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Single-cell advances in the investigation of the pathogenesis and treatment of brain metastasis

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Abstract

Brain metastasis remains a significant cause of morbidity and mortality for cancer patients with limited treatment options. Single-cell RNA-seq has greatly expanded our ability to study cancer and metastasis, particularly in our assessment of tumor heterogeneity and the tumor microenvironment. In recent years, there have been several single-cell studies that have applied this technology to brain metastasis. These studies have described transcriptional characteristics of brain metastatic tumor cells and characterized immune cell changes as well as contributions of vascular and stromal cells to the brain metastatic microenvironment. This review article summarizes these studies, how they contribute to our knowledge of the molecular and cellular steps that occur in brain metastasis and in response to therapy, and how they suggest promise for future treatments.

Key Points

- Primary tumors undergo molecular adaptations in the brain metastatic process including expressing brain-specific lineage markers and adopting a stem cell-like state.
- The tumor microenvironment is distinct in brain metastases and leptomeningeal disease.
- Immunotherapy shifts the T-cell population to an exhausted state in the central nervous system, although inflammatory activity is correlated with a more favorable therapy response.

Single-cell analysis has revolutionized the approach to tumor profiling. Separation of individual cells from a tumor sample can elucidate the various types of tumor, immune, stromal, and surrounding normal cells and profile their transcriptional state.[1,2] Following single-cell separation, transcriptional states are assessed by RNA-seq or methods to evaluate chromatin structure such as ATAC-Seq. The tumor microenvironment has become increasingly recognized as a crucial factor to whether tumor cells are suppressed, proliferate, or even metastasize.[3–5] Further, tumor microenvironmental factors are central to therapeutic response, especially in the age of immune checkpoint inhibitors, chimeric antigen receptor therapy, and other immune therapies.[6,7] Single-cell analysis has accordingly been an effective methodology to assess both molecular changes in tumor cells and the surrounding microenvironment.[1,2,8] A wide array of software has been developed for single-cell analysis, many of them specifically designed for cancer applications including the identification of tumors and associated immune cells.[2] While the advent of single-cell studies has queried many primary tumors and extracranial metastatic sites,[9–12] there have been only a handful of papers that have used single-cell technology to assess brain metastases.

Brain metastasis (BM) is a common complication of many types of tumors. They occur in up to 40% of patients with metastatic disease from solid tumors and are a frequent cause of morbidity and death as they progress.[13,14] Given the burden of brain metastatic disease and the fact that patients with intracranial metastases are often excluded from clinical trials, there is a great need for further study of the molecular features of BM.[15] While there has been incredible progress, the biology of

how primary tumors invade, adapt, and proliferate is still being elucidated.[16–18] Further, brain metastases are often resistant to initial treatment strategies, whether due to issues of blood-brain barrier penetration, unfavorable brain tumor microenvironmental factors, or other reasons.[19] Single-cell analysis provides an opportunity to study these types of biological questions. This review article describes the studies and results of single-cell technologies published through February 2025, focusing primarily on single-cell RNA-seq, that have assessed intracranial metastases in patients. Table 1 summarizes the major articles that have pursued single-cell RNA-seq analysis of brain metastases.

The Brain Metastatic Cascade

While the genetic and environmental changes that permit primary tumorigenesis have been extensively studied and described,[31] less is known about the steps required for tumors to undergo metastasis, including BM. Work that has been done so far has described a series of steps, known as the brain metastatic cascade.[3,13,17] In this paradigm, tumors acquire changes that allow intravasation into the circulatory system, extravasation and invasion into a new tissue microenvironment, and ultimately new cell growth.[32,33] Tumor cells in a new microenvironment may also undergo a period of dormancy where a subset of cells will be suppressed from further growth by protective intrinsic tissue mechanisms but some cells may ultimately proliferate leading to a new focus of metastasis.[34–36]

Single-cell studies have confirmed tenets of the brain metastatic cascade. From a genomic perspective, brain metastases tend to have more chromosomal instability, including whole genome duplication compared to primary tumors across multiple primary tumor histologies.[21,22,37] Across many studies of intracranial and extracranial metastasis, single-cell clustering of cells typically finds that tumor cells are transcriptionally unique in each individual patient sample, whereas nonmalignant cells cluster together across individuals.[21,22] Across multiple primary tumor histologies, single-cell analyses have also found that brain metastases tend to be relatively genetically and transcriptionally homogeneous compared to their primary tumor counterparts.[22,30] This idea is consistent with the "bottleneck" hypothesis with respect to brain tumor evolution, which hypothesizes that there is a narrow subset of cells that acquire the genetic and epigenetic characteristics that allow progression through the brain metastatic cascade and ultimate proliferation in the brain. Previous bulk sequencing approaches also support this hypothesis,[10,18,38] including studies that have shown that brain metastases are even more genetically homogeneous compared to lymph node metastases.[39]

Single-cell analysis of tumor samples along various stages in the brain metastatic cascade has deepened our understanding of the tumor and microenvironmental changes that happen from normal tissue to primary tumorigenesis, lymph node metastasis, and ultimately to BM. In a study of lung metastasis, Kim et al. analyzed single-cell samples at each of these timepoints to yield a "pseudotime" of transcriptional and microenvironmental changes that occur along these steps.[23] Epithelial and stromal cells gained pro-proliferative and angiogenic properties. T-cell phenotypes shifted from more naïve and cytotoxic T-cells in primary tumors to increased exhausted and regulatory T-cell phenotypes in BM that suppress anti-tumor immunity.[23] As single cells have been studied along the brain metastatic cascade, specific transcriptional properties are selected for to successfully cross the blood-brain barrier and proliferate in a new microenvironment.[22,23,30]

Brain Organotropism

Some primary tumors, particularly breast, lung, melanoma, and renal cell carcinoma, are known to metastasize to the brain, while other types of primary tumors rarely metastasize to the brain.[10,40] Paget originally coined the idea of the "seed and soil" hypothesis, suggesting that metastatic colonies grow in new environments with compatible molecular, metabolic, and immunologic characteristics.[41] The specific mechanisms for predilection for BM, sometimes called brain organotropism, have been studied but remain poorly understood.[42]

Previous studies using approaches other than single-cell sequencing have identified molecular changes that may allow for brain metastatic organotropism.[42] Broadly, it has been hypothesized that metastatic cells must cross the blood-brain barrier and subsequently adapt to the relatively hypoxic and immunologically distinct environment. Molecular factors have been identified in BM organotropism that fit along the brain metastatic cascade, including how primary tumors can express components of cell-cell communication pathways with resident brain cells, secrete factors to alter the blood-brain barrier, and even co-opt neuronal synapse pathways through the expression of neurotransmitter receptors.[43–45]

Single-cell studies of BM have also identified genes important for brain organotropism. Figure 1 summarizes the molecular pathways for brain organotropism that have been implicated by single-cell studies. For example, Biermann et al. compared melanoma brain metastases to extracranial metastases and found upregulation of neural differentiation, morphogenesis, and adhesion transcriptional programs in the brain including upregulation of neuronal growth factor receptor,[21] which has also been associated with melanoma phenotypic plasticity and drug resistance.[49] Synaptic function and formation were also upregulated,[21] supporting the idea that tumor metastases form pseudo-synapses with existing neurons (as do primary gliomas[47]) to support their malignant growth. By applying bulk Assay for Transposase-Accessible Chromatin (ATAC)-Seq to study cell lines generated from a subset of patients, they further demonstrated a neuronal program of transcription factor motifs upregulated and hypothesized that this program enabled melanoma brain metastases to adapt to brain-specific growth signaling pathways.[21] A recent study identified non-small cell lung cancer brain metastases also upregulate a variety of brain lineage factors, and that these factors are similar to but do not completely overlap with factors seen in melanoma brain metastases.[50]

Table 1. Key Single-Cell Studies Published Through February 2025.

Study	Samples	Main Methods Used	Main Conclusions
Alvarez-Breckenridge, C. et al. *Cancer Immunol Res.*[20]. PMID: 35706413	32 sequentially collected melanoma BMs from 27 patients (23 patients were post-ICI treatment)	Single-cell RNA-seq, whole exome sequencing, TCR sequencing	Identified features of myeloid and lymphocyte, including clonal expansion, in melanoma BMs (relative to extracranial metastases) after ICI, including markers of response to therapy
Biermann, J. et al. *Cell.*[21] PMID: 35803246.	22 melanoma BM (from 21 patients); 10 extracranial metastases.	Single-cell and single-nucleus RNA-seq, spatial transcriptomics	Compared treatment-naive melanoma BM to extracranial mets and found that BM expresses neuronal-lineage genes. Associated immune cells have an increased inflammatory meta state.
Gonzalez, H. et al. *Cell.*[22] PMID: 35063085	15 BMs across 7 primary histologies	Single-cell RNA-seq, mass cytometry by time-of-flight (CyTOF) for analysis of protein markers	Characterized genetic and transcriptional characteristics across many primary tumor types, including transcriptional "meta" states of BM-associated immune cells.
Kim, N. et al. *Nat Commun.*[23]. PMID: 32385277	44 patients with lung adenocarcinoma of various stages. 10 BM samples.	Single-cell RNA-seq; whole exome seq; IHC; flow cytometry	Performed a pseudotime analysis on samples along stages from lung adenocarcinoma to brain metastasis and identified trajectories of tumor and associated immune cells
Prakadan, S. M. et al. *Nat Commun.*[24]. PMID: 34642316	12 pretreatment and 25 serial posttreatment (ICI) CSF samples from 19 patients with LMD (various primary histologies)	Single-cell RNA-seq; cell-free DNA-Seq	Serial CSF examination of LMD patients post-ICI revealed CD8 + T-cells and IFN-γ as correlating with clinical response.
Rubio-Perez, C. et al. *Nat Commun.*[25]. PMID: 33686071	50 brain metastases of various histologies, 9 of which underwent single-cell RNA-Seq.	Bulk RNA-Seq, IHC, whole exome seq, single-cell RNA-seq. 6 patients with matched BM and CSF samples underwent single-cell RNA-seq and TCR-seq	Immunotherapy and BRAF-MEK inhibition in melanoma BM and LMD led to distinct post-therapy TIMEs, with specific T-cell and dendritic cell populations being predictive of therapy response.
Smalley, I. et al. *Clin Cancer Res.*[26]. PMID: 34035069	26 melanoma patients: 8 with skin metastases, 14 BMs, 6 patients with LMD (with sequential CSF sampling, 19 CSF samples total)	Single-cell RNA-seq Also re-analyzed and incorporated data from Biermann et al.[21]	Compared melanoma BM and LMD TIME and found distinct CD4 + T-cell, CD8 + T-cell, and myeloid proportions and phenotypes, some of which influence response to ICI
Song, Q. et al. *Commun Biol.*[27]. PMID: 37479733	5 samples from lung ($N = 2$) and breast ($N = 3$) BMs	Single-cell RNA-seq Also re-analyzed data from Nguyen et al.	In both lung and breast BM, tumor-associated fibroblasts were identified that express collagen genes and modify cell-cell interactions. An inflammatory pattern of immune cells was also found in BM.
Sudmeier, L. J. et al. *Cell Rep Med.*[28] PMID: 35584630	31 BMs of several histologies (14 lung, 4 melanoma, 6 breast, 7 other).	Single-cell RNA-seq, TCR-seq, Spatial Transcriptomics. Analysis focused on T-cell populations	Characterized CD8 T-cell subtypes across multiple histologies of primary tumor BMs and compared clonotypic profiles at the tumor site and in circulation.
Sun, H.-F. et al. *Clin Transl Med.*[29] PMID: 36336787	4 glioma and 10 lung BMs	Single-cell RNA-seq	In comparing TIME between lung BMs to primary gliomas, the lung BM TIME is characterized by immune cells and endothelial cells with invasive gene signatures. Epithelial cells have cancer stem cell-like traits that also are associated with invasiveness.
Wu, Y. et al. *Biomolecules.*[30] PMID: 36671569	23 primary lung adenocarcinoma; 16 lung adenocarcinoma BMs	Single-cell RNA-seq Also re-analyzed data from Nguyen et al. [10] and Brastianos et al.[14]	Compared cellular landscape between lung adenocarcinoma primary tumor vs brain mets as well as used novel approaches to infer cell-cell interactions and to predict ICI responses.

Abbreviations: BM, brain metastasis; ICI, immune-checkpoint inhibitor; LMD, leptomeningeal disease; TIME, tumor immune microenvironment.

Epithelial cells in lung adenocarcinoma BM samples also have upregulated transcription factor pathways that enable cancer stem cell-like states (Figure 1).[29] Cancer stem cells are characterized by specific cell surface markers, and characteristic changes in epigenetic, signaling, and metabolic pathways, as has been previously reviewed.[51] It has

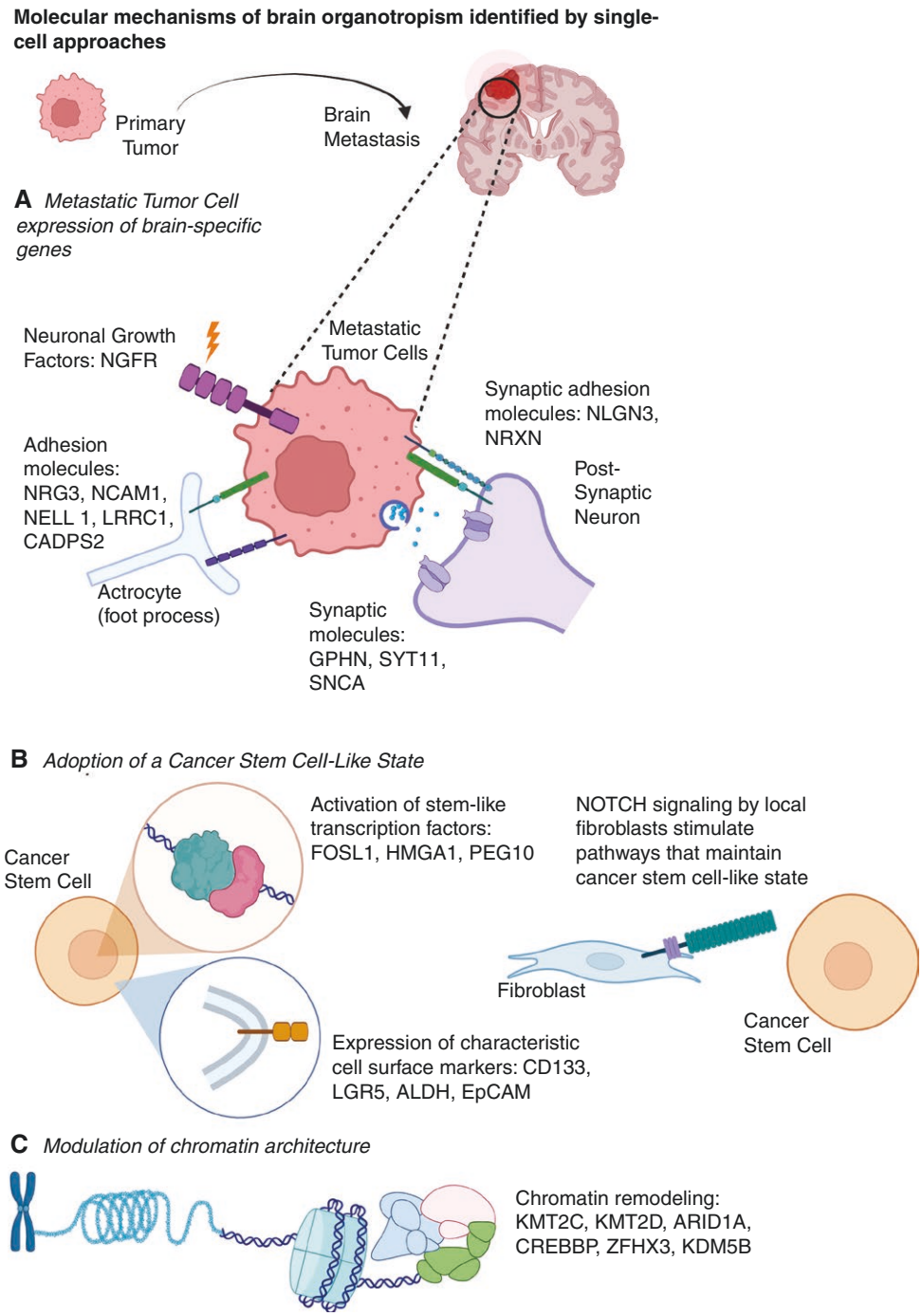

Figure 1. Single-cell studies identify molecular factors co-opted by brain metastases to adopt a brain-specific organotropism. (A) Tumor cells can express neuronal growth factors to promote growth in the new microenvironment, synaptic genes that allow communication with pre- or postsynaptic neurons, and adhesion molecules for inferred interactions with astrocytes or other brain support cells.[21] The direct interactions between metastatic tumor cells and resident brain cells are inferred, although some studies have used single-cell analytic methodologies to infer interactions through programs like CellPhoneDB.[46] There is also a rapidly growing field of cancer neuroscience studying the interactions between malignant and normal brain cells, as reviewed elsewhere.[47] (B) Cancer stem cell-like pathway transcription factors are upregulated and the NOTCH signaling pathway is increased to maintain a stem-like state.[29] (C) Chromatin-regulating genes are expressed that allow changes in epigenomic state for ongoing proliferation and/or adaptation to the brain microenvironment.[48] Created in BioRender. Nayyar N, and Porter, R. (2024) BioRender.com/z33z676

been hypothesized that adopting a stem cell-like state may give metastatic tumor cells the ability to modulate their transcription in a way that is favorable to the metastatic site microenvironment. Malignant epithelial cells identified in lung adenocarcinoma BM single-cell data have stem cell-like traits, including expression of stemness-like transcription factors, such as FOSL1, and characteristic cell surface markers including CD133 and LGR5.[29] Further, multiple studies have used single-cell bioinformatics programs to infer cell-cell interactions and demonstrated that malignant epithelial cells are able to interact with local endothelial cells, suggesting that this interaction could be necessary for invasion.[29,30] NOTCH signaling contributes to these cell-cell interactions,[29,30] a pathway known for maintaining the cancer stem cell-like state.[52] Consistent with these findings, whole exome and RNA sequencing approaches have found that BMs from colorectal carcinoma expressing the same cancer stem cell markers (including CD133 and LGR5) are enriched in chromatin-regulating genes including KMT2C, KMT2D, and ARID1A.[48] Single-cell sequencing of melanoma metastatic to extracranial sites also found high expression of another chromatin factor, KDM5B, particularly in slowly dividing stem-like cells.[53]

These studies have shown that cancer stem cell-like states allow various types of primary tumors to gain invasive characteristics that are able to interact with and invade the brain microenvironment (Figure 1). Across several primary tumor histologies, brain metastases upregulate neuronal proteins to interact with normal brain cells.[21,50] These brain-specific changes include alterations in both malignant tumor cells and associated epithelial and endothelial cells.[29,30] Associated cancer stem cells express cell surface markers, transcription factors, and epigenetic markers that allow for interaction with local cells and ongoing proliferation and invasion in a new microenvironment.[29,30,53]

Common Transcriptional Characteristics of Metastatic Tumor Cells

Brain metastatic tumor cells are genetically diverse and difficult to classify. Not only do they vary across primary histologies, but even within primary histologies, interpatient genetic variation is high which makes transcriptional and molecular classification challenging.[11,22] Within single-cell studies, metastatic tumor cells are often identified by the presence of chromosomal instability such as copy number variants.[37] Bulk sequencing approaches have found increased chromosomal instability in metastases compared to primary tumors.[10] Biermann et al. compared melanoma BM with melanoma extracranial metastases by whole exome sequencing and showed that BM had more chromosomal instability compared to extracranial metastases.[21] An in vitro assay of melanoma brain metastases showed that increased chromosomal instability led to increased migratory capacity, compared to melanoma extracranial metastases.[21]

Recent single-cell studies have attempted to identify clusters of gene expression to characterize metastatic tumor cells. Gonzalez et al. identified patterns of gene expression in metastatic tumor cells across 15 BM samples of varied primary histologies (melanoma, breast cancer, lung cancer, ovarian cancer, colorectal cancer, and renal cell carcinoma).[22] Their clustering analysis revealed 2 "meta"-gene expression programs, one defined by a proliferative state and the other an inflammatory state. Proliferating cells express a gene expression program with pre-mRNA splicing and DNA replication signatures. Opposingly, inflammatory cells exhibit gene expression programs with inflammation, stress responses, epithelial-to-mesenchymal transition, and increased regulation of extracellular matrix remodeling.[22] Genes within meta-programs are co-expressed, and this co-expression pattern appears to be unique in metastases compared to primary tumors.[22] Biermann et al., in their single-cell analysis, compared clusters of gene expression between melanoma BM and melanoma extracranial metastasis and demonstrated that the BM group was enriched in expression of lineage genes for neuronal differentiation while still maintaining melanocyte lineage gene expression.[21] Despite the intrinsic genetic and transcriptional heterogeneity, the identification of transcriptional "meta" states in groups of cells within brain metastases (of various primary histologies) shows that similar patterns of transcriptional dysregulation are necessary for proliferation in the brain metastatic niche.[21,22]

Immune Microenvironment

The immune microenvironment is a key factor that determines how metastatic cells invade and proliferate in the brain. This new era of single-cell studies is ideally positioned to evaluate and compare changes in tumor immune microenvironment (TIME), and previous reviews have summarized how single-cell studies have led to advancements in our TIME knowledge.[2,8,54] Interestingly, studies have revealed that the BM TIME shares characteristics regardless of primary tumor histology, even across brain metastases from lung adenocarcinoma, breast cancer, renal cell carcinoma, melanoma, and ovarian carcinoma.[22]

Lymphoid Cells

Earlier work used single-cell techniques to characterize the brain TIME in primary gliomas[55–57]; however, more recent work has compared the TIME in BM samples to primary glioma. Comparisons of lung BMs and primary gliomas demonstrated increased infiltration of T-cells, mast cells, and macrophages in lung BMs compared to gliomas which have more microglia and oligodendrocyte precursor cells.[29] Sajjadi et al. availed of publicly accessible single-cell RNA-seq data to compare gliomas, lung BMs, and breast BMs and found that each group had a unique composition.[58] The glioma microenvironment was enriched with macrophages and monocytes, the lung BM microenvironment had more cytotoxic CD4 +T-cells and more exhausted-type CD8 +T-cells, and the breast BM microenvironment identified novel subclusters of cancer-associated fibroblasts.[58] Such findings highlight the interplay between the primary

tumor and the brain microenvironment. While metastatic cells must adapt to a new microenvironment, metastatic cells also modulate lymphoid cell phenotypes and the composition of associated epithelial and endothelial cells.[29,58]

Single-cell studies have also shed light on mechanisms that brain metastases use for immune evasion. In comparing lung and breast BMs to primary gliomas, macrophage-mediated inflammation and T-cell malfunction, including the exhausted CD8 + T-cell phenotype, are suspected mechanisms by which BMs achieve immune evasion compared to gliomas which tend to have preserved cytotoxic T-cell function and antigen presentation.[30,58] Exhausted T-cells are thought to lack proliferative and cytotoxic properties due to stimulation of co-inhibitory cell surface markers such as PD-1, CTLA-4, and LAG3. However, recent single-cell studies of metastatic melanoma have found that exhausted T-cells may retain dysfunctional proliferative abilities and have a distinct group of cell surface markers.[53,59] T-cells in BMs have been characterized by single-cell sequencing, often in concert with T-cell receptor sequencing. In categorizing CD8 + T-cells within BMs of various primary histologies, Sudmeier et al. identified multiple clusters of T-cell phenotypes, including dividing cells, terminally differentiated cells, and multiple categories of progenitor-exhausted cells.[28]

CD8 T-cells with progenitor-exhausted phenotypes are suggested to be critical for tumor control in response to immune checkpoint inhibitors.[60,61] In BM sites, terminally exhausted CD8 + T cells carried more often a presumed tumor-specific antigen clonotype, while bystander CD8 T-cells had progenitor exhaustion gene signatures.[28]. Likewise, in a cohort of melanoma BM tumors resected post-immune-checkpoint inhibitor (ICI) treatment, CD8 T-cells carrying "private" clones (ie specificities unique to the patient and possibly including tumor-specific clones) were more frequent at the tumor locations and more likely to be exhausted.[20] Interestingly, a higher fraction of private clonotypes at the tumor was associated with clinical benefit, as was the presence of peripheral expansions of CD8 T-cells with "effector" phenotypes and non-tumor specificities.[20]

Taken together, both studies show that likely tumor-specific expanded clonotypes are located in the tumor and appear more exhausted presumably as a result of continued exposure to the antigen.[20,28] As is further described below with immunotherapy responses, the immune characteristics of the exhausted T-cell phenotype are associated with worse clinical outcomes in the setting of immunotherapy.[20,62] In these single-cell analyses, the presence of exhausted phenotypes that are associated with clinical benefit is evidence to the transient functionality of the immune system and the ability of ICI to boost the immune response, even in BM.[20,28] Current and future work using single-cell techniques will aid in understanding of which immune cells and their interactions are contributing to inevitable tumor progression or relapse in the brain.

Myeloid Cells

Classically, macrophages are characterized by either a pro-inflammatory "M1" state or an anti-inflammatory "M2" state.[63,64] However, this categorization seems more complex in tumor-associated macrophages, particularly with inflammatory subtypes in brain metastases.[65] In an analysis of pseudotime progression from lung adenocarcinoma primary tumor to BM, macrophages seem to differentiate from monoctyes into 2 phenotypes, a complement-containing (C1Q+) phenotype which promotes phagocytosis and is more anti-inflammatory versus a secreted phospho-protein-containing (SPP1+) phenotype which promotes ongoing inflammatory activation, cytokine release, migration, and pro-angiogenesis.[30] Alvarez-Breckenridge et al. found similar SPP1 + clusters of macrophages in their melanoma BM dataset, a marker that they note is associated with chronic viral infection.[20] Gonzalez et al. described a further step in metastasis-associated macrophages such that APOE + macrophages express both C1Q and SPP1, but a separate group of macrophages expresses S100A for a distinct inflammatory phenotype.[22] Across multiple histologies of brain metastases, distinct clusters of inflammatory macrophages are unique to the BM TIME compared to classical macrophage phenotypes and suggest mechanisms in which metastatic tumor cells evade the immune response.[20,22,30]

Microglia have also been identified in lung BMs that express a high proportion of checkpoint inhibitor ligand genes, suggestive of an immune evasion phenotype.[29] Alvarez-Breckenridge et al. identified a cluster of reactive microglia previously associated with models of neuronal injury and aging.[20] These observations start to show how chronic inflammation in brain metastases influences the BM TIME.

Dendritic cells have also been identified in single-cell BM analyses and have exceptionally high expression of markers associated with migration and transcription regulation (such as JUNB, NR4A2).[22] Conversely, a subset of dendritic cells tended to downregulate antigen-presenting molecules compared to various histologies of primary tumors.[22,30] Markers of dendritic cell activation were also decreased in BM samples compared to primary lung adenocarcinoma.[30] Dendritic cells associated with BMs have a transcriptional profile that appears to increase their migratory capacity and decrease their ability to react to external stimuli.[22,30]

Neutrophils in lung BMs (compared to primary gliomas) are characterized by transcriptional states leading to phenotypes that promote degranulation and formation of reactive oxygen species that favor angiogenesis.[29] Alvarez-Breckenridge et al. found 4 subclusters of neutrophils in their single-cell analysis of melanoma BMs.[20] A cluster of BM-associated neutrophils had elevated expression of interleukin (IL)-8, which was associated with gene expression that promotes angiogenesis. Further, this neutrophil phenotype was associated with metastatic tumor cells with higher expression of epithelial-to-mesenchymal transition pathways and an overall worse prognosis.[20] In their assessment of immunotherapy treatment response, they also found that an increased neutrophil-to-lymphocyte ratio was associated with increased tumor necrosis and a poor response to therapy.[20] These studies collectively suggest that, similar to macrophages, neutrophils may polarize between pro-tumor and anti-tumor phenotypes. Further, the specific transcriptional states in BM-associated neutrophils appear to be distinct from tumor-associated neutrophils in

gliomas, primary melanoma tumors, and melanoma extracranial metastases.[20,29]

Stromal Microenvironment

Vascular and Endothelial Microenvironment

Vascular cells, including endothelial cells and pericytes, are important cells in the brain metastatic microenvironment, particularly as they relate to the blood-brain barrier and angiogenesis.[5] Unsurprisingly, single-cell analysis demonstrated that vascular and endothelial cells have increased expression of angiogenic genes in BM samples across multiple primary tumor histologies.[22] Further, BM endothelial cells have increased expression of ABC cassette genes, which is hypothesized to be a cellular mechanism of treatment resistance via the efflux of chemotherapies,[22] although this idea remains to be tested experimentally. Endothelial cells in lung adenocarcinoma BMs also modulate their extracellular matrix protein expression in ways that change focal adhesion molecules and facilitate brain metastatic cell invasion.[29] Wu et al. analyzed pericytes from lung BM and found immature transcriptional signatures that were hypothesized to decrease blood-brain barrier integrity, increase vascular proliferation, and activate epithelial-to-mesenchymal transition transcriptional programs.[30] Beyond resident endothelial cells, brain metastases are also associated with tumor-derived endothelial cells which have properties that promote angiogenesis and tissue remodeling.[23] Single-cell study of endothelial cells has collectively identified mechanisms of drug resistance, angiogenesis, and alterations in cell-cell interactions that promote invasion.[22,23,29,30]

Epithelial Microenvironment

Fibroblasts, and specifically "cancer-associated fibroblasts," are crucial components of BM, particularly for tumor invasion into and out of the circulatory system.[66] Most single-cell studies distinguish between normal epithelial cells and malignant epithelial cells by the presence of copy number variants. In a single-cell RNA-seq analysis of 3 breast and 2 lung metastases, there were tumor-associated fibroblasts with high expression of collagen-related genes and tumor receptors, identifying ways in which the stromal environment interacts with metastatic tumor cells.[27] Cancer-associated fibroblasts in lung BMs have decreased expression of human leukocyte antigen (HLA), suggesting a deficiency in antigen presentation which may be another mechanism of immune evasion.[30] Upon re-analysis of lung BM single-cell data from Kim et al.,[23] Xiao et al. found that malignant epithelial cells (as defined by a copy number variant score) have properties associated with activation of coagulation and complement pathways, which is thought to promote tumor invasiveness.[67] Similar to the decreased genetic heterogeneity in brain metastases compared to primary tumors,[18,22] cancer-associated fibroblasts also have less genetic heterogeneity compared to cancer-associated fibroblasts seen in primary tumors.[30] These results collectively suggest that BM-associated epithelial cells evolve to express interaction molecules that invade the new environment and evade immune detection.[27,30,67]

CSF Sampling of BM and Leptomeningeal Disease

There is increasing interest in using serum samples as a means of "liquid biopsy" to capture circulating tumor DNA for diagnostic and prognostic purposes.[68] Analogously, there have been recent studies that have sampled cerebrospinal fluid (CSF) for cell-free/circulating tumor DNA in primary brain tumors, BM, and leptomeningeal disease (LMD).[69,70] LMD metastasis, characterized by the infiltration of tumor cells into the leptomeninges through the CSF, has a particularly poor outcome, even compared to BM.[71] More recently, several studies have performed single-cell analysis of CSF samples.[24–26] It is important to note that CSF samples may not be representative of the cells within BM, but they may offer central nervous system (CNS) microenvironment information that may be diagnostically or prognostically useful. One single-cell study of melanoma BM and LMD showed good concordance in the relative microenvironmental cell numbers and cell proportions of samples collected from CSF versus direct leptomeningeal sampling performed at autopsy.[26]

Rubio-Perez et al. directly compared the results of single-cell analysis of CSF samples to matched surgical BM specimens from 6 patients of various primary histologies.[25] Overall abundances of T and NK cells were similar across CSF and surgical BM specimens. While T cells overall showed a good correlation between CSF and surgical BM specimens, subtype compositions across T-cells varied such that there was increased ratio of CD8 + to CD4 + T-cells in the surgical BM samples compared to the CSF samples. In combining single-cell analysis with T-cell receptor sequencing, 4 out of 6 matched samples had identical T-cell receptor sequences suggesting direct communication between BM and the CSF space in those samples.[25] These results suggest that there are differences between the immune microenvironments between the CSF and within a BM, but that specific T-cell isotypes can be shared between the 2 compartments. Prognostically, the relative amount of T cells in either the CSF or surgical BM sample correlated with overall survival and was predictive of immunotherapy response.[25] In a clinical trial comparing both peripheral blood and CSF single-cell RNA-seq in LMD patients of various primary histologies, Prakadan et al. showed that inflammatory signatures in the CSF are greater than what is detected in the blood, and this correlated with overall prognosis in patients receiving immunotherapy.[24]

Studies have compared melanoma and breast BM with LMD and the associated TIME.[24,26] Previous work has shown examples of metastatic melanoma patients with both BM and LMD, where immunotherapy was only effective at the BM site but not the LMD site, or vice versa.[26] This clinical finding prompted the hypothesis that the BM and LMD TIMEs are distinct. Indeed, while both BM and LMD are within the CNS, they appear to have distinct TIMEs using single-cell sequencing. Extracranial metastases had

more phenotypic overlap with the BM compartment compared to the LMD compartment. LMD has a higher proportion of T and NK cells and the highest proportion of myeloid and monocytic cells compared to the BM compartment.[24,26] Contrastingly, the BM TIME appears to contain more B cells and plasma cells than the LMD compartment. Finally, compared to LMD, the myeloid cells in the BM group presented a more immunosuppressive phenotype and had the least amount of antigen-presenting dendritic cells.[26] As noted above, while there are shared immune cells between BMs and throughout the CSF space, there are differences in the local TIME between BM and LMD. The above studies show how TIME differences have diagnostic and prognostic importance.[24–26]

Posttreatment Changes in BM and LMD

Immunotherapy, and particularly ICIs, has become key therapeutics for primary and metastatic cancers including BM.[72–74] As ICIs have become more widely used, studies have begun to investigate the cellular and molecular changes associated with ICI use. Single-cell analysis of ICI response is useful to measure T-cell infiltration and immune effector function.[30] Reviews of the TIME field have attempted to predict ICI response based on the primary tumor microenvironment.[54] Profiling has characterized immunologically "hot" tumors (often characterized by a high proportion of CD8 + T-cells) that would theoretically be more susceptible to ICI compared to immunologically "cold" tumors (often characterized by a high percentage of tumor-associated macrophages).[12,54] Single-cell molecular characterization of melanoma extracranial metastasis has suggested that tumor mutational burden, neoantigen load, and T-cell infiltration are important molecular factors in ICI response.[62] However, it is unclear if these conclusions apply to intracranial brain metastases given the distinct brain TIME. Figure 2 summarizes the TIME changes that occur post-ICI treatment in the BM and LMD compartments, as outlined in the studies described next.

Alvarez-Breckenridge et al. analyzed 27 patients with melanoma BM and characterized the TIME after ICI treatment with single-cell RNA-seq and T-cell receptor sequencing, factoring in whether there had been a treatment response, partial response, or no response.[20] Although there was great systemic clonal expansion of T-cell populations outside of the CNS, this expansion was limited in the BM samples. As described earlier, post-ICI treatment BM T-cells showed expansion of the exhausted phenotype.[20]

Prakadan et al. sampled CSF from patients with LMD of breast and other primary tumors at several time points following ICI treatment and subjected the samples to single-cell RNA-seq. An early (< 30 days posttreatment) and transient influx of CD8 + T-cells was observed, as well as an early transient increase in interferon-γ and antigen

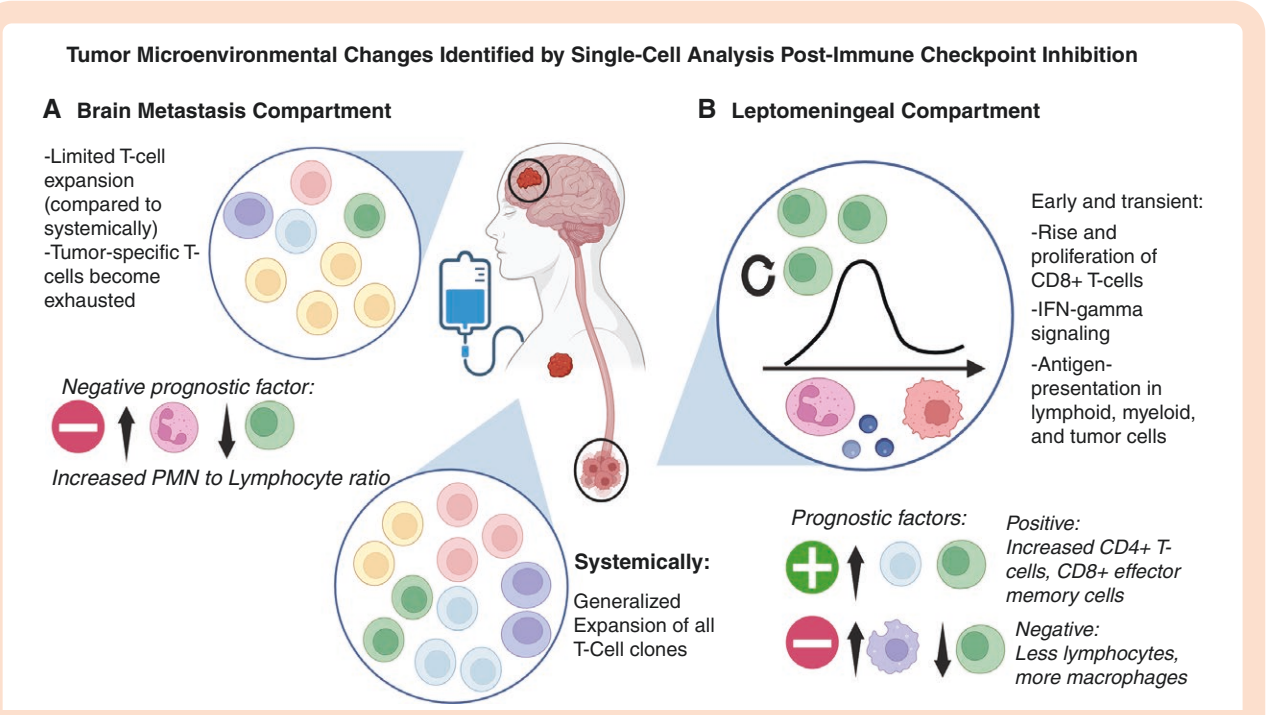

Figure 2. Tumor microenvironmental changes identified by single-cell sequencing studies performed after immune checkpoint inhibitor treatment. Systemically, ICI causes broad expansion of immune cells and T-cell clones. (A) In brain metastases, T-cell expansion is limited, and CD8 + T-cells are predominantly of the exhausted phenotype. An increased neutrophil-to-lymphocyte ratio is a negative prognostic sign.[20] (B) Post-ICI, there is a transient increase in CD8 + T-cells in the LMD compartment. There is also increased expression of IFN-gamma signaling and antigen-presenting signatures in lymphoid, myeloid, and tumor cells. Increased CD4 + T-cells and CD8 + effector memory T cells are a good prognostic factor, whereas increased macrophages and decreased lymphocytes relatively are a negative prognostic factor in LMD post-ICI.[24,26] Created in BioRender. Nayyar N and Porter R. (2024) BioRender.com/a58k470. ICI, immune-checkpoint inhibitor; LMD, leptomeningeal disease.

presentation response signatures across lymphoid, myeloid, and tumor cells.[24] This result underscores the importance of longitudinal sampling to understand the effect of treatment on the TIME. Increased activity of interferon-γ signaling was associated with overall prognosis and durability of response to ICI.[24] One particularly striking result came from the analysis of a patient with rapid progression in LMD. Compared to other analyzed patients, there was increased copy number variation heterogeneity suggesting the presence of tumor subclones. Ultimately, a subclone with less interferon-γ response activity was selected for which likely led to disease progression.[24] Their results show that both the magnitude and persistence of CD8 +T-cell influx and interferon-γ response are important prognostic markers in ICI response for LMD patients.[24]

Smalley et al. compared melanoma BM and LMD samples including samples after ICI treatment. Particularly interesting was one patient sample with LMD who had a remarkable response to ICI and lived well beyond the median survival time and was particularly enriched with CD4 +T-cells.[26] In a separate patient with melanoma BM that underwent serial BM resections, both ICI and targeted therapy (with tyrosine kinase inhibitors) greatly increased the number of infiltrating immune cells; however, targeted therapy led to an increased ratio of CD8 + to CD4 +T cells in BM, whereas ICI was characterized by a more diverse lymphocytic landscape as well as a marked increase in B cells and plasma cells in BM.[26] Larger cohorts will be necessary to validate this result to compare the immunologic effect of BRAF-MEK inhibition to immune checkpoint inhibition.

In all 3 studies, across various primary tumor histologies, CNS infiltration of specific populations of T-cells (particularly CD8 +T-cells) appears to be required for immunotherapy response (Figure 2).[20,24,26] Profiling of the pretreatment TIME by single-cell analysis may have prognostic value. Future work could use such profiling to inform a personalized treatment strategy that optimizes a patient's response to immunotherapy.

Perspective and Future Directions

Single-cell RNA-seq studies of BM have already led to important findings about the molecular and cellular steps required for BM, the brain metastatic TIME, and the response to treatments, particularly immunotherapy. Several important overarching conclusions arise from this group of studies: (1) Primary tumors use distinct intrinsic strategies to adapt and proliferate in the brain metastatic niche. (2) The BM TIME is different from the primary glioma TIME. Distinct inflammatory characteristics of myeloid cells and CD8 +T-cells allow for immune evasion in the BM TIME. (3) Immunotherapy modulates the BM TIME including a shift in T-cell phenotypes toward the exhausted phenotype; however, the exhausted T-cell phenotype requires further study as it has more proliferative potential than previously appreciated. (4) CSF studies hold promise as a method to less invasively interrogate the CNS TIME. Notably, BM samples have a distinct TIME compared to LMD samples.

There are multiple similarities seen in BMs regardless of the original primary tumor histology. Brain metastases of distinct primary tumor histology appear to have different transcriptional characteristics and unique genetic and epigenetic mechanisms of adapting to the brain microenvironment,[21,22] although the specific similarities and differences merit additional study. Contrastingly, the associated immune microenvironmental changes have much in common across distinct primary tumor histologies, and these were the focus of most of the studies reviewed here. Studies across 7 different primary tumor types identified a polarization of immune cell phenotypes such that immune cells associated with BMs have an inflammatory "meta" state. This inflammatory state is associated with increased cytokine release, increased myeloid cell migration, and a predominance of CD8 +T-cells of the exhausted phenotype.[21–24] Overlap in immune phenotypes is also seen in LMD from both melanoma and breast cancer: in both types of cancer, the influx of CD8 +T-cells and inflammatory cytokines is associated with ICI treatment response, although this seems to decline when CD8 +T-cells shift to the exhausted phenotype.[24–26] Similarities in the TIME across multiple tumor types may be useful diagnostically or therapeutically and could constitute a clinical application for single-cell technologies.

Single-cell analysis has several limitations. The cost of the technology remains high (typically ranges between $500 and $10 000 per sample, depending on the application) relative to bulk sequencing methods. While there are many clinical trials studying the role of single-cell sequencing in clinical settings, its use has not yet become widely available or considered standard-of-care. Single-cell RNA-seq samples must be processed immediately after tissue collection, making sample collection challenging in many scenarios. Single-nucleus RNA-seq, however, does allow evaluation of fixed or frozen samples, as some of the studies reviewed here performed. Extracellular matrix components or other noncellular components of the BM microenvironment are not necessarily captured by single-cell analysis. Further, there is a biased selection of certain cell types during the steps required to prepare a single-cell RNA-seq library. In brain tissue, for example, there are well-known perturbations in gene expression in microglia and other immune cells that alter accurate interpretation of transcriptional states.[75] Gene representation may also be biased, such as with "drop-outs," or difficulty in detecting low-abundance genes that are poorly amplified during library preparation.[76]

While most of these initial studies are limited in their power due to small sample numbers, further work will expand both common molecular features and the complex tumor heterogeneity seen in BM. Some of the studies described here included samples that are paired between a primary tumor and metastatic site, adding additional value in inferring the molecular steps from the primary tumor to an established BM. In addition, some studies included both pre- and posttreatment samples allowing inference of the treatment effect including tools that may help predict treatment effect in the future. Further work and greater power in such paired studies will offer diagnostic and prognostic information.

Future work using spatial transcriptomics that combines sequencing approaches with spatial context within a histologic sample will add knowledge about how the

immune system interacts with the tumor and stroma in functional niches and will assess cell-cell interactions. These approaches are also advantageous as they can be performed on formalin-fixed tissues, increasing the ease of the timing at which samples can be analyzed and offer the possibility of retrospectively recovering key information on prior interesting cases. These technologies are becoming increasingly powerful as they approach single-cell resolution. Single-cell studies tend to focus on either primary tumor biopsies or BM biopsies, which allow inference of the molecular steps in between the 2 samples, but do not directly assess intermediate steps of the brain metastatic cascade. Recent work assessed autopsy samples of breast cancer patients and found brain micro-metastases that may represent initial steps in BM.[77] Serum or CSF liquid biopsies in the future may be noninvasive ways in vivo to elucidate these intermediate steps in patients.

Multi-omic studies in the future will also add to our knowledge of the molecular and cellular characteristics in brain metastases. Further studies will continue to elucidate how brain metastases acquire drug resistance, change their signaling pathways, and modulate the TIME in response to therapies. Combining single-cell RNA-seq with genomic or epigenomic approaches will show how mutations influence expression, and this could be further related to proteomic data. Such multi-omic approaches will define molecular and cellular pathways that brain metastases use and can ultimately serve as potential treatment targets.

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brain metastasis | organotropism | single-cell sequencing

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References

1. Suvà ML, Tirosh I. Single-cell RNA sequencing in cancer: lessons learned and emerging challenges. *Mol Cell*. 2019;75(1):7–12.
2. Huang D, Ma N, Li X, et al. Advances in single-cell RNA sequencing and its applications in cancer research. *J Hematol Oncol*. 2023;16(1):98.
3. Fidler IJ. The pathogenesis of cancer metastasis: the "seed and soil" hypothesis revisited. *Nat Rev Cancer*. 2003;3(6):453–458.
4. DeClerck YA, Pienta KJ, Woodhouse EC, Singer DS, Mohla S. The tumor microenvironment at a turning point knowledge gained over the last decade, and challenges and opportunities ahead: a white paper from the NCI TME network. *Cancer Res*. 2017;77(5):1051–1059.
5. de Visser KE, Joyce JA. The evolving tumor microenvironment: from cancer initiation to metastatic outgrowth. *Cancer Cell*. 2023;41(3):374–403.
6. Sharma P, Allison JP. The future of immune checkpoint therapy. *Science*. 2015;348(6230):56–61.
7. Davis-Marcisak EF, Deshpande A, Stein-O'Brien GL, et al. From bench to bedside: single-cell analysis for cancer immunotherapy. *Cancer Cell*. 2021;39(8):1062–1080.
8. Du Q, An Q, Zhang J, Liu C, Hu Q. Unravelling immune microenvironment features underlying tumor progression in the single-cell era. *Cancer Cell Int*. 2024;24(1):143.
9. Tirosh I, Suva ML. Cancer cell states: lessons from ten years of single-cell RNA-sequencing of human tumors. *Cancer Cell*. 2024;42(9):1497–1506.
10. Nguyen B, Fong C, Luthra A, et al. Genomic characterization of metastatic patterns from prospective clinical sequencing of 25,000 patients. *Cell*. 2022;185(3):563–575.e11.
11. Robinson DR, Wu YM, Lonigro RJ, et al. Integrative clinical genomics of metastatic cancer. *Nature*. 2017;548(7667):297–303.
12. Hong F, Meng Q, Zhang W, et al. Single-cell analysis of the pan-cancer immune microenvironment and scTIME portal. *Cancer Immunol Res*. 2021;9(8):939–951.
13. Kim AE, Nieblas-Bedolla E, de Sauvage MA, Brastianos PK. Leveraging translational insights toward precision medicine approaches for brain metastases. *Nat Cancer*. 2023;4(7):955–967.
14. Brastianos PK, Curry WT, Oh KS. Clinical discussion and review of the management of brain metastases. *J Nat Comprehensive Cancer Network*. 2013;11(9):1153–1164.
15. Vogelbaum MA, Brown PD, Messersmith H, et al. Treatment for brain metastases: ASCO-SNO-ASTRO Guideline. *J Clin Oncol*. 2022;40(5):492–516.
16. Kienast Y, von Baumgarten L, Fuhrmann M, et al. Real-time imaging reveals the single steps of brain metastasis formation. *Nat Med*. 2010;16(1):116–122.
17. Lambert AW, Pattabiraman DR, Weinberg RA. Emerging biological principles of metastasis. *Cell*. 2017;168(4):670–691.
18. Brastianos PK, Carter SL, Santagata S, et al. Genomic characterization of brain metastases reveals branched evolution and potential therapeutic targets. *Cancer Discov*. 2015;5(11):1164–1177.

19. Eichler AF, Chung E, Kodack DP, et al. The biology of brain metastases-translation to new therapies. *Nat Rev Clin Oncol.* 2011;8(6):344–356.
20. Alvarez-Breckenridge C, Markson SC, Stocking JH, et al. Microenvironmental landscape of human melanoma brain metastases in response to immune checkpoint inhibition. *Cancer Immunol Res.* 2022;10(8):996–1012.
21. Biermann J, Melms JC, Amin AD, et al. Dissecting the treatment-naive ecosystem of human melanoma brain metastasis. *Cell.* 2022;185(14):2591–2608.e30.
22. Gonzalez H, Wei M, Robles I, et al. Cellular architecture of human brain metastases. *Cell.* 2022;185(4):729–745.
23. Kim N, Kim HK, Lee K, et al. Single-cell RNA sequencing demonstrates the molecular and cellular reprogramming of metastatic lung adenocarcinoma. *Nat Commun.* 2020;11(1):2285.
24. Prakadan SM, Alvarez-Breckenridge CA, Markson SC, et al. Genomic and transcriptomic correlates of immunotherapy response within the tumor microenvironment of leptomeningeal metastases. *Nat Commun.* 2021;12(1):5955.
25. Rubio-Perez C, Planas-Rigol E, Trincado JL, et al. Immune cell profiling of the cerebrospinal fluid enables the characterization of the brain metastasis microenvironment. *Nat Commun.* 2021;12(1):1503.
26. Smalley I, Chen Z, Phadke M, et al. Single-cell characterization of the immune microenvironment of melanoma brain and leptomeningeal metastases. *Clin Cancer Res.* 2021;27(14):4109–4125.
27. Song Q, Ruiz J, Xing F, et al. Single-cell sequencing reveals the landscape of the human brain metastatic microenvironment. *Commun Biol.* 2023;6(1):760.
28. Sudmeier LJ, Hoang KB, Nduom EK, et al. Distinct phenotypic states and spatial distribution of CD8+ T cell clonotypes in human brain metastases. *Cell Rep Med.* 2022;3(5):100620.
29. Sun HF, Li LD, Lao IW, et al. Single-cell RNA sequencing reveals cellular and molecular reprograming landscape of gliomas and lung cancer brain metastases. *Clin Transl Med.* 2022;12(11):e1101.
30. Wu Y, Kang K, Han C, et al. Single-cell profiling comparisons of tumor microenvironment between primary advanced lung adenocarcinomas and brain metastases and machine learning algorithms in predicting immunotherapeutic responses. *Biomolecules.* 2023;13(1):185.
31. Hanahan D, Weinberg RA. Hallmarks of cancer: the next generation. *Cell.* 2011;144(5):646–674.
32. Massagué J, Ganesh K. Metastasis-initiating cells and ecosystems. *Cancer Discov.* 2021;11(4):971–994.
33. Massagué J, Obenauf AC. Metastatic colonization by circulating tumour cells. *Nature.* 2016;529(7586):298–306.
34. Steeg PS. Targeting metastasis. *Nat Rev Cancer.* 2016;16(4):201–218.
35. Goss PE, Chambers AF. Does tumour dormancy offer a therapeutic target? *Nat Rev Cancer.* 2010;10(12):871–877.
36. Lawson DA, Bhakta NR, Kessenbrock K, et al. Single-cell analysis reveals a stem-cell program in human metastatic breast cancer cells. *Nature.* 2015;526(7571):131–135.
37. Bakhoum SF, Ngo B, Laughney AM, et al. Chromosomal instability drives metastasis through a cytosolic DNA response. *Nature.* 2018;553(7689):467–472.
38. Priestley P, Baber J, Lolkema MP, et al. Pan-cancer whole-genome analyses of metastatic solid tumours. *Nature.* 2019;575(7781):210–216.
39. Reiter JG, Hung WT, Lee IH, et al. Lymph node metastases develop through a wider evolutionary bottleneck than distant metastases. *Nat Genet.* 2020;52(7):692–700.
40. Hess KR, Varadhachary GR, Taylor SH, et al. Metastatic patterns in adenocarcinoma. *Cancer.* 2006;106(7):1624–1633.
41. Paget S. The distribution of secondary growths in cancer of the breast. 1889. *Cancer Metastasis Rev.* 1989;8(2):98–101.
42. Yuzhalin AE, Yu D. Brain metastasis organotropism. *Cold Spring Harb Perspect Med.* 2020;10(5):a037242.
43. Zeng Q, Michael IP, Zhang P, et al. Synaptic proximity enables NMDAR signalling to promote brain metastasis. *Nature.* 2019;573(7775):526–531.
44. Chen Q, Boire A, Jin X, et al. Carcinoma-astrocyte gap junctions promote brain metastasis by cGAMP transfer. *Nature.* 2016;533(7604):493–498.
45. Valiente M, Obenauf AC, Jin X, et al. Serpins promote cancer cell survival and vascular co-option in brain metastasis. *Cell.* 2014;156(5):1002–1016.
46. Efremova M, Vento-Tormo M, Teichmann SA, Vento-Tormo R. CellPhoneDB: inferring cell-cell communication from combined expression of multi-subunit ligand-receptor complexes. *Nat Protoc.* 2020;15(4):1484–1506.
47. Mancusi R, Monje M. The neuroscience of cancer. *Nature.* 2023;618(7965):467–479.
48. Desette A, Guichet PO, Emambux S, et al. Deciphering brain metastasis stem cell properties from colorectal cancer highlights specific stemness signature and shared molecular features. *Cell Mol Gastroenterol Hepatol.* 2023;16(5):757–782.
49. Liu D, Lin JR, Robitschek EJ, et al. Evolution of delayed resistance to immunotherapy in a melanoma responder. *Nat Med.* 2021;27(6):985–992.
50. Tagore S, Caprio L, Amin AD, et al. Single-cell and spatial genomic landscape of non-small cell lung cancer brain metastases. *Nat Med.* 2025;31(4):1351–1363.
51. Lin S, Li K, Qi L. Cancer stem cells in brain tumors: from origin to clinical implications. *MedComm.* 2023;4(4):e341.
52. McGowan PM, Simedrea C, Ribot EJ, et al. Notch1 inhibition alters the CD44hi/CD24lo population and reduces the formation of brain metastases from breast cancer. *Mol Cancer Res.* 2011;9(7):834–844.
53. Tirosh I, Izar B, Prakadan SM, et al. Dissecting the multicellular ecosystem of metastatic melanoma by single-cell RNA-seq. *Science.* 2016;352(6282):189–196.
54. Binnewies M, Roberts EW, Kersten K, et al. Understanding the tumor immune microenvironment (TIME) for effective therapy. *Nat Med.* 2018;24(5):541–550.
55. Patel AP, Tirosh I, Trombetta JJ, et al. Single-cell RNA-seq highlights intratumoral heterogeneity in primary glioblastoma. *Science.* 2014;344(6190):1396–1401.
56. Tirosh I, Venteicher AS, Hebert C, et al. Single-cell RNA-seq supports a developmental hierarchy in human oligodendroglioma. *Nature.* 2016;539(7628):309–313.
57. Tirosh I, Suvà ML. Dissecting human gliomas by single-cell RNA sequencing. *Neuro Oncol.* 2018;20(1):37–43.
58. Sajjadi SF, Salehi N, Sadeghi M. Comprehensive integrated single-cell RNA sequencing analysis of brain metastasis and glioma microenvironment: contrasting heterogeneity landscapes. *PLoS One.* 2024;19(7):e0306220.
59. Li H, van der Leun AM, Yofe I, et al. Dysfunctional CD8 T cells form a proliferative, dynamically regulated compartment within human melanoma. *Cell.* 2019;176(4):775–789.e18.
60. Liu Z, Zhang Y, Ma N, et al. Progenitor-like exhausted SPRY1+CD8+ T cells potentiate responsiveness to neoadjuvant PD-1 blockade in esophageal squamous cell carcinoma. *Cancer Cell.* 2023;41(11):1852–1870.e9.
61. Siddiqui I, Schaeuble K, Chennupati V, et al. Intratumoral Tcf1+PD-1+CD8+ T cells with stem-like properties promote tumor control in response to vaccination and checkpoint blockade immunotherapy. *Immunity.* 2019;50(1):195–211.e10.
62. Hugo W, Zaretsky JM, Sun L, et al. Genomic and transcriptomic features of response to anti-PD-1 therapy in metastatic melanoma. *Cell.* 2016;165(1):35–44.

63. Stein M, Keshav S, Harris N, Gordon S. Interleukin 4 potently enhances murine macrophage mannose receptor activity: a marker of alternative immunologic macrophage activation. *J Exp Med*. 1992;176(1):287–292.
64. Mills CD, Kincaid K, Alt JM, Heilman MJ, Hill AM. M-1/M-2 macrophages and the Th1/Th2 paradigm. *J Immunol*. 2000;164(12):6166–6173.
65. Klemm F, Maas RR, Bowman RL, et al. Interrogation of the microenvironmental landscape in brain tumors reveals disease-specific alterations of immune cells. *Cell*. 2020;181(7):1643–1660.e17.
66. Chhabra Y, Weeraratna AT. Fibroblasts in cancer: unity in heterogeneity. *Cell*. 2023;186(8):1580–1609.
67. Xiao Y, Hu F, Chi Q. Single-cell RNA sequencing and spatial transcriptome reveal potential molecular mechanisms of lung cancer brain metastasis. *Int Immunopharmacol*. 2024;140(1):112804.
68. Cescon DW, Bratman SV, Chan SM, Siu LL. Circulating tumor DNA and liquid biopsy in oncology. *Nat Cancer*. 2020;1(3):276–290.
69. Shankar GM, Balaj L, Stott SL, Nahed B, Carter BS. Liquid biopsy for brain tumors. *Expert Rev Mol Diagn*. 2017;17(10):943–947.
70. Rehman AU, Khan P, Maurya SK, et al. Liquid biopsies to occult brain metastasis. *Mol Cancer*. 2022;21(1):113.
71. Glitza IC, Smalley KSM, Brastianos PK, et al. Leptomeningeal disease in melanoma patients: an update to treatment, challenges, and future directions. *Pigment Cell Melanoma Res*. 2020;33(4):527–541.
72. Twomey JD, Zhang B. Cancer immunotherapy update: FDA-approved checkpoint inhibitors and companion diagnostics. *AAPS J*. 2021;23(2):39.
73. Tawbi HA, Forsyth PA, Algazi A, et al. Combined nivolumab and ipilimumab in melanoma metastatic to the brain. *N Engl J Med*. 2018;379(8):722–730.
74. Long GV, Atkinson V, Lo S, et al. Combination nivolumab and ipilimumab or nivolumab alone in melanoma brain metastases: a multicentre randomised phase 2 study. *Lancet Oncol*. 2018;19(5):672–681.
75. Marsh SE, Walker AJ, Kamath T, et al. Dissection of artifactual and confounding glial signatures by single-cell sequencing of mouse and human brain. *Nat Neurosci*. 2022;25(3):306–316.
76. Kharchenko PV, Silberstein L, Scadden DT. Bayesian approach to single-cell differential expression analysis. *Nat Methods*. 2014;11(7):740–742.
77. Gan S, Macalinao DG, Shahoei SH, et al. Distinct tumor architectures and microenvironments for the initiation of breast cancer metastasis in the brain. *Cancer Cell*. 2024;42(10):1693–1712.e24.