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Leveraging translational insights towards precision medicine approaches for brain metastases

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Abstract

Due to increasing incidence and limited treatments, brain metastases (BM) are an emerging unmet need in modern oncology. Development of effective therapeutics has been hindered by unique challenges. Individual steps of the brain metastatic cascade are driven by distinctive biological processes, suggesting that BM possess intrinsic biological differences compared to primary tumors. Here, we discuss the unique physiology and metabolic constraints specific to BM, as well as emerging treatment strategies that leverage potential vulnerabilities.

INTRODUCTION

Brain metastases (BM) are the most common centra nervous system (CNS) malignancy, and occur in up to 40% of patients in metastatic cancer[1]. Approximately 200,000 patients are diagnosed with BM annually in the United States. This number will likely increase in the modern era of targeted therapies and immune checkpoint inhibitors (ICI), as patients are living longer with improved extracranial disease control and the risk of developing BM increases with duration of disease[2,3]. Lung (39–56%), breast (11–19%), and melanoma (6–11%) are among the most common primary tumors to spread to the brain[4–6]. As progression of BM is the cause of death in 50–75% of patients with BM[6], they represent an unmet need in modern oncology and an emerging public health crisis.

Surgical resection followed by radiation to the resection cavity is generally the standard of care for solitary or large (>3 cm) symptomatic lesions. Stereotactic radiosurgery (SRS) is recommended for small (<3 cm) asymptomatic BM and for oligometastatic disease. Hippocampal-sparing whole brain radiation can be considered in more challenging cases such as multiple disseminated BM and leptomeningeal carcinomatosis[7]. Systemic therapies

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are generally reserved for patients with an oncogene-addicted BM (e.g. EGFR-mutation, ALK-rearrangement) or those with smaller asymptomatic BM not in urgent need of local control and active concomitant extracranial disease.

Due to improved resolution of commercial MRI's and recent guidelines that recommend increased screening for BM[8], an increasing number of patients present with intracranial lesions that are relatively small and minimally symptomatic. Other patients possess BM in an inoperable location. These cases represent an emerging opportunity for CNS-penetrant systemic therapy, which would reduce the morbidity associated with radiation-induced neurotoxicity or neurosurgical resection. Unfortunately, to date, many agents have limited intracranial efficacy. While targeted therapy and immunotherapy have displayed early promise for BM, the response rates are generally lower than than those observed in ECM[9]. Often, differential responses for intracranial and extracranial disease are observed, where systemic disease may be adequately controlled with concurrent progression of intracranial tumors.

Arguably the most promising way to revolutionize care for BM likely lies in a better understanding of BM biology in order to identify brain-specific targets. To stimulate future investigation in this field, we have focused this Review on three topics: 1) blood-brain barrier (BBB) physiology, 2) physiologic differences of BM tumor cells compared to extracranial tumors, and 3) mediators of immunosuppression from cell populations specific to the BM microenvironment. We note, however, that our review of these subjects is not meant to be comprehensive, and instead is intended to inspire discussion and collaboration among investigators rather than to present a dogmatic view. Finally, we infuse our thoughts on how these investigations may translate into early-phase trials, biomarkers of treatment response, and precision-based paradigms for BM patients.

BIOLOGY OF BRAIN METASTASES

The brain metastatic cascade

For patients at high risk of developing BM, a preventative strategy may lie in targeting molecular mediators of critical steps of CNS dissemination of solid tumors. To this end, a study using *in vivo* laser scanning microscopy in a murine model outlined discrete steps of the brain metastatic cascade,[10] vascular arrest of tumor cells in intracranial capillaries, extravasation into perivascular space, brain parenchyma invasion, and perivascular growth with angiogenesis (Figure 1). Initial steps appear to be dependent on integrins, which are transmembrane ligands that mediate adhesion between tumor cells and the extracellular environment[11–13]. Integrin-mediated interaction of tumor cells with the basement membrane modulates vascular arrest through adhesion of tumor cells to the endothelium. However, as 95–99% of brain-arrested cancer cells fail to develop into a macro-metastasis, the metastatic cascade likely involves multiple biological processes, as penetrating the BBB does not necessarily translate into the ability to transition from a micro-metastasis to a macro-metastasis[10]. Using multimodal correlative microscopy, multiple aspects of the metastatic cascade (e.g. endothelial remodeling, extravasation, BM growth) was found to be dependent on cancer-cell derived matrix metalloprotease 9 (MMP9)[14]. Additionally, vascular co-option is essential for proliferation of cancer cells of diverse histologies. To

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our knowledge, clear molecular drivers of vascular co-option have yet to be identified. Another complementary factor to vascular co-option is angiogenesis, as aberrant vascular physiology[13,15] and increased VEGF expression are associated with enhanced metastatic potential and higher rates of BM[16]. However, there have been mixed results with VEGF inhibition in preclinical models and clinical practice[17], suggesting that angiogenesis is likely only one, albeit important, step for the brain metastatic cascade. Moving forward, we encourage additional efforts in identifying 'bottlenecks' and mediators of these steps within the brain metastatic cascade.

Interestingly, a large fraction of cancer cells enter a dormant state after extravasation and rest as single cells along blood vessels without proliferation or regression for long periods of time[10]. Using an in vivo MR technique that enabled tracking of breast cancer cells in a murine model, three dormant tumor cells for every brain macrometastasis were visualized, serving as a considerable reservoir for tumor cells to awaken[18]. Release of TGF-beta 1, periostin, and thrombospondin-1 from the basement membrane activate vascular endothelial cells and may drive the transition from dormancy into a proliferative state[19,20]. Treatment modalities aimed at reducing levels of these proteins may be beneficial in preventing development of macro-metastases.

Other clinically impactful questions include: what biological factors mediate organ-specific metastasis of cancer? Do these traits arise in the primary tumor, circulating tumor cells, extracranial metastases or the affected organ site? Cancers exhibit clear organ-specific patterns of metastasis; for example, prostate cancer commonly metastasizes to the bone and rarely to the brain, whereas melanoma and lung cancer frequently spread to the CNS. Recent studies, which we present below, suggest that brain-specific metastasic growth results from complex interactions between cancer cells and CNS resident cells that modify the brain TME into a tumor-supportive immunosuppressive niche. Understanding these mechanisms may form the basis for a CNS preventative treatment paradigm for histologies at risk of metastasizing to the CNS that both targets the primary tumor cell and protects the susceptible organ microenvironment.

The blood-brain barrier

Understanding BBB dynamics is critical towards identifying mechanisms of tumor cell extravasation and maximizing on-target effects of candidate therapeutics. In homeostasis, the BBB is a tightly regulated neurovascular unit including endothelial cells, pericytes, and astrocytic end-feet that controls molecular and cellular transport into the CNS[21,22]. Tumor cells express cell-surface ligands that alter the BBB and facilitate extravasation of cancer cells into the parenchyma. Consequently, the BBB is remodeled into a brain-tumor barrier (BTB), which is characterized by aberrant and dysfunctional pericytes, astrocytic endfeet, and neuronal connections[23]. The BTB is functionally more permeable than an intact BBB, which permits infiltration of cancer cells, peripheral immune cells, and therapeutics (Figure 2)[24,25]. Genomic and functional analysis of BM identified the prostaglandin-synthesizing enzyme COX2, heparin-binding epidermal growth factor (HBEGF), and the alpha 2,6-silayltransferase ST6GALNAC5 as mediators of cancer cell passage through the BBB[26]. ST6GALNAC5 exerted activity through augmenting adhesion to endothelial cells. Another

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study noted that tumor cells secrete the protease cathepsin S, which degrades junctional adhesion molecules on the BBB and facilitates transmigration[27]. Interestingly, increased physical activity has been associated with higher BBB integrity and decreased BM formation in murine models[28]; however discrete molecular mediators that augment BBB integrity are still being explored.

Intracranial efficacy of many systemic therapies is negatively impacted by poor penetration into the CNS. In patients with breast cancer BM, the ratio of trastuzumab levels in the CSF and serum was 1/420 at baseline and rose to only 1/49 – 1/76 following radiation of the BM – which still qualifies as sanctuary site levels[29]. Another study evaluated drug uptake in a murine BM model through an injection of radiolabeled paclitaxel and doxorubicin and autoradiography of tissue sections[30]. BM uptake of the drug in question was usually greater than normal brain but only reached cytotoxic concentrations in less than 10% of BM. Given these findings and heterogenous BTB permeability, there is a concerted effort to design brain-permeable compounds. Medicinal properties of successful drugs able to pass the BBB are listed in Figure 2[31].

Further complicating the issue of CNS drug delivery is considerable heterogeneity in BTB function[31–37]. Preclinical models of BM have demonstrated differences in permeability within a single BM[34,37], across spatially separated BM[32], and across different subtypes within the same histology. This heterogeneity results in an uneven and unpredictable distribution of systemic therapies, especially if a patient has multiple BM, and can hamper consistent adequate delivery of a therapeutic agent to BM. However, recent studies indicate that BTB permeability can be modified through select BTB proteins and metabolites, suggesting therapeutic potential in maximizing drug penetration and efficacy. Upregulation of astrocyte-mediated sphingosine 1-phosphate receptor 3 (S1PR3) loosens the BTB through downregulation of tight and adherens junction proteins by secretion of IL-6 and chemokine-ligand 2 (CCL2)[33]. HER-2 positive breast cancer BM, compared to triple-negative or basal subtypes, possess a less disrupted BTB, which is associated with increased expression of glucose transporter 1 (GLUT1) and breast cancer resistance protein (BCRP)[31].

Another set of clinically relevant targets are endothelial drug efflux transporters, which transports substances out of the brain into the blood. Expression of the ATP-binding cassette (ABC) efflux transporters, such as P-glycoprotein, multi-drug resistance (MDR) proteins, and BCRP, have been linked to decreased drug uptake and treatment resistance[38,39]. Many of these targets have not been evaluated in early-phase trials. Identifying other mediators of drug or effector T cell efflux across the BTB are important considerations to maximize efficacy of immune-based therapies. Other strategies in development include drug-ligand compounds or agents targeting BTB receptor peptides to either trigger receptor-mediated endocytosis across the BTB or prevent efflux of the drug out of the BTB (Figure 2C)[22]. Moreover, techniques such as drug administration with radiation or focused ultrasound in an attempt to ‘open up’ the BTB are being evaluated[22].

Genomic mediators of brain metastases

A logical step towards developing effective systemic therapies for BM is to leverage genomic differences between primary, extracranial, and intracranial metastases to identify

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molecular drivers of CNS tropism (Figure 3)[40]. In recent years, increasing availability and size of whole exome (WES) and genome sequencing (WGS) datasets for metastatic tumors have facilitated the identification of candidate variants for functional validation. [41]. An analysis of BM of diverse histologies and patient-matched primary tumors and ECM demonstrated near-universal genomic divergence between primary tumor and BM (Figure 3) with more than 50% of BM harboring clinically actionable mutations not detected in the primary tumor. Clinically actionable somatic variants such as *CDK, PI3K,* and *MAPK* pathway alterations, were common in BM[41,42]. Another study in NSCLC patients found that *CDK* or *PI3K* pathway alterations or WNT pathway activation were associated with a higher incidence of BM in NSCLC patients[42]. Furthermore, anatomically distinct BM from the same patient were concordant for 97% of clinically informative driver mutations[41]. This genomic homogeneity of BM across histologies suggests that unique histology-agnostic physiologic changes are needed for tumors to colonize the CNS.

Extensive efforts have been devoted to identifying the molecular traits that enable cancer cells to metastasize to the CNS. A WGS analysis of primary and metastatic breast cancer tissues noted that spatially distant metastases often possessed potential driver mutations (e.g. *JAK-STAT* inactivating mutations) not detected in the primary tumor[43]. A similar analysis for melanoma metastases revealed increased activation of the *PI3K/AKT* pathway within BM[44]. Another study employing a targeted NGS panel from 25,000 patients with metastatic cancers to identify associations between somatic variants and metastatic dissemination[45]. Metastases, compared to primary tumors, had higher chromosomal instability. Certain somatic alterations (e.g. *TP53* mutation, *PTEN* loss) were associated with increased metastatic burden. Additionally, associations between genomic features and metastasis to specific target organs were detected. For example, lung adenocarcinoma patients with BM had higher frequencies of *TP53* mutations, *TERT* amplification, and *EGFR* mutations, and a lower frequency of *RBM10* mutations.

However, while differences in somatic mutations or copy number aberrations have been reported for diverse genes between BM and ECM, only a few pathways have been functionally validated using pharmaceutical inhibition, or genetic manipulation in brain-tropic cell lines and animal models - namely, the *PI3K/mTOR*[46,47], OXPHOS pathway[48], and matrix metalloproteinase family[49]. Recent clinical studies have shown intracranial efficacy with CNS-penetrant targeted therapies in patients with BM and oncogenic drivers[50–56]. CNS-penetrant *PI3K* inhibition in patients with *PIK3CA*-mutant breast cancer BM has shown promising activity[57] and is now being evaluated in clinical trials (NCT04192981; NCT03994796). Similarly, *CDK* inhibitors have demonstrated activity in BM of diverse histologies that harbor alterations in the *CDK* pathway[40]. These findings have inspired the creation of CNS basket trials to evaluate CNS-penetrant targeted therapies for other druggable targets in BM[58].

Other analyses suggest that the molecular makeup of the cancer cell, by itself, may not be the only driver of metastasis. A study using WGS data from 2500+ metastatic tumors did not find recurrent cancer-causing mutations associated with either metastasis or organ-specific spread[59]. Furthermore, there was a high degree of genomic concordance and minimal driver gene heterogeneity between patient-matched spatially distinct metastases.

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While mutations of metastatic tumors varied widely, commonly implicated genes included: *TP53* (52%), *CDKN2A* (21%), *PIK3CA* (16%), *APC* (15%), *KRAS* (15%), and *PTEN* (13%). These findings have been corroborated[60]. WGS analysis of 250 anatomically distinct biopsy pairs collected at different times during a patient's treatment revealed >90% concordance for a targeted panel of 219 potential oncogenic drivers[61]. We emphasize, however, that these studies had either a limited number or no BM and some of these findings may not be generalizable for BM physiology. Due to genomic heterogeneity between ECM and BM, we interpret these studies as further evidence that BM possess important biological differences from extracranial tumors. Extracranial organs generally possess similar cellular and metabolic traits with the organ from which the primary tumor originated[10,62]. Conversely, the brain contains electrically-active cell populations (e.g. astrocytes, microglia, oligodendrocytes) unique to the CNS[63]. As more than 99% of cancer cells that reach the brain die[10], tumors must assume a unique set of physiologic adaptations, different from those seen in extracranial sites, to thrive within the brain parenchyma. Furthermore, as many genomic alterations recurrent within BM (e.g. *CDK*, *PI3K* pathway alterations) also occur in ECM[41,59], brain colonization probably results from a complex combination of general metastatic pathways, brain-specific vulnerabilities, and cancer-host cell interactions. For example, the molecular makeup of a metastatic tumor can be dependent on its TME. Astrocyte-derived exosomes secrete microRNAs that induce *PTEN* loss within metastatic tumor cells in the brain[64]. This loss is reversed when cancer cells leave the brain. These conclusions are supported by an analysis of spatially separated metastases in a breast cancer murine model illustrating unique dynamic organ-specific transcriptional and metabolic signatures, which are dependent on the local TME[65].

TME MODULATORS OF BRAIN METASTASES

A growing body of work illustrates synergistic interactions between astrocytes[66], macrophages[67–69], neutrophils[67,70], and natural killer cells[71] with cancer cells to potentiate immunosuppression and perpetuate BM growth. With growing availability of single-cell and spatial profiling techniques, there is great potential to identify molecular or transcriptional activation states that can serve as hypotheses for functional validation. As a detailed characterization of each cell population within the BM TME is beyond the scope of this Review, we have focused our attention on highlighting studies of non-malignant cell populations with high translational potential. For a more comprehensive discussion of CNS resident cells in the context of malignancy, we acknowledge expert work by our colleagues[72–75]. Furthermore, recognizing that BM TME investigation is largely a nascent field, we summarize important unresolved questions.

T lymphocytes

The brain has historically been regarded as an immune privileged organ, given the relative paucity of peripheral immune surveillance due to the BBB and lack of conventional lymphatic drainage. As described above, due to pathologic remodeling of the BBB, BM possess variable levels of antigen-presenting dendritic cells as well as CD4+ and CD8+ T-cells, which play key roles in dictating treatment outcomes. CD8+ T cell infiltration within tumors is necessary for ICI response[76]. Furthermore, the degree of CD3+ or

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CD8+ lymphocytes and production of inflammatory cytokines correlates with treatment response[77,78].

While the bulk of studies evaluating intracranial T-cell dynamics are in in glioblastoma, available data suggests that the BM TME is more immunosuppressive than that of primary tumors or ECM. Several studies comparing patient-matched primary tumors and BM found reduced T cell infiltration and expansion, as well as inhibition of dendritic cell maturation and helper T cell signaling pathways, in BM[48,79–81]. Similarly, single-cell transcriptomic characterization for patient-matched primary lung cancer, ECM, and BM illustrated a shift towards immunosuppressive T cell phenotypes in metastatic sites[82]. In metastases, normal myeloid cell populations were replaced with immature dendritic cells, regulatory T cells (Tregs), and exhausted T cells[82]. Therefore, a logical strategy towards improved treatments for BM are to identify mechanisms that augment T cell entry and cytotoxicity into BM.

However, recent studies present a translational conundrum. As a complicated array of biological factors have been linked to recruitment, trafficking, function, and activation of T cells, it is probable that a multi-target strategy would be needed to fully augment a tumor's immunogenicity. Interestingly, the presence of extracranial disease can impact intracranial T cell activity. Effector T cell recruitment into BM is augmented by the presence of extracranial tumor lesions via peripheral expansion of effector cells and activation of endothelial cells of the BBB to enhance lymphocyte trafficking into BM[83]. In addition, dendritic cells and Tregs play a key role in immunosuppression. Intra-tumoral dendritic cells tend to be more immature as the TME inhibits their differentiation. These immature cells can have downstream effects of suppressing T-cell proliferation and recruiting Tregs. Release of regulatory cytokines by Tregs can prevent activation of helper T cells and impair effector T cells. In a pre-clinical model of glioblastoma, targeting glucocorticoid-induced TNFR-related receptor (GITR) on Tregs augments the effect of pembrolizumab by blocking Treg activity and results in a potent anti-tumor effect[84]. To our knowledge, similar strategies have not yet been tested for BM. Importantly, there is potential in non-invasively monitoring these dynamic changes. A paired single-cell RNA- and T-cell receptor sequencing effort of patient-matched BM and CSF samples identified strong correlations between T-cell clonotypes and phenotypes, suggesting that T-cell dynamics within the BM can be monitored through serial lumbar punctures[85].

While enhancing T-cell activation through ICI has demonstrated remarkable intracranial response rates for melanoma and non-small cell lung cancer, most patients with BM eventually progress within the CNS. Current efforts revolve around identifying mechanisms for acquired or innate ICI resistance and then using these putative targets to enhance BM immunogenicity. There is a pressing need to define the molecular factors that mediate T-cell recruitment or shift towards a pro-tumor (Treg) vs. anti-tumor (effector) phenotype. One difficulty in studying these questions is disentangling the roles that distinct TME cell types play in dictating T cell phenotype. While correlative studies using single-cell or spatial techniques have been explored, robust functional valdation is needed. Other strategies in development include using dendritic cell vaccines or chimeric antigen receptor (CAR) T cells to augment T-cell cytotoxic effects against tumor antigens in BM, or using intracranial radiation or targeted therapy in conjunction with ICI to increase T-cell recruitment and

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limit the effect of Tregs. However, it remains to be seen whether directly augmenting T-cell cytotoxic phenotypes can overcome the immunosuppressive effects of the glial and/or myeloid compartment.

Astrocytes

Astrocytes are the most abundant cell population within the CNS and have diverse homeostatic functions including maintenance of the BBB, neurotransmission, regulation of cerebral blood flow, and synaptic plasticity[86,87]. In homeostasis, they block the entry of peripheral immune cells into the CNS and mediate the local innate immune response to promote tissue repair. As astrocytes are found only in the brain, metastases that thrive within the brain likely acquire a set of adaptations that allow tumor cells to synergistically interact with an unfamiliar cell type.

Activated astrocytes are prevalent within BM[88,89] and play a role in tumor invasion and proliferation. The physiologic factors that mediate the functional shift of astrocytes from the neuro-inflammatory anti-tumor ‘A1’ state to the neuro-protective pro-tumor ‘A2’ state are poorly characterized. In the non-diseased state, astrocytes produce plasmin to promote clearance of tumor cells that enter the brain[90]. Tumor cells can produce serpins, which are anti-plasminogen activators, to prevent plasmin generation and its metastasis-suppressive effects. Serpin levels in tumors and blood are associated with poor outcomes[90]. Astrocyte function is also state-dependent; for example metastatic breast cancer cells secrete inflammatory cytokines such as IL-1-beta which ‘activates’ surrounding astrocytes[91]. Interactions between these activated astrocytes and tumor cells result in an upregulation of stem cell signaling pathways, which ultimately render tumor cells resistant to the deleterious effects of chemotherapy and reactive oxygen species.

In the pro-tumor state, astrocytes facilitate brain invasion through expressing the matrix-degrading enzyme heparinase[92]. Astrocytes relax endothelial cell junctions through secretion of inflammatory cytokines, such as interferon-alpha and tumor necrosis factor[64]. Additionally, extravasated cancer cells employ the cadherin-related protein PCDH7 to form connexin 43 (CX43) gap junctions with surrounding astrocytes[93]. Afterwards, cancer cells exhibit a gap junction-dependent up-regulation of survival genes (GSTA5, BCL2L1, TWIST1) that correlates with chemoresistance[94,95], neovascularization, and a shift towards a pro-tumor astrocyte phenotype[96,97]. Interestingly, in pre-clinical models, brain-permeable gap junction inhibitors reduced intracranial disease burden when given in a therapeutic schedule and augmented the efficacy of chemotherapy[93].

Single cell profiling is facilitating the identification of functionally relevant subpopulations of astrocytes and their roles in brain tumors. Available data illustrate substantial region-specific heterogeneity and functional diversity[98,99] as astrocytes can be re-programmed from a metastasis-hostile environment to a tumor-promoting immunosuppressive environment[33,100]. Further complicating this study is that these diverse astrocytic transcriptional signatures do not operate in a vacuum, and interact with microglia, monocytes, and T cells to potentiate these effects. Nonetheless, astrocytes have promising translationally-relevant features. RNA microarray analysis of murine breast cancer BM revealed differential expression of astrocytic sphingosine-1 phosphate receptor 3 (S1PR3)

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in the neuroinflammatory response of low and high permeability metastases. S1PR3 activation directly resulted in increased BTB permeability to systemic therapy and recruitment of lymphocytes from the peripheral circulation[33]. Moreover, analysis of single-cell profiling and cell culture models revealed astrocytes within breast cancer BM expressed brain-derived neurotrophic factor and tumor cell tropomyosin kinase receptor B in an estrogen-dependent manner[101,102]. These factors increased the invasive and tumor-initiating capabilities of breast cancer. This process was inhibited by estrogen depletion, suggesting that astrocytes could be modulated using hormonal therapies. Another study identified an anti-inflammatory subset of astrocytes driven by STAT3 that blunt microglial and tumor-specific T-cells through secreting immune-suppressive cytokines[66]. Inhibition of STAT3 resulted in impaired viability of tumor cells and reduced outgrowth of BM, suggesting STAT3 may be a therapeutic target. However, as STAT3+ astrocytes are also associated with neurodegenerative disorders[103,104], additional investigation is needed to identify mechanisms of BM-specific immunosuppression. Furthermore, as STAT3+ astrocytes are frequently located at BM tumor margins, newer techniques such as spatial transcriptomics may be helpful to identify astrocyte interactions with other resident cell populations that contribute to treatment resistance. These insights will enable rational development of astrocyte-directed therapies for BM.

Tumor-associated macrophages

Tumor-associated macrophages (TAM) for BM consist of microglia and bone-marrow derived macrophages (BMDM). Microglia are macrophages native to the CNS and function as the first line of defense for the innate immune system within the brain parenchyma. BMDM do not usually infiltrate healthy brain but can be recruited to the CNS through mechanisms mediated by CCL2 and CXCL12[105,106]. BMDM and microglia can comprise up to half of all cells within the TME[67,107,108] and illustrate temporal and region-specific transcriptional diversity, dictated by the tissue environment[109,110]. A thorough mechanistic understanding of TAM-mediated tumor potentiation may identify candidate interactions of therapeutic interest. Here, we summarize available TAM data of therapeutic interest in BM, recognizing that the bulk of TAM exploration in brain tumors, to date, have been in gliomas.

In the initial response to inflammation, microglia repair the damage incurred on the BBB by transmigration of tumor cells, which can protect micrometastases from the tumoricidal effect of systemic therapies. TAM can also transform its phenotype to phagocytose the BBB to facilitate leakage of peripheral immune cells and cytokines into the CNS, which cause widespread inflammation[111]. After tumor cell extravasation, microglia accumulate at the point of contact between tumor cells and brain parenchyma. Tumor cells use microglial processes in a WNT-dependent fashion to invade and proliferate within the brain parenchyma[112]. Once macrometastases are established, BM control the recruitment of BMDM to the TME through chemokines such as CCL2 and CX3CL1[106,113]. BMDM subsequently lose phagocytic activity and acquire tumor-supportive microglial-like states to adapt to the BM TME. In gliomas, this shift to a pro-tumoral TAM state is associated with immunosuppressive gene expression signatures and cytokines (e.g. PD-L1[114–116], TGF-beta[114,115], IL-10), which have gained traction as potential therapeutic targets. More studies specific for BM are needed.

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Until recently, single cell profiling of TAM subsets in brain tumors have been hindered by challenges in differentiating between microglia and BMDM and the perturbation of microglial phenotype with conventional tissue dissociation and isolation methods. Discovery of unique markers for microglia and BMDM[117–119] (e.g. TMEM119, CX3CR1, and CD49D) have enabled study of these respective populations. In a comparison of TAMs, BM and IDH-wild type gliomas generally possessed a more activated microglial phenotype compared to IDH-mutant gliomas. BM-associated microglia had greater type I interferon signaling and inflammatory nuclear factor-KB (NFKB) signaling, as well as increased expression of CXCL8, a neutrophil attractant. Histopathology review of BM confirmed substantial infiltration of T cells and neutrophils, compared to gliomas[67]. The functional significance of these transcriptional programs is still being explored. In BM, TAM subsets were differentiated by varying activity of antigen presentation pathways, and expression of pro-inflammatory genes (e.g. hypoxia-inducible factor 1a, IL-1B, *VEGFA*)[120]. This transcriptional heterogeneity hints at unique pro-tumoral or anti-tumoral roles performed by different TAM subsets. One of these subpopulations exhibited upregulation of CXCL10, which then promoted an immunosuppressive niche - associated with the molecules VISTA and PD-L1 - and enhanced BM growth. In a transgenic murine model, CXCL10 or VISTA inhibition in combination with anti-PDL1 treatment reduced immune suppression and resulted in BM regression[69]. These results highlight the dynamic flexibility of CXCL10, as this molecule promotes T-cell recruitment in certain contexts but also immunosuppression through microglial expression of VISTA and PD-L1. Understanding what mediates this plastic phenotypic shift would have high therapeutic potential.

THERAPEUTIC STRATEGIES UNDER DEVELOPMENT

Recent studies have shown intracranial benefit with CNS-penetrant targeted therapies and ICI. These advances have been covered in prior clinical reviews[25,121,122]. However, the majority of patients with BM exhibit intrinsic or acquired resistance to these therapies. At present, two major categories of BM treatments under investigation include: 1) anti-neoplastic agents that overcome the BBB and 2) targeted or immune-based therapies that interrupt tumor-host cell interactions. A list of select ongoing trials designed specifically for BM patients is listed in Table 1.

As BM harbor biological differences from primary tumors or ECM, BM genomic characterization and functional studies are instrumental to identify therapies effective for this patient population. Recent studies[40,50–52,54–57,123] suggest that targeting oncogenic drivers within BM is a viable therapeutic strategy. Larger multi-institutional studies are now validating this hypothesis[58]. However, the majority of BM do not possess a genomic alteration that can be targeted with currently available CNS-penetrant targeted therapies[41]. Pre-clinical studies have identified the *PI3K* pathway[46,124], OXPHOS pathway[48], and ST6GALNAC5[26] are druggable targets in BM. CNS-penetrant inhibitors for these targets are either under development or being evaluated in clinical trials. Furthermore, cancer cells adapt to the BM TME by altering gene and protein expression profiles in situ[125,126]. Another strategy might be to identify and target the specific vulnerabilities that arise in the process of adapting tumor cells to the BM TME.

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While selectively targeting activated oncogenic pathways may result in high initial efficacy, single-agent molecular therapeutics eventually fail in metastatic cancers as tumors will inevitably develop resistance mechanisms. Drugs aimed instead at the BM TME may circumvent this - a prominent example being programmed death ligand (PD-L1), which is expressed on TAM[127,128] and mediate CD4+ T-cell suppression[129] and Treg expansion[114]. [130] Combined blockade of CTLA-4 and PD1-PDL1 recently demonstrated increased intracranial efficacy, compared to single-agent ICI[130,131]. However, many BM patients do not respond even to combination ICI; thusrecent efforts have proposed combining PD1-PDL1 blockade with other CNS TME-targeting agents, such as inhibitors of of TAM receptor tyrosine kinases (RTK - e.g. TYRO3, AXL, MERTK). TAM RTK suppress innate immune responses within the TME. In vivo TAM RTK inhibition with anti-PD1 ICI extended survival in preclinical models of melanoma BM through increasing CD8 T-cell infiltration[132]. Gap junction and STAT3 inhibitors aimed at tumor astrocytes have shown activity for BM in pre-clinical models[66,93]. STAT3 inhibitors are now being evaluated in phase I trials for melanoma BM (NCT01904123). As TAM characterization for BM continues to mature, we anticipate study of astrocyte-TAM-tumor interactions will yield potential mechanisms that tumor cells employ to reprogram TAM and astrocytes to an immunosuppressive pro-tumor state. This knowledge can be used to propose immune-based therapies that revert these native cells to an anti-tumor state.

Ultimately, given distinct molecular differences and a highly unique TME in BM, compared to ECM, treatments effective for BM may not always be effective for other metastases. In cases where BM harbor a distinct oncogenic driver not detected within the primary tumor or ECM, systemic agents targeting the BM may not result in extracranial responses[40,41]. As more CNS-specific targets are identified, treating a metastatic cancer patient will likely necessitate a combination of BM-specific drugs, local therapies (e.g. radiation) as well as treatments to target extracranial metastases. However, such combinatorial approaches will require adequate clinical investigation to understand and minimize toxicities. We thus encourage multi-organ TME-based studies to identify shared contributors of metastasis, as well as increased study of BM prevention paradigms, as BM may be easier to treat prophylactically compared to once they have already formed.

BM prevention models are a relatively understudied concept. Prophylactic cranial irradiation was previously recommended for patients with solid tumors at high risk of spreading to the CNS, but has recently fallen out of favor due to neurocognitive sequelae. Therefore, pharmacologic prevention could be attractive to high-risk patients. There is an increasing body of pre-clinical evidence that systemic therapies given at preventative dosing may have higher efficacy than given at therapeutic dosing when macro-metastases have already formed[10,133,134]. Post-hoc analyses from clinical studies also support this hypothesis. Patients with metastatic RCC treated with sorafenib showed a 75% reduction in development of BM, compared to a 4% clinical response rate on established BM[135]. Similarly, lapatinib exhibited an intracranial response rate of 6% in patients with HER-2 positive breast cancer BM[136], but follow-up analyses noted a significant reduction in the brain as the first site of relapse, which qualifies as a CNS preventative effect[137]. Recently, pre-clinical efforts have demonstrated preventative efficacy with temozolomide for breast cancer BM[138] and *PI3K/AKT* inhibition for melanoma BM[139]. A phase 1 trial evaluating intracranial

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preventative potential of low-dose temozolomide for HER2-positive breast cancer patients is now enrolling (NCT03190967; Table 1).

Pharmacologic prevention is especially appealing due to the poor BBB penetrance of many systemic therapies. Furthermore, in a prevention scenario, it may be possible to lower doses of drugs, and therefore reduce the risk of toxicity, to achieve the desired effect. Targeting essential steps (e.g. vascular co-option) of the brain metastatic cascade may control the outgrowth of micro-metastatic tumor cells and reduce the risk of developing BM. To test candidate therapeutics, we encourage planning of prevention trials enrolling patients at high risk of developing BM (e.g. small cell lung cancer, patients with previously treated BM). A primary prevention trial measures a drug's ability to prevent development of BM in patients without CNS disease[140]. These trials, however, can require years of follow-up, and CNS events following the initial development of a BM are ignored. Secondary prevention trials examine the efficacy of an intervention at preventing BM in patients who have been diagnosed with BM and are therefore at a high risk of developing further BM[140]. This trial design may be a more feasible way of providing initial evidence of a preventative effect. In this scenario, a possible endpoint would be time to development of a new BM.

Finally, another exciting effort is the investigation of non-invasive biomarkers reflective of BM physiology. While precision medicine approaches for BM have demonstrated promising responses, many patients are not able to benefit from this treatment paradigm as molecular or transcriptional analysis of BM is not usually feasible due to the morbidity associated with brain biopsy. One emerging technique to inform the selection of genotype-directed therapies is liquid biopsy. Multiple studies have demonstrated that CSF cell-free tumor DNA (cfDNA) provides a high-fidelity representation of the glioma genome[141–143]. However, tumor-derived DNA is shed into the CSF for only 40–60% of patients with malignant brain tumors[142–144]. While the amount of tumor-derived DNA within the CSF correlates with brain tumor burden[144], more studies assessing the worth of CSF cfDNA as a prognostic and therapeutic biomarker for BM (e.g. concordance of cfDNA to brain and ECM) are needed. If these issues are optimized, liquid biopsy-based approaches hold promise to increase patient enrollment in future CNS basket trials, particularly those who are poor surgical candidates.

CONCLUSION

Treatment of BM has dramatically improved over the past decade owing to advances in molecular profiling of BM, targeted agents, and immunotherapy. To bolster this progress, we encourage study of diverse facets of BM biology - from individual steps of the brain metastatic cascade to cell populations within the TME – to reveal potential vulnerabilities and prognostic biomarkers (Figure 3. To date, the role of the BM TME and its contribution to the immune response and BM growth are poorly defined. The investigation of the BM TME at the level of single cells to identify candidate gene, protein, or metabolic changes is of great therapeutic interest. Furthermore, burgeoning advances in spatial transcriptomics and proteomics hold promise to transform our understanding of synergistic cross-talk between cell populations in the TME with cancer cells. By understanding the influence of these facets of BM biology on clinical outcomes, we hope our vision of personalized treatment for BM is becoming closer to reality. Clinical applicability of these translational

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studies will facilitate the development of effective precision-based treatment paradigms and the identification of future targets for BM-directed therapies.

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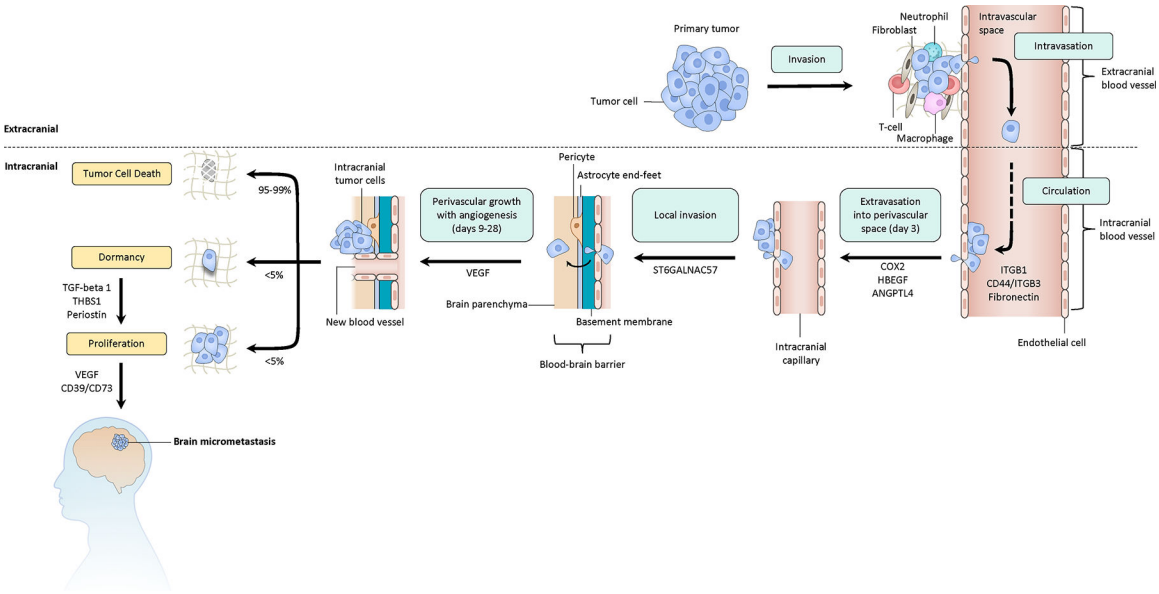

Fig 1. The brain metastatic cascade.

Major steps include: invasion of the tumor cells from the primary site into the extending stroma; intravasation of tumor cells into the peripheral circulation; extravasation of tumor cells into an intracranial perivascular space; local invasion of tumor cells, perivascular growth, and angiogenesis. We note that peri-vascular growth and angiogenesis are two separate steps in the formation of a macrometastasis, and that VEGF is therapeutically relevant are targeting angiogenesis. The majority of tumor cells do not survive following extravasation, with less than 5% lying in a dormant state with the ability to proceed to a proliferative state. This schematic may not recapitulate many nuances of *in vivo* brain metastasis, as this work was mostly done on cell lines.

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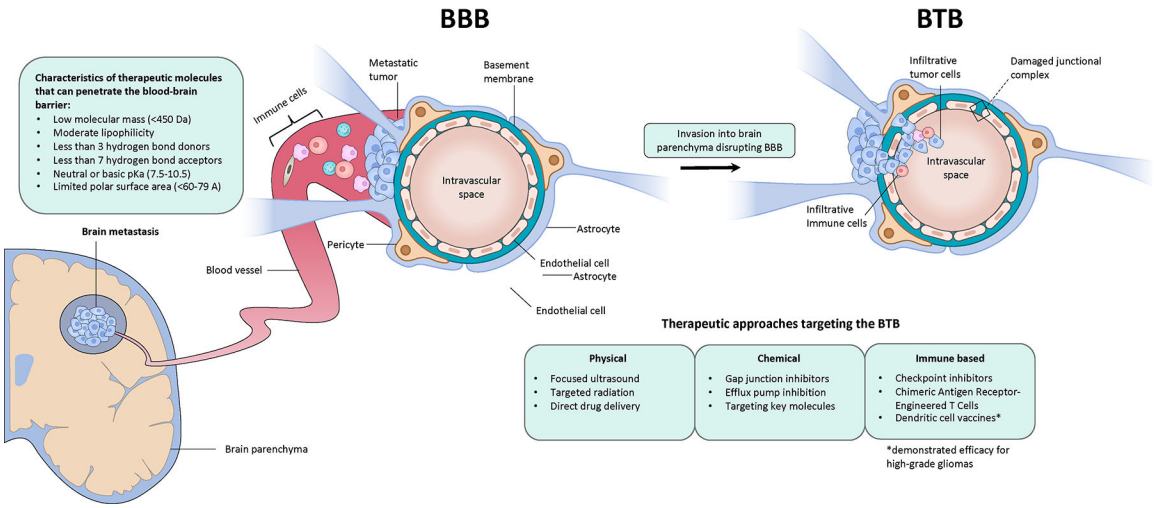

Figure 2: Simplified schematic of BBB and BTB physiology and potential therapeutic strategies.

We illustrate biological changes exerted onto the BBB as a result of the brain metastatic cascade. Characteristics of therapeutic molecules that penetrate the BBB are listed. We also note therapeutic strategies augmenting drug efficacy (e.g. increasing immune cell efflux), as well as therapeutically relevant targets within the BTB.

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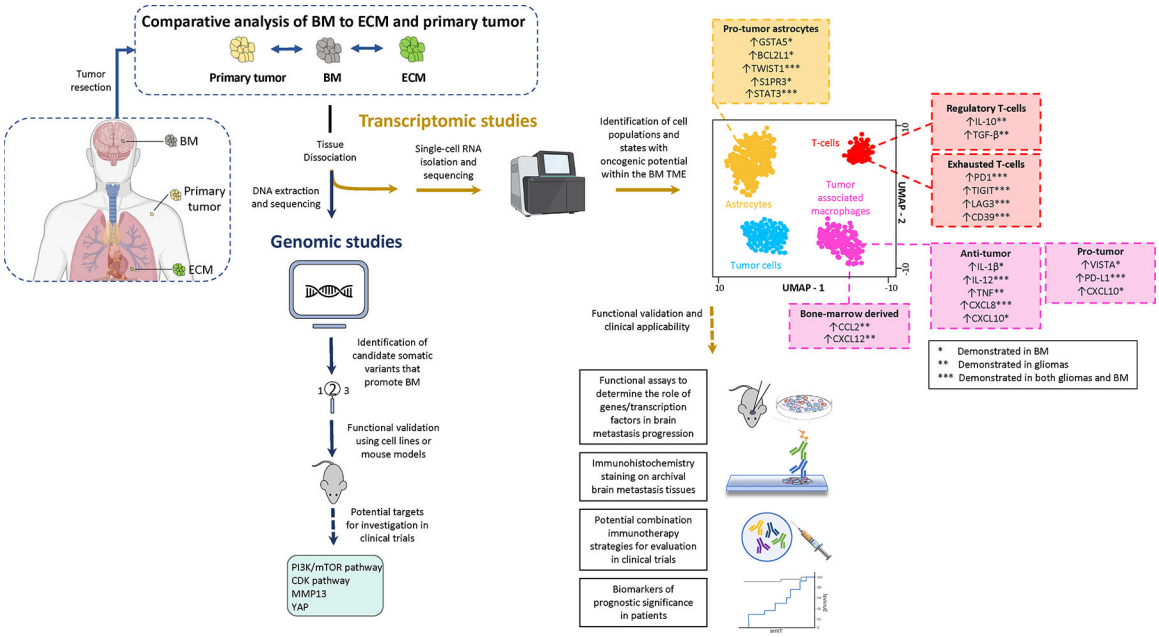

Figure 3: A translational workflow in analyzing patient-derived brain metastasis tissue and identifying new therapeutic targets.

Comparative genomic, transcriptomic, immunogenic, and proteomic analyses of BM to ECM/primary tumor may result in a privileged view of mechanisms of CNS dissemination and therapeutic resistance. BM tissue can be submitted for either genomic or transcriptomic analysis. Following DNA sequencing, comparative analyses between BM and primary tumor WES data may reveal somatic variants associated with increased frequency of BM. Functional validation of these candidate variants have revealed potential targets that are being investigated in trials. Similarly, single-cell expressing profiling has potential to understand TME cell populations or states that play a role in treatment response. While the majority of single-cell profiling efforts in brain tumors have been in gliomas, we anticipate some overlap between immunosuppressive signatures of gliomas to that of BM. These immunosuppressive and tumor-promoting facets of T, glial, and myeloid cell populations may provide new therapeutic opportunities.

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Table 1:

Select clinical trials evaluating systemic therapies for patients with BM

NCT (Phase)	Inclusion / exclusion criteria	Planned Enrollment	Intervention	Study Design	Primary Outcome
Targeted Therapy					
NCT04992013 / (2)	CNS basket trial; presence of progressive BM and BRCA1, BRCA2, PARP, DNA repair pathway, or homologous recombination gene alteration within BM	20	Niraparib	Single arm open-label	Intracranial clinical benefit rate
NCT03994796 / (1/2)	CNS basket trial; presence of progressive BM and CDK pathway, PI3K pathway, or NTRK/ROS1 alteration within BM	150	Abemaciclib Paxalisib Entrectinib	Non-randomized open-label parallel assignment: Arm 1: CDK pathway alteration Arm 2: PI3K pathway alteration Arm 3: NTRK/ROS1 alteration	Intracranial clinical benefit rate
NCT02896335 / 2	CNS basket trial; presence of progressive BM and CDK pathway alteration within BM	30	Palbociclib	Single arm open-label	Intracranial clinical benefit rate
NCT03898908 / 2	BRAF-mutant metastatic melanoma without prior local treatment in the brain	38	Encorafenib / binimetinib	Non-randomized open-label parallel assisgnment: Arm 1: asymptomatic BM Arm 2: symptomatic BM	Intracranial response rate
NCT04158947 / 1/2	HER-2 positive breast cancer BM	130	Afatinib and trastuzumab emtansine (T-DM1)	Randomized two-arm study: Arm 1 – T-DM1 + afatinib in dose escalation Arm 2 – T-DM1 + afatinib vs. T-DM1	Maximum tolerated dose of afatinib when used in combination with T-DM1; intracranial response rate
NCT04752059 / 2	HER-2 positive breast cancer BM	15	Trastuzumab deruxtecan (T-DxD)	Single arm open-label	Intracranial response rate
NCT04185883 / 1b/2	Basket trial for KRAS G12C-mutant solid tumors (progressive BM are allowed)	1054	Sotorasib and histology-specific therapeutic	Non-randomized, open-label, multi-arm (for each histology)	Maximum tolerated dose of sotorasib in combination with other therapeutics; extracranial response rate
Astrocyte-directed Therapy					
NCT01904123 / 1	Progressive melanoma BM or glioma	8	WP1066 (STAT3 inhibitor)	Single arm open-label	Maximum tolerated dose; incidence of adverse events
NCT02429570 / pilot	Progressive BM of any histology	30	Meclofenamate (CX-43 inhibitor)	Single arm open-label	Feasibility
Chemotherapy (prevention trial)					
NCT03190967 / 1/2	Histology-confirmed HER-2 positive breast cancer BM	125	T-DM1 Temozolomide (TMZ)	Randomized open-label sequential assignment Arm 1 – T-DM1 + TMZ in dose escalation Arm 2 – T-DM1 alone	Median time to developing new BM; maximum tolerated dose of TMZ when

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NCT (Phase)	Inclusion / exclusion criteria	Planned Enrollment	Intervention	Study Design	Primary Outcome
					used in combination with T-DM1;
Combination immunotherapy regimens					
NCT04789668 / 1/2	Progressive BM of any histology	36	Bintrafusp alfa + pimasertib	Single arm open-label	Intracranial clinical benefit rate; incidence of dose-limiting toxicities
NCT03175432 / 2	BRAF V600 wild type melanoma BM	60	Atezolizumab Bevacizumab Cobimetinib	Non-randomized open-label parallel assignment Arm 1: atezolizumab, bevacizumab Arm 2: atezolizumab, bevacizumab, cobimetinib	Intracranial response rate
NCT03131908 / 1/2	Metastatic melanoma with PTEN loss (progressive BM can be enrolled)	36	GSK2636771 (PI3K inhibitor) Pembrolizumab	Single arm open-label	Maximum tolerated dose of GSK2636771 in combination with pembrolizumab; extracranial and intracranial response rate
NCT02910700 / 2	BRAF V600 mutant metastatic melanoma that has progressed on prior PD-1 inhibition (progressive BM can be enrolled)	51	Binimetinib Dabrafenib Encorafenib Trametinib Nivolumab	Non-randomized open-label parallel assignment Arm 1 – nivolumab, dabrafenib Arm 2 – nivolumab, trametinib Arm 3 – nivolumab, encorafenib, binimetinib	Extracranial and intracranial response rate
NCT02886585 / 2	CNS metastasis of any histology Arm 1 – untreated BM Arm 2 – progressive BM Arm 3 – neoplastic meningitis Arm 4 – 1–4 melanoma BM	102	Pembrolizumab Stereotactic radiosurgery	Non-randomized open-label parallel assignment Arm 1, 2, 3 – pembrolizumab Arm 4 – pembrolizumab + SRS	Intracranial response rate
NCT02816021 / 2	Metastatic melanoma (progressive BM can be enrolled) Arm 1 – PD-1 naïve Arm 2 – post PD-1 progression	24	Azacitidine Pembrolizumab	Non-randomized open-label parallel assignment	Extracranial and intracranial response rate
CAR T-cell therapy					
NCT03696030 / 1	CNS metastasis of any histology with HER2-overexpression	39	CAR T cell targeting HER2 antigen	Single arm open-label	Treatment-related adverse events
Dendritic cell vaccine					
NCT04348747 / 2	Triple-negative or HER-2 positive breast cancer BM	23	Anti-HER2 / HER3 dendritic cell vaccine	Single arm open-label	Intracranial response rate