents opportunities for disruption to create an inhospitable environment both before and after the initial metastatic spread. Current research points to some shared pathways across primary tumor sources but indicates a vast range of diversity within the BrM TME. Investing in research that explores how BrMs induce change in the surrounding native CNS is a promising avenue to progress in a dire clinical context.

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Table 1. Pivotal clinical trials of targeted or immunotherapies in patients with brain metastases. *Pre-specified subgroup of trial, [†]Prior local therapy allowed to non-target lesions, [‡]6 month PFS rate, [§]Pooled analysis of two phase II trials, [|]12 month PFS rate. N.E. – not estimable, N.R. – not reported, NSCLC – non-small cell lung cancer, O.S. – overall survival, PFS – progression-free survival, TKI – tyrosine kinase inhibitor, WBRT – whole brain radiotherapy.

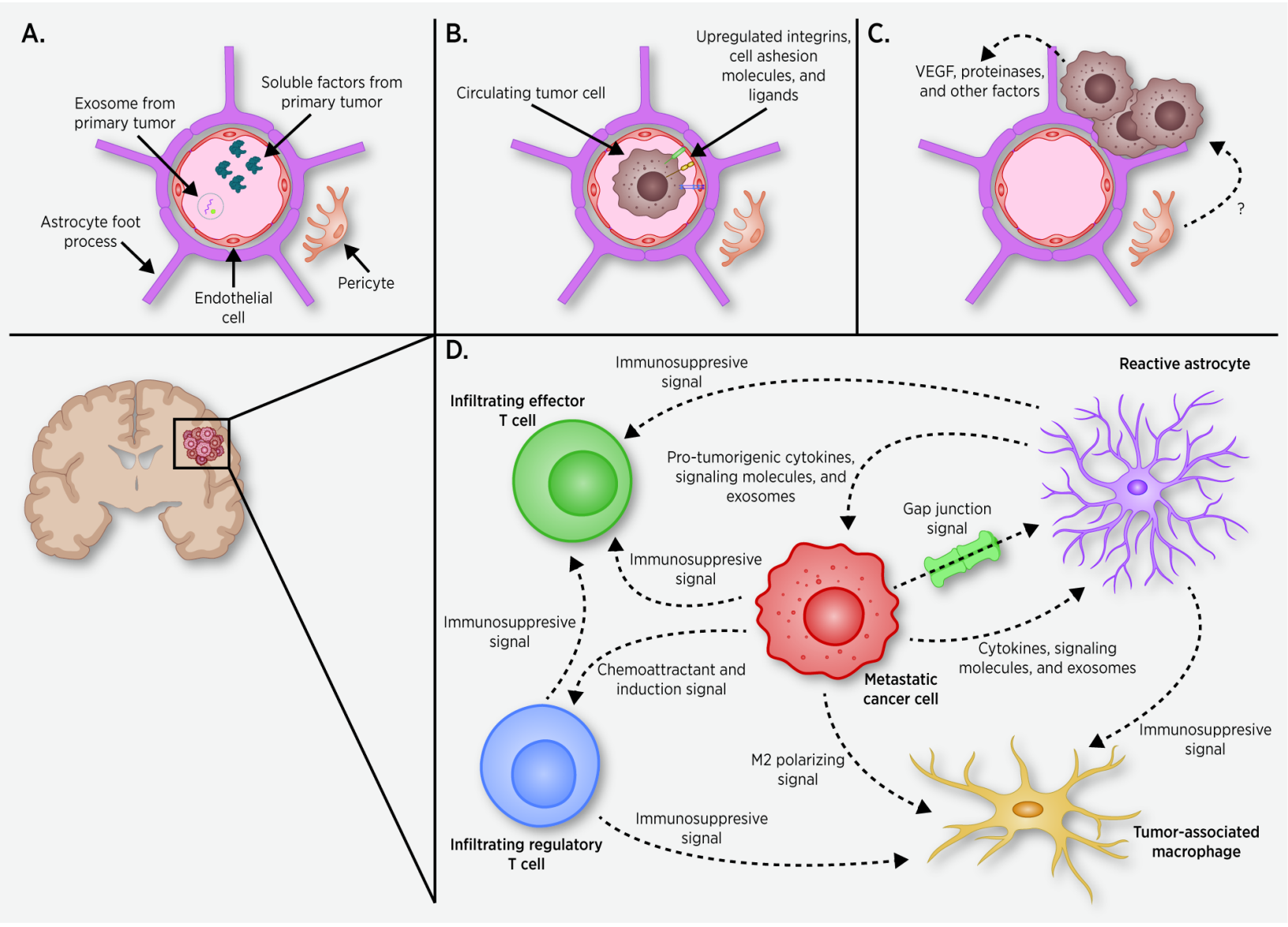
Intervention	**Patient Population**	**Phase**	**Symptomatic**	**Prior Local Therapy**	***n***	**Intracranial Response (%)**	**Median PFS (mths)**	**Ref**
Breast Cancer								
HER2 Targeted Therapy								
Lapatinib	*HER2* positive, prior trastuzumab	II	No	Allowed	39	5	3.0	[163]
Lapatinib plus capecitabine	*HER2* positive, prior therapy allowed	II	Allowed	No	45	66	5.5	[152]
Neratinib	*HER2* positive, prior therapy allowed	II			40	8	1.9	[164]
Neratinib plus capecitabine	Cohort 3A: *HER2* positive, lapatinib-naïve Cohort 3B: *HER2* positive, prior lapatinib	II	Allowed Allowed	Yes Yes	37 12	49 33	5.5 3.1	[153]
Trastuzumab deruxtecan	*HER2* positive, prior trastuzumab emtansine	II*	No	Yes	24	NR	18.1	[165]
Tucatinib, trastuzumab and capecitabine	*HER2* positive, prior trastuzumab, pertuzumab, trastuzumab emtansine	III*	No	Allowed	198	NR	7.6	[166]
Melanoma								
BRAF ± MEK TKI								
Dabrafenib	Cohort A: *BRAF* V600E mutation *BRAF* V600K mutation Cohort B: *BRAF* V600E mutation	II	No No No	No No Yes	74 15 65	39 7 31	3.7 1.9 3.8	[167]

	BRAF V600K mutation		No	Yes	18	22	3.7	
Dabrafenib plus trametinib	Cohort A: *BRAF* V600E mutation	II	No	No	76	58	5.6	[155]
	Cohort B: *BRAF* V600E mutation		No	Yes	16	56	7.2	
	Cohort C: *BRAF* V600D/K/R mutation		No	Allowed	16	44	4.2	
	Cohort D: *BRAF* V600D/K/R mutation		Yes	Allowed	17	59	5.5	
Vemurafenib	*BRAF* V600 mutation	II	Yes	Yes	24	37	4.4	[168]
Vemurafenib	Cohort 1: *BRAF* V600 mutation	II	Allowed	No	90	18	3.7	[169]
	Cohort 2: *BRAF* V600 mutation		Allowed	Yes	56	20	3.9	
Immunotherapy								
Ipilimumab	Cohort A: no prior immunotherapy	II	No	Allowed	51	25	1.9	[170]
	Cohort B: no prior immunotherapy		Yes	Allowed	21	10	1.2	
Ipilimumab plus nivolumab	No prior immunotherapy (unless given as adjuvant therapy)	II	No	No[†]	94	57	64%[‡]	[159]
Ipilimumab plus nivolumab	Cohort A: no prior immunotherapy	II	No	No	25	44	50%[‡]	[160]
Nivolumab	Cohort B: no prior immunotherapy	II	No	No	26	20	29%[‡]	
	Cohort C: no prior immunotherapy		Yes	Yes	16	6	0%[‡]	
Pembrolizumab	Prior immunotherapy allowed	II	No	Allowed	18	22	NR	[161]
NSCLC								
ALK TKI								
Alectinib	*ALK* rearranged, no prior TKI	III*	No	Allowed	64	59	NE	[156]
Alectinib	*ALK* rearranged, prior crizotinib	II[§]	No	Allowed	50	64	10.8	[171]
Brigatinib	*ALK* rearranged, no prior TKI	III*	No	Allowed	43	67	67%[‖]	[172]
Ceritinib	*ALK* rearranged, no prior TKI	III*	No	Allowed	44	73	10.7	[173]
Ceritinib	Arm 1: *ALK* rearranged, prior crizotinib	II	No	Yes	42	39	9.2	[157]
	Arm 2: *ALK* rearranged, prior crizotinib		No	No	40	28	10.1	

	Arm 3: *ALK* rearranged, no prior TKI		No	Yes	12	29	NE	
	Arm 4: *ALK* rearranged, no prior TKI		No	No	44	52	7.5	
Crizotinib	*ALK* rearranged, no prior TKI	III*	No	Allowed	58	26	3.7	[156]
Crizotinib	*ALK* rearranged, no prior TKI	III*	No	Allowed	47	17	21%[‖]	[172]
Lorlatinib	*ALK* rearranged, prior ALK TKI	II*	No	Allowed	81	63	14.5	[174]
EGFR TKI								
Gefitinib or erlotinib	*EGFR* mutation, no prior TKI	III*	No	No	67	43	71%[‡]	[158]
Osimertinib	*EGFR* mutation, no prior TKI	III*	No	No	61	66	87%[‡]	[158]
Immunotherapy								
Pembrolizumab	No prior immunotherapy	II	No	Allowed	18	33	NR	[161]

Figure 1. Summary of interactions within the brain metastasis microenvironment: A) Priming of the blood-brain-barrier and establishment of the premetastatic niche through soluble factors and exosomes from the primary tumor site. B) Initial arrest of the circulating tumor cell within the brain microvasculature, mediated by a number of integrins, cell adhesion molecules, and secreted factors. C) Initial perivascular tumor niche with angioadaptive signaling. D) General case of interactions between the metastatic cancer cell and reactive astrocytes, tumor-associated macrophages, infiltrating effector T cells, and regulatory T cells. Created with BioRender.com.

Figure 1



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Molecular Cancer Therapeutics

Salting the soil: Targeting the microenvironment of brain metastases

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