Review

The Journey of Cancer Cells to the Brain: Challenges and Opportunities

Marzena Łazarczyk[1,#] ,Michel Edwar Mickael[1,#], Dominik Skiba[1], Michał Ławiński[1,2], Jarosław Olav Horbańczuk[1], Piotr Religa[2] and Mariusz Sacharczuk[1,4]

[1]Department of Experimental Genomics, Institute of Genetics, Animal Biotechnology and Academy of Sciences, Postępu 36A, 05-552 Poland.
[2] Department of General Surgery, Gastroenterology and Oncology, Medical University of Warsaw, Warsaw, Poland.
[3] Department of Medicine, Solna, Karolinska Institute, Stockholm, Sweden
[4] Department of Pharmacodynamics, Faculty of Pharmacy with the Laboratory Medicine Division, Medical University of Warsaw, Banacha 1B, 02-091 Warsaw, Poland
* Correspondence: m.mickael@igbzpan.pl, m.lazarczyk@igbzpan.pl
[#]These authors contributed equally to the manuscript.

Abstract

Cancer metastasis into the brain constitutes one of the most severe but not uncommon development of cancer growth. Several factors control how cancer types interact with the brain to establish metastasis These factors include system of migration, system of infiltration of the blood-brain barrier and the interaction with host cells (e.g., neurons, astrocytes) and the immune system fight against metastasis in the brain. Although, brain radiotherapy is the main treatment procedure carried out to treat, new therapies are emerging that constitute a glimpse of hope for increasing the diminutive life expectancy currently forecasted to cancer patients who are facing the prospectus of brain metastasis. However applying these therapeutic strategies has been has not been effective. Thus there is a need for better understanding of the metastasis process in order to suggest novel therapeutic targets. In this review, we follow the journey of various cancer cells from their primary location through the various process that they undergo including EMT, penetration of the ECM, intravasation, extravasation and infiltration of the blood brain barrier ending up with colonization and angiogenesis. In each phase, we focus on molecules that could potentially be drug target candidates.

Keywords: T cells; metastasis

1. Introduction

Brain metastasis (BM) is the most common type of intracranial neoplasm in adults. In addition to end-of-life status, brain metastasis patients suffer from various distressing symptoms such as headache, cognitive impairment, ataxia, and seizures. It has been revealed that the incidence of BM has doubled between 1987 and 2006 [1]. The increase in BM incidence is due to a combination of various factors including better diagnostic imaging and successful treatment modalities resulting in longer survival following the diagnosis of primary cancer. However, the prognosis of brain metastasis is currently devastating [2]. The median survival period for BM patients is between 5 weeks and 18 months. Several factors control the length of the survival period of BM patients. These factors include; the source of the primary tumor, the number of metastatic foci in the brain, the treatment method, and the BM location**. (1) There is a slight variation among BM patients based on the source of the primary cancer** (Table 1). Notably, it was reported that lung cancer cells were responsible for 80 % of the cases of reported BM cases, while melanoma, breast cancer, kidney cancer were responsible for 3.8,3.7 and 3% of the BM cases respectively [3]. However, in another study it was reported

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that secondary brain tumors are most likely to spread from breast cancer cells followed by lung, renal cell, and melanoma [4]. The reasons behind these discrepancies could be related to the age of patients, where it was shown that kidney/renal pelvis and melanomas were the most frequent in children [3]. Gender could also be playing a significant role in determining the origin of the BMs cells where it was reported that cancers most responsible for BM are breast in women and lung in both genders (Table 1). **(2) The number of metastatic loci was shown to be inversely proportional to prognosis outcome**, with a worse prognosis predicted based on the detection of one or more metastatic sites[5]. Due to constant improvements in diagnostic and therapeutic strategies, the median survival among patients with metastatic disease of the brain has been reported to increase [6]. **(3)The type of treatment influences the survival rate** as it was shown that patients undergoing specified therapy, including surgery or radiotherapy have a higher chance of surviving longer [7]. **(4) BM is not uniformly distributed among different brain regions.** Several studies have revealed that 85 % of metastasis cases were reported in the cerebrum including the posterior areas of the two hemispheres as well as the anterior border zone between the anterior and middle cerebral arteries. Interestingly only 10% to 15% of metastasis cases were detected in the cerebellum followed by 3% in the brainstem [8]. The reasons behind these differences are not yet clear.

Table1. Cancer cell types grouped by rate of brain metastasis and rate of survival[2]

Cancer type	Rate of brain metastasis	Rate of survival by month
Colorectal cancer	**0.27**	**3-17**
Breast	**0.41**	**3-36**
Lung	**12%**	**7-46**
Kidney	**1.48**	**4-35**
Melanoma	**0.65**	**5-34**

2. The metastatic cells journey to the brain

The molecular course of BM development follows various stages. The process starts with phenotypic changes of the tumor cell, detachment from the primary tumor, followed by invasion of the extracellular matrix (ECM), intravasation into blood or lymphatic arteries in a form of circulating tumor cell (CTC), dissemination via the blood or lymphatic system, extravasation, and colonization. To survive, cancer cells have to overcome several immune cells such as microglia, macrophages, CD4+ T cells, CD8+ T cells, and natural killer T cells. In this review, we will cover the stages the cancer cells follow to migrate from the primary cancer site to their metastatic site in the brain. We will also discuss current knowledge addressing how these cells challenge their surroundings.

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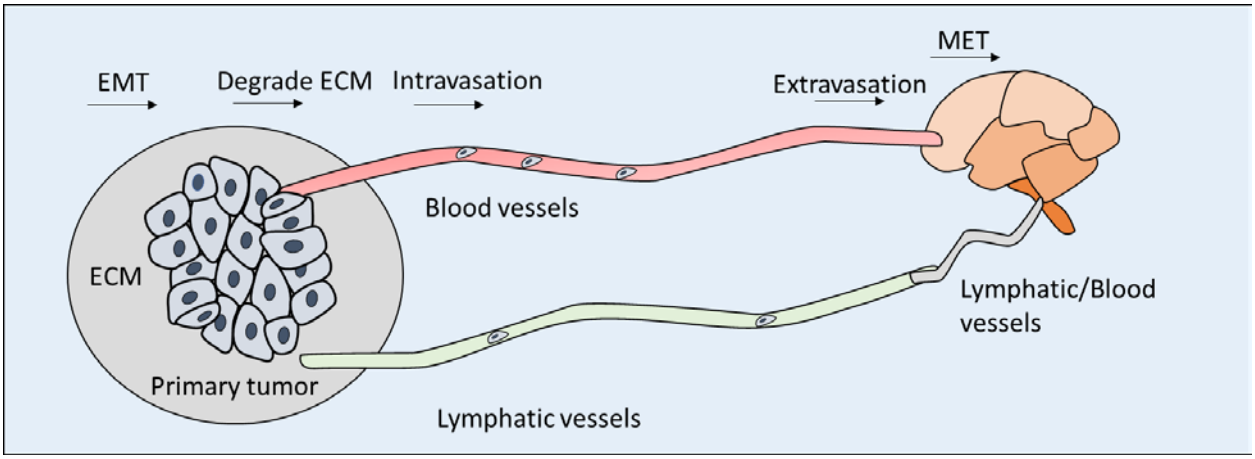

Figure 1. The main phases of cancer metastasis from primary cancer sites into the brain. Metastatic cancer cells pass through several phases in order to colonize the brain. First they acquire a stem cell like phenotype and shed their epithelial phenotype in a process called epithelial-mesenchymal transition (EMT). Once the EMT phases started, cancer cells begin degrading the ECM and reshaping it to form a hole approximately the size of one cancer cell. After that they have to choose which intravasation passage they take. There is a need of better understanding of the factors affecting cancer cells migrtaion route. Nevertheless, after finishing the intravasation phase, the cancer cells initiate the extravasation phase, where they infiltrate the blood-brain barrier (BBB) and finally they reverse their status from stem cell like cells back to epithelial cells as they start to colonize the brain. The interaction between cancer cells and their environment constitute a large mine of therapeutic opportunities hoping to halt cancer cells invasion of the brain.

2.1 Epithelial-Mesenchymal Transition (EMT)

Cancer cells preparing to invade the brain undergo epithelial-mesenchymal transition (EMT) [9][10]. This process constitutes shedding its epithelial phenotype to become less differentiated, more aggressive, and more stem-cell-like [11]. To perform this crucial transition, cancer cells utilize a large group of genes and transcription factor networks. These transcription factors include; Slug, Twist, Snail, Zeb1, and Zeb2. These transcription factors are activated in a response to signaling cascades regulated by TGFβ, BMP, EGF, FGF, PDGF, Wnt, Shh, NOTCH, and integrins [12]. Overall, these genetic networks collaborate to acquire various mesenchymal properties, such as increased cytoskeletal reorganization and decreased cell adhesion among other factors. Notably during EMT, cancer cells use several strategies to protect themselves against destruction by immune cells.

2.1.1 Cytoskeletal reorganization

Cytoskeletal reorganization takes place through the upregulation of the expression of mesenchymal cell markers (e.g., type III intermediate filament-vimentin). During EMT, vimentin networks not only play a role as a passive scaffold for organelles in the cytoplasm but also display an active commitment to the EMT-related signaling pathways. It has been shown that vimentin supports the extracellular signal-regulated kinase (ERK) kinase-mediated phosphorylation of the

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Slug transcription factor, which actively participates in EMT [13]. Vimentin also plays a role in the assembly of filaments in the polypoidal giant cancer cells (PGCCs). Similarly, vimentin supports oriented migration [14]. MALAT1(Metastasis-Related Lung Adenocarcinoma Transcript 1) is a regulatory long non-coding RNA. MALAT1 has been shown to play various roles including regulating over two hundred genes. One of its main functions is supporting cancer cells migrtaion through targeting Rho/ROCK signaling pathway[15][16][17] [18]. MALAT1 can also induce transcription factors, such as ZEB1, leading to TGF-β-mediated EMT in human keratinocytes [19]. In patients with non-small cell lung cancer (NSCLC), brain metastases and poor prognosis are associated with the presence of MALAT1 in primary tissues. MALAT1 was demonstrated to change the differentiated cobblestone-like shape of epithelial cells into a spindle-like mesenchymal phenotype with increased motility, thus promoting EMT and brain metastasis [20]. Furthermore, MALAT1 silencing in highly invasive human lung cancer H1915 cells prevented brain metastasis formation in athymic BALB/c mice compared to control animals. Interestingly, in 2018 a report appeared highlighting an anti-metastatic effect of MALAT1 in breast cancer [21].

2.1.2 Regulation of cell adhesion molecules

Cells transitioning into a mesenchymal phenotype utilize multiple genes in order to achieve their target. One of the main genes used by cancer cells in both cytoskeletal organization and cell adhesion is CADM2 (Cell adhesion molecule 2) as it play a fundamental role in aggregation and forming of cell clusters. This adhesion molecule modulates axonal myelination in oligodendrocytes and participates in the arrangement of synapses [22]. In NSCLC patients with brain metastases, CADM2 was up-regulated over 2-fold, as compared to non-metastatic NSCLC patients. Silencing of CADM2 in several NSCLC cell lines (e.g., A549 and H322) resulted in lower vimentin levels accompanied by a decrease in cell migratory ability and an increase in the expression of epithelial marker E-cadherin. Hence, CADM2 both induces EMT and supports migration of lung cancer cells to the CNS. Cells transitioning into a mesenchymal phenotype also upregulate N-cadherins expression and down-regulate E-cadherins and the epithelial cellular adhesion molecule-EpCAM [23]. One of the regulators of E-cadherin is SNORA17B. SNORA17B belongs to snoRNAs, non-coding RNAs and is involved in ribosomal RNA methylation. SNORA17B is highly expressed in the brain metastasis of breast cancer patients and correlated with poorer prognosis [24]. SNORA17B-transfected human breast cancer cell lines showed higher invasion ability and reduced E-cadherin. Thus suggesting that SNORA17B could be directly/ indirectly controlling E-cadherin levels. However the exact mechanism is not yet known. Another gene that also controls cell adhesion is HER2. HER2 is a transmembrane protein containing a tyrosine kinase domain. HER2 promotes phosphorylation reactions leading to the initiation of several signaling pathways mediated by STAT3, RAS-MAPK, that results in the inhibition of pro-apoptotic proteins and an increase in the expression of cell proliferation-related genes, supporting metastasis to the brain [25]. Breast cancer expressing HER2 is well-known to be highly invasive [26]. Recently, HER2-overexpressing human breast cancer cells have been evidenced to induce greater expression of SNAIL, SLUG, and ZEB-1 transcription factors, greater TGF-β production, and increased level of N-cadherins, followed by a decrease in expression of E-cadherin and cytokeratin-18, HER2 could constitutes one of the main drug targets in designing therapies to inhibit brain metastasis as it was shown that HER2 inhibition reduced metastases to the brain, lungs, and livers in nude mice by 86%, 50%, and 64%, respectively. Additionally overexpression of AEG-1 (Astrocyte

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Elevated Gene-1) upregulated N-cadherin and Slug but decreased epithelial markers E-cadherin and ZO-1 (Zonula occludens-1) expression. AEG-1 is an endoplasmic reticulum (ER)-associated cytoplasmic RNA binding protein, interacting with numerous mRNAs encoding secretory, cytosolic, and organelle proteins [27]. AEG-1 has been identified for the first time in primary astrocytes of the human fetus, particularly in endoplasmic reticulum, but also near the cell nucleus. In time, this oncogene was found in the cell membrane of breast cancer cells. Functions that were assigned to AEG-1 in cancer include: malignant transformation, resistance to chemotherapy (being a co-inducer of chemoresistance-associated genes) and anoikis, angiogenesis and metastatic spread [28][29]. Notably, cancer cells undergoing EMT also down-regulate several epithelial markers such as β-catenin. β-catenin is involved in creating cell junctions [30]. Metastatic cancer cells down-regulate several genes involved in creating desmosomess, such as desmoplakins and resisting mechanical stress[31] such as cytokeratins [32].

2.1.3 EMT cells interaction with immune cells

EMT Cancer cells have to protect themselves from the effect of immune cells. It has been shown that cancer cells deplete tryptophan in their microenvironment by producing IDO (indoleamine 2,3-dioxygenase) which metabolize tryptophan to kynurenine. Tryptophan deficiency causes T-cells to undergo a stress response, which prevents them from proliferating. Kynurenine induces the development of Tregs and enhances the production of anti-inflammatory cytokines such as IL-10 and TGF-B[33][2].

2.2. Cancer cells infiltration of the ECM

2.2.1 The ECM infiltration process

One of the first obstacles that migrating cancer cells face is the ECM. For cancer cells to overcome this powerful barrier, they use follow several phases. First, they form invadopodia which is transformed to a pseudopod protrusion afterwards This is followed by the construction of focal contacts, focalized proteolysis, actomyosin contraction, and finally, the detachment of the trailing edge [34] [35].

Tumor cells start their journey as invadopodia. One of the main characteristics of invadopodia is the high enrichment of fast-polymerizing filamentous actin (F-actin) bundles [36]. Src family kinases and their substrates play a pivotal role in the formation of the disassembly of the invadopodia and pseudopodia. For example, Tks5 acts as a protein scaffold. Cortactin is known a monomeric protein which is found in the cytoplasm of cells. Once activated it was shown to enhance actin cytoskeleton polymerization and reorganization promotes the formation of actin networks. AFAP110 (Actin Filament Associated Protein 1) regulates actin-binding and cross-linking proteins. In addition, recent reports have indicated that the Src-dependent regulatory mechanisms are mediated by several other pathways such as reactive oxygen species, and microRNA [37] [38]. **Focal adhesions (FAs)** are sites of integrin clustering that link the actin cytoskeleton to the ECM. The primary function of FAs is to provide physical attachment to the ECM and transduce force between the cell and the ECM. The FA component focal adhesion kinase (FAK) regulates FA signaling by providing a scaffolding function for the protein-protein interactions and regulating the cross-talk between integrins and growth factor signaling. Proteolysis of the ECM via recruitment of surface proteases is the primary goal of this phase. Proteases such as MT1-MMP, MMP2, MMP9, ADAM12, ADAM15, ADAM19, cathepsins, and seprase can accumulate

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in Invadopodia. These proteases, once secreted, contribute to localized pericellular proteolysis. cancerous cells upregulate genes that are responsible for the expression of metalloproteinases. The main aim of this step is to degrade the extracellular matrix. Interestingly, cancer cells can also exploit normal fibroblasts to produce significant amounts of MMP2 [39][40]. Secondary remodeling via ECM component secretion results in realigning ECM fibers to remain stable in a new form that contains a hollow tube the size of the cell's diameter [35][41]. **Actomyosin contractility** promotes cancer cell colonization and outgrowth. Contractility is under the control of a wide variety of pathways, including SRF/MRTF, TGFβ-SMAD-CITED1, MMP-9, BRAF-V600E, and CDC42 signaling. Similarly, it was speculated (v) **that detachment of the trailing** edge is calcium-dependent.

2.2.2 Cancer cells interaction with immune cells during ECM infiltration.

Notably it was shown that various extracellular matrix (ECM) components play a role in protecting migrating cancer cells through preventing antigen presentation to T–cells [42]. Fibroblasts produce matrix metalloproteases (MMPs) to degrade the ECM [43]. Mesenchymal stromal cells produce TGFβ to weaken the immune response. Necrosis caused by ECM disruption by cancer cells induces MDSCs to downregulate the antitumor immune response by degrading various amino acids in the tumor microenvironment. This starves the T cells and inhibits the signaling pathways required for their activation. Another pathway that MDSCs utilizes to inhibit T cells proliferation and activation is through the production of NO which inhibit IL-2 signaling. MDSC produces reactive oxygen species (ROS), which increase T-cell apoptosis. ROS can combine with NO to generate peroxynitrite, that disrupt the TCR-MHC interaction, resulting in increasing cancer cells resistance to cytotoxic T-cell responses [44].

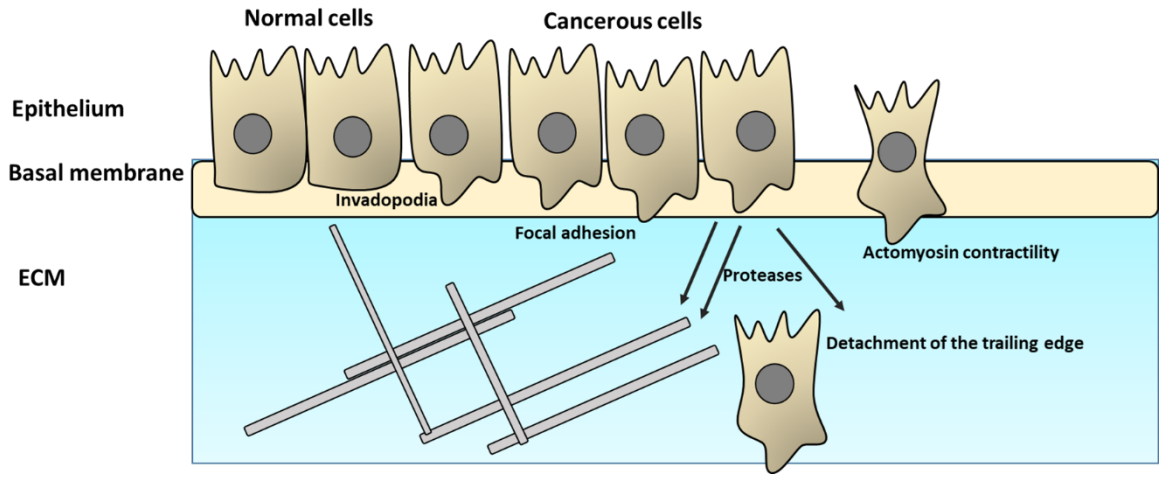

Figure 2. Cancer cells infiltration of the ECM. This process constitutes several phases such as (1) pseudopod protrusion formation, (2) construction of focal contacts, (3) focalized proteolysis, (4) actomyosin contraction, and finally, the (5) detachment of the trailing edge.

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2.3 Intravasation

2.3.1 factors controlling intravasation pathway

During their migration to the brain, cancer cells can either use blood circulation pathway or the lymphatic vessels pathway [45][46]. The factors that govern the decision of the cancer cells to migrate through the blood vessels or the lymphatic vessels are still being investigated. **They include (i) differences in function between blood vessels and lymphatic vessels.** The lymphatic system's main functions include transferring various macromolecules, fluids, and immune cells to the blood, and maintaining plasma volume. The composition of the lymph fluid is almost identical to that of the interstitial tissue fluids, which promotes the survival of migrating tumor cells. **(ii) Blood and lymphatic vessels differ considerably in their structure**[46]**.** Whereas, blood circulation constitutes a closed system lymphatic system that flows in one direction from the peripheral tissues to the blood. To achieve its function, the lymphatic system uses lymphatic capillaries and pre-collectors followed by lymphatic vessels and trunks that flow into the bloodstream. Lymphatic vessels are threefold the size of blood vessels with an incomplete discontinuous basal lamina and lack pericytes and smooth muscles. This difference in structure means that the migrating cells could face lower mechanical resistance in traveling through lymphatic vessels compared to blood vessels. Thus lower energy would be needed by the cancerous cells to travel through lymphatic vessels. Lymphatic vessels are leakier thus supporting cancer cells to spread more. However, blood vessel remodeling is correlated with cancer spreading, where novel fragile blood vessels lacking basal membrane are being created to support cancer cell growth. **(iii) role of EMT** Surprisingly, malignancies isolated from sentinel lymph nodes show a strong invasive potential but lack mesenchymal markers. This finding supports the notion that, while EMT is essential for metastasis progression through blood vessels, cancer cells spreading through lymph node does not require MT. This hypothesis may be explained by the fact that the structure of the lymphatic system does not force mesenchymal cells to become more invasive. Furthermore, EMT increases intravasation, whereas cells exiting the arteries do not need it. As a result, unlike blood vessel spread, successful lymphatic migration may not involve EMT. **(iv) the role of ECM** Cancer cells migrating through the blood and lymphatic vessels may use the ECM to their advantage. Endostatin, tumstatin, canstatin, arresten, hexastatin, and type IV and type XVIII ECM collagens all have a significant influence on the angiogenesis of both blood and lymphatic vessels [46][47]. Integrin 91, an ECM receptor, has been linked to the development of lymphangiogenesis [48]. Similarly, low molecular weight hyaluronan promotes lymphangiogenesis by interacting with its Lymphatic vessel endothelial hyaluronan receptor 1 (LYVE-1), promoting lymphatic endothelial cell proliferation and tube formation. Lymphangiogenesis and tumor invasion are tightly connected to MT1-MMP-mediated proMMP-2 activation and the production of ECM1 and EMILIN1, an elastic microfibril-associated protein[49][50]. (v) expression of various genes and receptors specific to blood or lymphatic vessels, as well as cross-talk between lymphatic and blood vessels could also contribute to the decision of cancer cells in choosing its route.

2.3.2 Interaction of cancer cells with its surrounding during intravasation

After infiltrating the lymphatic or blood vessel wall, tumor cells are known as circulating tumor cell (CTC). Notably, a small portion of the CTCs survives in the bloodstream and forms metastasis. There are several stress factors CTC face in the blood including: hemodynamical forces, anoikis (e.g., programmed cell death), encountering immune cells, as

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well as narrow microcapillares posing a risk for deformation or being entrapped. Hemodynamical forces may trigger mechanical damage of the cell. Two biomechanical forces act on the vascular wall: tensile stress resulting from blood pressure and shear stress tangent to endothelium. Additionally, turbulent blood flow at arterial bifurcations, collisions with vascular wall and other morphotic elements of blood, all are noxious factors that may results in CTC deformation, apoptosis or death [51]. To avoid anoikis, CTCs form clusters that display higher metastatic potential than single cells [52][53]. The third obstacle CTC must overcome are immune cells, particularly natural killers which are able to lyse cell intruder. CTCs also expresses tissue factor on their surface. TF activates coagulation process and formation of platelets-rich envelope around CTC. Within this coating CTC forms connection with thrombocytes via integrins and it is considered such barrier protects CTC from shear stress or NK cells. Worth of notion is the fact that coagulation and thrombocyte activation are known mechanism that accelerate metastases formation [54][55]. By producing TGF- and downregulating NKG2D on NK cells, platelets also inhibit natural killer cells. Platelets can provide normal MHC class I molecules to tumor cell surfaces, shielding them from cytotoxic T-cells. One explanation for how macrophages boost CTC survival is the production of 4-integrin, which interacts with VCAM-1 on the surface of CTCs and provides a survival signal.

2.4 Extravasation through the blood brain barrier

The next stage of cancer cells' invasion of the brain is infiltration of the blood-brain barrier (BBB). The BBB is a complex barrier formed of endothelial cells, astrocytes, basal membranes, and pericytes. During their attempt to cross the BBB, cancer cells aim at exploiting the surrounding microenvironment. Cancer cells attack leukocytes and regulate their production of various cytokines to facilitate their infiltration of the brain [56]. Chemokines play a central role in directing cancer cells toward the endothelial cells surface and guiding the process of infiltration of the brain. To be able to home to the endothelial cells of the brain, cancer cells were shown to express CXCR4 which can increase the chemotaxis attraction of cancer cells to the BBB. Notably, cancer cells seem to enter a state of arrest with no recorded proliferation during the time preceding the infiltration[57][58]. The morphology of the cancer cells changes based on their location within the extravasation process. At first, while in the arrest state, they take an elongated shape. After that, before the start of the transmigration process, they acquire a round morphology, where they exert pressure on the vessel walls.

The infiltration process includes three main stages, namely rolling, adhesion, and transmigration. This process seems similar to that of leukocyte infiltration of the brain, albeit less well described. Interestingly, it was found that the time needed for extravasation is cancer-type dependent. For example, lung cancer cells were reported to take around 2 days to infiltrate the brain, while breast cancer cells were shown to need between 2–7 days. During the transmigration process, cancer cells morphology exhibits a flexible structure with parts of the cell outside and other parts within the vascular wall. Additionally, they show changes in their cellular extensions, showing a dynamic interaction between invasive cancer cells and endothelial cells [59][60].

2.4.1 The rolling process

The rolling process seems to depend on selectin. Cancer cells express selectin ligands, which mediate the tethering and rolling of cancer cells on the surface of the endothelial cells. They also could be used to adhere to leukocytes which in turn support the

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attachment of cancer cells to the vessel walls. Heparin inhibitions of selectin interactions slow the process of extravasation of melanoma, confirming a central contribution by selectin to the success of the rolling process [61][62].

2.4.2 The adhesion process

Once the rolling stage has ended, cancer cells begin binding to the endothelial cells. Endothelial cells express ICAM1 and VCAM1 which are important in the attachment between lung cancer cells and the endothelial cells [63]. Similarly, cadherins including E and N subtypes have been shown to contribute to lung cells' interaction with endothelial cells of the BBB. Other genes that seem to be playing a role in the interaction between cancer cells and endothelial cells include endothelial cells receptors. Inhibition of endothelial cells receptors resulted in inhibition of the interaction between invading cancer cells and the endothelial cells and reduction of brain metastasis. Integrins are cell receptors that interact with ECM components such as collagen, laminin, and fibronectin. Integrins can also mediate the production of cells' survival signals. One of the main integrins that were demonstrated to be expressed by lung metastatic cancer cells in the brain is A3b1. A3b1 inhibition resulted in decreasing brain metastases in nude mice. However, the complete pathways controlling the adhesion process are yet to be determined.

2.4.3 The transmigration route

Interestingly, it was shown that metastatic cancer cells have two main choices of transmigration namely (i) paracellular transmigration and (ii) transcellular transmigration in way similar to leukocytes transmigration mechanisms [64] [65]. For example, melanoma cells use a paracellular route while breast cancer cells follow a transcellular route. MMPs are particularly important for paracellular transmigration because of their role in degrading junctions between cells. Another critical alteration by the migrating cancer cells is the rho/ rho kinase signaling, which was shown to support paracellular infiltration of the brain. In the case of the paracellular route, downregulation of genes responsible for stabilizing BBB integrity such as β-catenin and zonula occludens (ZO)-1 as well as CX43 has been reported. Furthermore, rearrangements of the cytoskeleton also have been reported through increasing myosin light chain kinase (MLCK) and phosphorylated myosin light chain (p-MLC) resulting in endothelial cells contraction. On the other hand in the case of transcellular migration, CAV1 levels are upregulated. One gene that could be playing a role in the specificity of brain metastasis is PI3K, where it was shown that its inhibition leads to inhibiting breast cancer metastasis but not lung cancer cells. If it plays a role in controlling the transmigration route is still to be found. Additionally, the complete map of genes determining the transmigration route is yet to be drawn. Interestingly, after finishing the extravasation process, breast cancer cells favor localizing in proximity to the endothelial cells. Conversely, lung cancer cells seem to favor the formation of novel blood vessels and migrating further into the brain. The reason behind their choices remains speculative.

BBB permeability

Various reports suggest that the BBB is often compromised during brain metastasis. Tumor cells aim to increase leakage of the BBB. One of the mechanisms they use is to alter lipid metabolism by inhibiting the expression of the endothelial cell fatty acid transporter Mfsd2a. Conversely, some strategies for disrupting the BBB to enhance drug delivery to the brain have been reported [66]. The treatment of CNS tumors by radiation often results in increased BBB permeability [67]. Cytostatics used in breast cancer therapy, such as taxane docetaxel, can disrupt the BBB. Taxane docetaxel usage was correlated with an increase in brain metastasis [68]. Thus, the strategy of increasing brain permeability could need to be tuned based on personal conditions, the stage, and the type of cancer.

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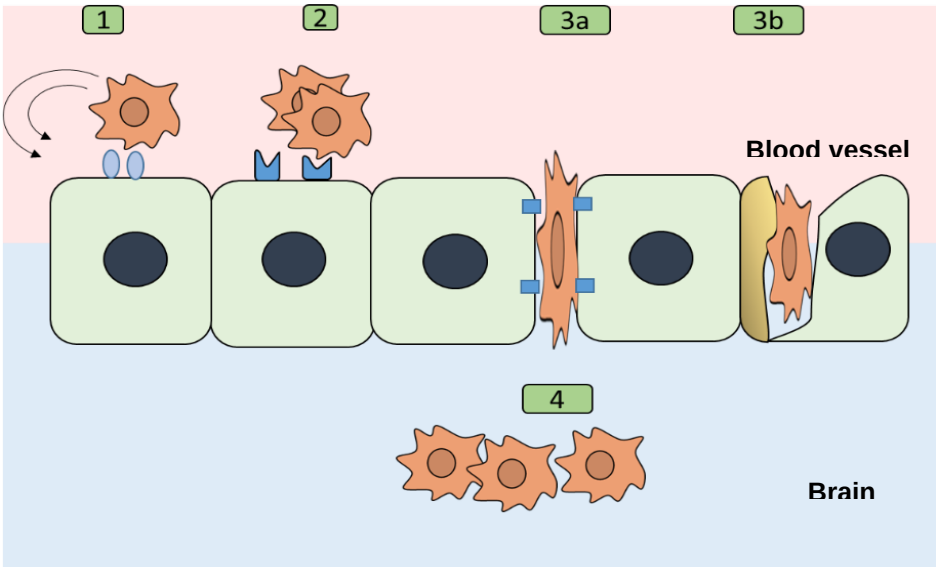

Figure 3. Cancer cells infiltrating the blood brain barrier. The infiltration process comprises three primary phases: (1) rolling, (2) adhesion, and (3) transmigration (colonization). Transmigration can take a parcellaur route (between the cells), or (3b) transcellular route (through the cell). (4) After infiltration of the BBB, cancer cells choose either to colonize the areas proximal to endothelial cells or to migrate further into the brain.

2.5 Mesenchymal to epithelial transition (MET)

Mesenchymal to epithelial transition is a reprogramming process that aims to reverse the process of EMT. MET could be considered an early stage of the adaptation of CTC to its novel conditions. The MET transition is characterized by the re-expression of the genes that were silenced during EMT, followed by changes in the cancer cell morphology to a more polarized shape. One of the hallmarks of EMT is the restoration of E-cadherin expression at cell-cell junctions. To achieve that, intracellular domains of E-cadherin are bound to the cytoskeleton through the linking "bridges " such as β-catenin. This structure modulates the interactions between cells and ensures epithelial phenotype maintenance. Interestingly, investigators exploring metastatic tissues uncovered that the tumor cells could exist in an intermediate state between EMT and MET. This transient phase of cellular reprogramming involves both apparent co-expression of the epithelial marker such as E-cadherin with the mesenchymal markers such as vimentin. These observation seems to take place regardless of the primary cell type [10][69]. Molecular reprogramming requires a switch in expression of many other genes followed by intense metabolic processes as protein synthesis, intracellular transport and secretion. Endoplasmic reticulum (ER) protein 29 (ERp29), is highly expressed throughout mammalian tissues. It predominantly acts as an ER to Golgi escort/chaperone protein[70] [71] [72] [73]. Cancer research revealed additional properties of the ERp29, including cell growth arrest and involvement in MET. Moreover, ERp29-transfected human breast cancer MDA-MB-231 cells changed the morphology into more epithelial, and stimulated them to grow in clusters, while control cells

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retained their predilection for scattered growth in vitro. When injected in nude mice, breast cancer cells overexpressing Erp29 demonstrated delayed tumor formation, as compared to control.

Besides the endogenous, intracellular mechanisms controlling MET, some of the external factors may contribute to the transition towards epithelial phenotype. Few years ago it was discovered, that in a response to human cytomegalovirus infection, breast carcinoma and glioma stem cells inhibited EMT and induced MET [74]. The question, if HCMV supports colonization of metastatic cells or rather renders them less malignant is still open. It is however possible, that CMV realizes the first scenario due to earlier reported evidence indicating the virus entanglement in enhancing the metastatic spread [75][76]. Induction of the MET state seems to have some therapeutic implications in oncology. Targeted and coerced transformation of malignant cells into less proliferative and migratory ones would be an attractive approach. First attempts to reverse EMT have been already made on human mesenchymal mammary epithelial cells. Exposure them on cholera toxin or forskolin induced increase in the intracellular levels of adenosine 3′,5′-monophosphate (AMP), followed by an activation of protein kinase A (PKA). As a result, mesenchymal-to-epithelial transition was observed in treated culture [77]. Reaching primary tumors or metastasis in the brain with drugs that would be able to change phenotype selectively appears to be challenging, but worth of further exploration.

2.6 Angiogenesis

Angiogenesis refers to the sprouting of new vessels from preexisting ones. Both primary and secondary tumors need constant delivery of nutrients and oxygen for growth and expansion. However, at the initial stages of their development, vasculature construction is less frequently observed. The vascular network density usually correlates with tumor grade. The higher number of formed microvessels in the cancerous lesion, the more advanced the disease. Migration of endothelial cells is essential for new vessel creation, as well as their communication with ECM. These interactions are provided by ECM fibronectins and endothelial integrins, among others [78]. The importance of integrins in the angiogenesis of brain metastasis was demonstrated in SCID mice, inoculated with human MDA-MB-435 breast cancer cells with activated integrin αvβ3. As a consequence, instead of tumor growth in the primary site (mammary pad), metastatic spread to the brain has been observed. The mechanism involved activated integrin αvβ3-induced translation of VEGF mRNA [79]. To prepare the space for the developing tumor vasculature, ECM decomposition is initiated, through tumor cells releasing proteases activating plasmin (tissue-type plasminogen activator and urokinase-type plasminogen activator), and subsequently MMPs [80][81]. Several other molecules involved in angiogenesis have been described including vascular endothelial growth factor (VEGF), fibroblast growth factors (FGFs), angiopoietin-1 (Ang1), and angiopoietin-2 (Ang2) acting through the Tie2 receptors, interleukin-8, matrix metalloproteinase-2, and many more [82]. It has been shown that VEGF is highly pro-angiogenic specially under limited availability of oxygen. The structure of tumor vessels is fragile, with increased permeability and a tendency to collapse, which results in incidences of local hypoxia, followed by necrotic core development. Oxygen deficiency is a strong accelerator of angiogenesis and cancer progression. One of the mechanisms induced by lack of oxygen is the increase in the cellular level of hypoxia inducible-factor 1 (HIF-1). HIF1 activates the expression of various genes related to angiogenesis as such as angiopoietin 1 and 2, matrix metalloproteinase 2 and 9 (MMP-2 and -9), platelet-derived growth factor B (PDGFB) and vascular endothelial growth factor A (VEGFA). Regular tumor vessel collapse and repeated hypoxia may lead to the continued release of VEGF by cancer cells and angiogenesis. The described process, seen in gliomas, is an example of how the tumor supports its growth in an autocrine manner. Together with the increase of VEGF in progressed

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tumors, a rise in regulatory molecules takes place. This modify the expression of potent pro-angiogenic factors. Among them are microRNAs that may function as oncogene or gene suppressors. In relation to secondary brain tumors, for instance, miR-378 has been evidenced to be upregulated in thoracic specimens of the patients with NSCLC and brain metastases, as compared to NSCLC cases without metastatic spread in the CNS. Further in vitro and in vivo experiments confirmed that miR-378 binds to VEGF 3′ UTR and promotes VEGF expression 45. Considering HIF-1α, examination of clinical specimens from patients with lung, breast, kidney, colorectal, and melanoma cancers, expression level and microvascular density were documented to be the highest in brain metastasis of renal cell carcinomas, but the lowest in the metastatic lesion in the CNS of the melanoma patients. Simultaneously, the same markers were overexpressed in brain metastasis than in matched primary tumors. These observations are in line with another investigation, evidenced that brain metastasis of melanomas demonstrated lower tumor microvasculature counts than of breast and lung carcinomas. The extent of vasculature formation in metastases depends on various factors, such as primary cancer type, disease stage, and the specific growth rate of given tumors. In the course of cancer invasion/metastasis, both matrix metalloproteinases (MMPs) and tissue inhibitors of matrix metalloproteinases (TIMPs) balanced action is required to provide both vascular remodeling and prevention from structural abnormalities due to extensive outgrowth, respectively. Interestingly, implantation of the human lung cells or their TIMP-1–overexpressing counterparts into the brain of nude mice resulted in the development of larger tumors along with a 2-fold increase in the number of vessels in the latter group, as compared to the control [83]. These results are consistent with in vitro studies on human umbilical vascular endothelial cell culture growing in the presence of a higher concentration of TIMP-1, that enhanced tubule formation. It seems that the cited in vivo study is an example of the beneficial effect of inhibition of ECM degradation for tumor progression, probably due to reduced vascular malformations. Last but not least aspect of angiogenesis in brain metastases is the specific microenvironment rich in neuronal- (or glial-)-derived trophic factors, including nerve growth factor (NGF), neurotrophin-3, neurotrophin-4, and brain-derived neurotrophic factor (BDNF), binding to different Trk kinases. For example, BDNF was found to induce VEGF expression in neuroblastoma cells upon interaction with TrkB and downstream activation of HIF-1α [84].

3. Survival of cancer cells in the metastatic environment.

Migratory CTCs must adapt to the distinct microenvironment to survive and colonize, forming metastasis. Further spread of metastatic cells following colonization requires extracellular matrix remodeling. After BBB invasion, it is hypothesized that these cells proliferate along the microvasculature rather than into the brain parenchyma, a tendency described as "vascular cooption". This is necessary to establish microcolonies.48. In the brain, metastatic cancer cells face the opposition of the glia, microglia, and neurons counteracting the penetrating intruders. However, the effect of the response of the host may not always be favorable.

3.1 Neurons

Cancer cells are capable of exploiting neurons to achieve their goal of colonization. To ensure their glutamate supply, breast cancer cells replace astrocytes in the tripartite synapses, forming pseudo–tripartite synapses. This process ensures NMDAR activation, which in turn supports cell division. NMDA receptor antagonists, such as MK-801 (dizocilpine), have been shown to possess anti-proliferative and anti-invasive effects. Perplexingly, it was shown that NMDAR

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activation using glycineB site ligands also resulted in anti-proliferative effects. This might indicate that the NMDAR role could be cancer-specific. Furthermore, the glutamine glutamate cycle is more complicated than previously thought. We and others have found that one transporter could regulate the passage of glutamine and glutamate from cancer cells. Thus, using a single drug would not necessarily be sufficient for blocking glutamine uptake by cancer cells [85]. Another dimension of complexity arises from the ability of metastatic cells to alter their phenotype. This change includes the ability to produce GABA-associated proteins such as GABA transaminase (ABAT), GABA A receptor (GABA A R), glutamate decarboxylase (GAD67), GABA transporter, reelin, and parvalbumin. In that sense, cancer cells utilize GABA as a source of energy by metabolizing GABA into succinate. This was demonstrated by the observation that inhibiting GABA uptake in metastatic cancer cells decreased NADH levels, indicating a reduction in cellular proliferation [86].

3.2 Astrocytes

Astrocytes seem to play a dual role in cancer metastasis. Astrocytes were reported to support endothelial cells in resisting cancer cells infiltration. It has been reported that astrocytes secrete laminin-211 in the parenchymal basement membrane. Laminin-211 transmits pro-dormancy signals via dystroglycan and promotes quiescence by inhibiting YAP in the cytoplasm of cancer cells. YAP is a well-known transcription regulator which plays a central role in cell proliferation. This process prevents the activation of growth-promoting mechanisms, which appear to be necessary for brain metastases [87]. However, there are various pathways through which astrocytes support cancer metastatic colonization. For example, extravasation of cancer cells penetrating the brain is associated with the astrocytes activation and upregulation of the heparanase and **matrix metalloproteinase-9** that promote further invasion and colonization of metastatic cells by degrading the ECM. Astrocytes can produce transforming growth factor-beta 2 (**TGF-β2)**, which in turn regulates SMAD-mediated ANGPTL4 expression in cancer cells contributing to successful colonization. Astrocytes were demonstrated to support lung and breast cancer cells metastasis by producing IL1B, and TNFα [88]. Xing et al showed that brain metastases express high levels of IL1β under the influence of surrounding astrocytes, which leads to increased Notch signaling in cancer stem cells, promoting their stemness and growth in the metastatic niche. In addition, interleukin-1 beta (IL-1β) and tumor necrosis factor-alpha (TNFα) released by cancer cells increases TGF-β2 expression in astrocytes52. Evidence has also shown the formation of gap junctions between astrocytes and tumor cells that allows for the passage of cGAMP, which activates the STING pathway in astrocytes and promotes the expression of IFNα and TNFα to further facilitate tumor growth. Other factors released by astrocytes that supported cancer cell survival in the CNS include cytokines, heparanase, stromal cell-derived factor 1 (SDF-1), sphingosine-1 phosphate, and glial-derived neurotrophic factor. Astrocytes can protect tumor cells from chemotherapy by sequestration of calcium from, cancer cells and by regulating survival genes. The threshold of astrocytes switching sides is not yet known. However in the case of breast cancer metastasis, it was shown that along with the growth of metastatic lesions astrocytes were gradually expelled to the border of the tumor[89]. We can speculate that the process is time specific and is cunningly regulated by cancer cells not only to neutralize astrocytes' effect but also to exploit their abilities for the metastatic cells' gain. At the early stages of brain invasion, cancer cells could be releasing migratory signals that encourage astrocytes to free the space needed for tumor growth. After that, the production of proinflammatory cytokines such as IL1B could be over-activating astrocytes. After the cancer cells trick the astrocytes into over activation, astrocytes enter a vicious cycle of continuous over activation by IL1B and TGFβ2.

Several opportunities might be available to target this relationship such as targeting migratory elements produced by the cancer cells in the early stages of brain metastasis. It could also be viable that the reduction of inflammatory signals could prevent astrocytes from being overactivated. However, experimental validation is still needed to support this hypothesis.

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3.3 Pericytes

Pericytes seem to support cancer metastasis in the brain. They produce chemokines that can attract breast cancer cells. Pericytes also secret high volumes of extracellular matrix proteins, supporting the adhesion of melanoma and triple-negative cancer cells. Recently, it was discovered that pericytes produce insulin-like growth factor 2 (IGF2). IGHF2 supports the proliferation efforts of mammary carcinoma but not melanoma cells. Inhibiting IGF2 signaling using silencing or picropodophyllin (PPP) decreases the size of brain tumors in mice inoculated with triple-negative breast cancer cells. These results indicate that brain pericytes have significant pro-metastatic features, especially in breast cancer [90]. However, several questions remain unanswered. Why are cancer cells capable of exploiting pericytes to their advantage without any resistance from the pericytes' side? It has been reported that tumor cells through the production of PDGF-BB lead to pericytes removal from the vessel and vessel sprouting. Tumor cells also can alter pericytes differentiation through the production of VEGF. Also, cancer cells produce several growth factors such as PDGF-B, VEGFA, and TGFβ1, which may trigger the prevalence of a PDGFR+/desmin+ pericyte phenotype and not the PDGFR+/CD13+ phenotype. Interestingly, PDGFR+/desmin+ pericytes, but not PDGFR+/CD13+, have been associated with metastasis and proliferation of cancer cells. However, these findings highlight the importance of further investigations that aim to identify various pericytes subpopulations and compare their contribution to cancer metastasis. Pericytes subpopulations have been proposed to be highly plastic, which means they can change their phenotype to other types of cellular lineage, such as microglial or vascular lineages. Thus, further investigations are needed to unravel the capabilities of pericytes subpopulation plasticity and how they influence metastasis [91].

3.4 Interaction with stromal cells

Upon colonisation, disseminating metastatic cells interact with the exploit surrounding to support their growth possibly through autocrine and paracrine loops [92]. For example, fibroblasts can increase metastases through the production of tissue growth factors that in turn can enhance tumor growth. Fobroblats can also produce MMPs that can degrade the ECM [43]. Additionally, mesenchymal stromal cells can produce TGF–β and soluble decoy T–cell ligands that inhibit T cells interaction with tumor cells [93].

3.5 Immune cells interaction with the cancer cells

3.5.1 Microglia

The microglial contribution to cancer metastasis is still obscure. There is evidence that supports a pro-metastatic role for the microglia. However, there are various observations that draw a more complex picture. The tumor microenvironment is dominated by microglia and infiltrating macrophages. Microglia remain quiescent during normal homeostatic conditions but become activated in response to malignant cell infiltration. Factors supporting the hypothesis that microglia support tumor growth include: **(i) Activation of microglia in the areas surrounding metastatic sites has been widely documented**[94][95]. These activated microglia are characterized by increased anti-inflammatory cytokine production, decreased phagocytic activity, growth factor

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release, a chemo-attractive effect on peripheral monocytes, and inhibition of T-cell proliferation within the tumor microenvironment. However, they exhibit upregulation of proteins that support tumor cell adhesions such as; cellular adhesion molecules, CAMs, LFA-1, ALCAM, and E-selectin. It has been demonstrated that metastatic lung cancer cells produce IL6 that in turn could be inducing anti-inflammatory action of microglia by switching JAK2/STAT3 signaling. Furthermore, macrophages could supply matrix degrading enzymes, where certain cancer class produce CSF-1 that in turn activate macrophages. While macrophages produce EGF that promote tumor mass proliferation. **(ii) In glioblastoma, the most aggressive form of solid cancer in the CNS, microglia contribute significantly to the total tumor mass, indicating it plays a primary role in tumor progression within the CNS**. However, recent findings are revealing that the microglia's contribution to metastatic growth is affected by various other factors, including (i) Reports have shown that microglia utilizes NO to combat and lyse tumor cells, and that inhibiting microglial activation using neurotrophin NT-3, is associated with an increase in brain metastastsis formation. **(ii) the observation that microglia represent a heterogeneous combination of different groups of subpopulations that differ in their inflammatory profiles considerably**. These subpopulations contribute differently to the tumor microenvironment. Those immune cells with a pro-inflammatory phenotype have been described as exerting an anti-tumorigenic effect, while those with an anti-inflammatory profile have shown tumor-supporting activity. Furthermore, the exact role of certain microglial subpopulations is still not clear. For example, disease-associated microglia (DAM) deep transcriptomic analysis reveals an upregulated profile of several microglial genes such as Lgals3, Trem2, NOS2, and COX1. However, how these different pathways interact with the invading cells is yet to be found. **(ii) The same subpopulation of microglia can transit between pro- and anti-inflammatory states**. The procedures underlying the phenotypic switch between these two states and the potential coexistence of both identities during the brain are poorly understood. **(iii) The phenotypes of microglia and brain-infiltrating BMDMs (e.g., bone marrow-derived macrophages) are comparable**. Microglia develop from embryonic yolk sac progenitor cells and remain in the CNS, but bone marrow-derived macrophages (BMDMs) penetrate the brain during metastasis. Up to date, no unique markers that can distinguish between the activities of microglia and BMDM have been discovered.

3.5.2. Dendritic cells

Dendritic cells contribution to brain metastasis could be classified based on their subtype. For example, plasmacytoid dendritic cells have been shown to promote mutations. It was shown that pDC produce IDO and ICSOL which supports Tregs recruitment. On the other hand dendritic that have risen from the myeloid linage are known to be pro-inflammatory and thus supporting antitumor immunity. Interestingly, it was shown that tumor cells within the brain are capable of inhibiting the maturation of DC. This prevent them from proper antigen presentation and enhance their immune suppression activity by inducing the production of Il10 and TGFβ and hence Treg recruitment.

3.5.3 TILs (Tumor infiltrating lymphocytes)

Further investigation of TILs' interactions with metastatic tumors is still needed. During the process of tumor metastasis to the brain, the BBB becomes compromised, increasing its permeability. This in turn increases the number of immune cells accessing the brain and trying to infiltrate the metastatic brain tumor. Conversely, we found that number of CD4+

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T cells infiltrating the brain increase during peripheral immune conditions, with no significant disintegration of the BBB[96]. Furthermore, CD4+ T cells infiltration of the brain could also be through areas lacking a BBB such as the median eminence of the hypothalamus[96]. Once in the brain, TILs infiltration of the metastatic tumors has been suggested to follow one of three main mechanisms, namely, matrix infiltration, peritumoral infiltration, or diffuse infiltration. However, it is still not known if these mechanisms are cancer type-specific. Moreover, the molecular pathways controlling these mechanisms are still not well understood. Furthermore, it has been reported that TILs in brain metastases are positively correlated with the prognosis [97]. Studies investigating metastatic brain tumors found that the numbers of CD3+ T cells, CD8+ T cells, and CD45+ T cells are positively correlated with the prognosis. However, this hypothesis overlooks the complexity within each T cell population. For example, CD4+ T cells are a heterogeneous group of cells composed of pro-inflammatory cells such as Th1 and anti-inflammatory cells such as Tregs, as well as cells that could switch between different inflammatory conditions such as Th17[98][99][100]. CD4+ T cells are also highly plastic, thus they can switch from one state to the other based on the presence of specific cytokines [101][102]. Furthermore, Tregs are composed of different subtypes that differ in their function and their immune suppressive effect [96][103]. Our understanding of how these TILs function is based on their interaction with the primary tumor. However, there is a significant difference between the immune profiles of TILs infiltrating the primary cancer locations and the parenchyma of brain metastases. For example, it has been shown that TILs localized in brain metastases caused by lung cancer show stronger immunosuppressive abilities compared to the TILs isolated from the primary tumor. Furthermore, TCR repertoire comparison between T cells from metastasis regions and those of the primary regions showed difference in abundance and diversity. Moreover the interaction between B cells and the migration metastatic cancer cells has not been yet investigated especially on the level of B cells subpopulations such as Bregs [104].

4. Future prospectors and conclusion

The journey of cancers cells from their primary location to the brain is still not filly mapped. We know that cancer cells follow a typical route starting from EMT passing through infiltration of the ECM, blood vessels and the BBB and ending up with angiogenesis and colonization. However our review raises several unanswered questions such as the factors controlling the choice of the intravasation route (e.g., blood vessels versus lymphatic). Notably the choices behind the cancer cells final destination within the brain regions are also not known. Similarly, our review highlights the need to better understand the interaction of cancers cells with its microenvironment in the brain on a single cell level. Several attempts had been initiated to investigate the interaction between microglia and cancer cells suing single cell strategies[105]. However, less is known about the interaction between cancer cells and the rest of immune cells in the brain. Understanding these relationships will shed more light on the possible candidates that could be therapeutically targeted to halt the progression of cancer cells during their metastatic journey and after they reach their niche in the brain.

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