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Brain metastasis models: what do we need to aim for better treatments

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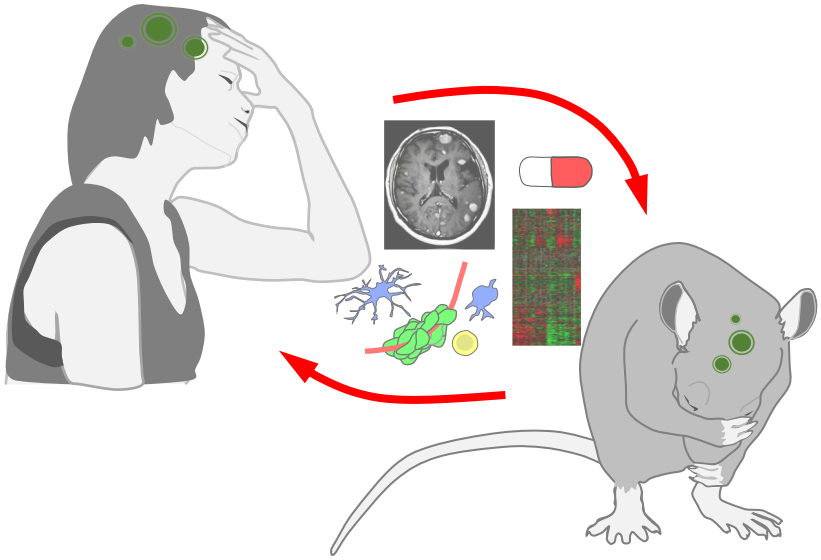

Graphical abstract

Brain metastasis models: what do we need to aim for better treatments.

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Highlights

-A large repository of brain metastasis experimental models is available

-Multiple aspects of the biology must be considered to develop effective drugs

-Many clinically-relevant aspects have not been modelled experimentally

Conflict of interest statement

The authors declare no potential conflict of interest

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Abstract

Brain metastasis is emerging as a unique entity in oncology based on its particular biology and, consequently, the pharmacological approaches that should be considered. We discuss the current state of modelling this specific progression of cancer and how these experimental models have been used to test multiple pharmacologic strategies over the years. In spite of pre-clinical evidences demonstrating brain metastasis vulnerabilities, many clinical trials have excluded patients with brain metastasis. Fortunately, this trend is getting to an end given the increasing importance of secondary brain tumors in the clinic and a better knowledge of the underlying biology. We discuss emerging trends and unsolved issues that will shape how we will study experimental brain metastasis in the years to come.

Keywords

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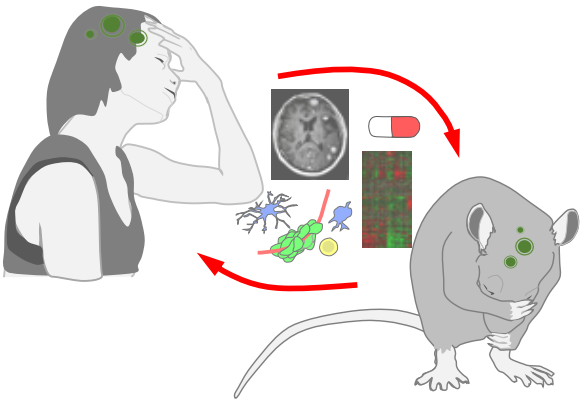

1. The relevance of brain metastasis as a unique entity in oncology

1.1. Why treating metastasis is not as treating a primary tumor

Analysis of metastasis in different organs and patients have depicted a landscape of massive genomic intra- and inter-patient heterogeneity [21,95,197,207,281]. The exponential increase of this complexity might derive from the highly selective multistep process that transforms a local tumor into a systemic disease, which is unequivocally much more difficult to control therapeutically. Besides the evident limitations to apply local therapies (i.e. surgery, radiation) to metastases in a way that could yield complete responses, intra-patient genomic heterogeneity between the primary tumor and derived metastases also raise the question whether an effective therapeutic approach against the first entity would be equally effective against the various secondary tumors. In addition to genetic abnormalities, the plasticity of individual cancer cells is modelled differently according to their surrounding environment [145]. Each metastatic cell must successfully transit through very different stages (i.e. migrating out of the tumor, intravasation, survival in circulation, extravasation, colonization) that involve their ability to constantly rewire themselves to adapt and survive. For instance, the very last step of the metastatic cascade, organ colonization, involves specific constrains from a new environment, which could be more or less similar to the one at the primary location, where the cancer cell must resume their aggressive growth after reallocating key aspects of the cellular intrinsic machinery towards this end.

1.2. Not even as treating any other type of metastasis

The nature of the brain as a vital organ unable to regenerate upon damage involves major limitations for every type of therapy. For instance, neurosurgery cannot be applied always and radiotherapy has the risk of limiting brain plasticity irreversibly that could even evolve into a potentially lethal radionecrosis.

Not only local therapies have limitations. Although the presence of a tumor in the brain might alter the blood-brain barrier (BBB), this does not involve an absence of a selective filter for the entrance of drugs administered systemically. Indeed, the vascular barrier gets remodelled and evolves into a blood-tumor barrier (BTB), an entity that requires further characterization in comparison to the BBB [12]. Drug concentrations in the brain affected by metastasis are generally lower than those reached in other organs affected by disseminated cancer cells [132]. This could favour

the emergence of resistant clones in an organ-specific manner given that only sub-therapeutic doses will be reached in the brain parenchyma [95].

Additionally, metastatic outgrowth in the brain frequently impairs patient neurocognition [78], which is a major contributor to the morbidity of this type of metastasis. Although this problem is clinically well-documented and there are established tests to evaluate neurocognitive decline in patients with brain metastasis [264], there is no knowledge on the underlying pathophysiology rather than the mass effect, which does not always explain the variability among patients regarding the degree of cognitive impact [100]. Emerging interests to understand this aspect that applies to many brain tumors could generate additional organ-specific strategies to improve the prognosis of patients with brain metastasis [153].

2. A general overview of available brain metastasis models

2.1. Organotropic cell lines

Brain metastases models mainly consist on the use of organotropic cell lines, which involve the inoculation of human (xenograft models) or mouse (syngeneic models) derived cancer cells into murine hosts. Frequently, inoculation of available cancer cell lines (parental cell lines) into mice circulation do not show prominent ability to generate brain metastasis [27,196]. However, parental cancer cell lines are heterogeneous and could contain clones with the intrinsic or acquired ability to target the brain. To select out clones prone to develop brain tropism, researchers have derived additional cell lines from the parental cancer cells using an *in vivo* selection strategy. As broadly reported elsewhere [27,163], systemic inoculation of parental cancer cells via intracarotid or intracardiac injection is followed by the selection of the rare clones able to populate the brain from a discrete number of mice who might have certain metastatic load in the brain. Cancer cells recovered from these brains are expanded *in vitro*, established as a cell line and re-injected into mice. Typically, repeating the process of *in vivo* selection multiple times, between 3 to 5, increases the efficacy of selected cancer cells to target the brain. Thus, the final cancer cell line established after *in vivo* selection through the brain is termed brain metastatic (BrM) derivative and upon injection consistently generate established metastases in the majority of inoculated mice [27,163,196,251]. To track metastasis colonization BrM cells are frequently engineered with different reporters (e.g. luciferase or GFP). Alternatively,

the use of reporters could be avoided and metastasis tracked with magnetic resonance imaging (MRI) or simply by identifying metastatic deposits at the experimental endpoint using histology [29].

Most BrM cell lines derive from the three cancer types that are top sources of brain metastasis in patients including lung cancer, breast cancer, and melanoma [249]. The inoculation routes that could be used are not only systemic (intracardiac injections through the left ventricle and intracarotid through the internal carotid artery) but also local (intracranial) and orthotopic (intradermic or subcutaneous for melanoma, mammary fat pad for breast cancer, intrathoracic for lung cancer) approaches. Systemic inoculation recapitulates several steps of the metastatic cascade such as survival in circulation and the extravasation of cancer cells through the BBB as well as all organ colonization. However, they neglect previous stages such as the presence and influence of primary tumors on the formation of the pre-metastatic niche [221,268]. Local inoculation by direct injection of cancer cells into the brain should only be used when the object of study is present in brain macrometastases since intracranial models do not faithfully reproduce the initial steps of organ colonization (i.e. extravasation of cancer cell through the BBB, clonal initiation of metastasis). However, an advantage of this type of inoculation is that the exact site where metastases develop can be predetermined. Finally, orthotopic inoculations (injection of cancer cells in the organ where they were generated) recapitulate better all the stages of the metastatic cascade. They involve not only the colonization but the previous steps such as the presence of a tumor that will be the source of the metastasis that will need to succeed in the intravasation of migratory cancer cells into circulation and all subsequent steps. However, working with orthotopic models that generate spontaneous brain metastasis involves assuming a high experimental variability. These models have low success rate of developing spontaneous brain metastasis that usually require long incubation time to generate brain metastasis, which. In addition, the removal of the orthotopic tumor mass from the site of inoculation it is usually required in order to increase the time available for brain metastases to develop [221].

2.1.1. Of human origin (xenografts models)

In xenografts models, BrM cell lines derived from human lung cancer [41,106,121,271], breast cancer [41,121,132,137,224,225,271,290], and melanoma [106,173] are inoculated into immune-deficient mice in order to prevent human tumor

cell rejection by the host. In these models, brain metastases are fully developed and mice reach the endpoint of the disease (i.e., extensive weight loss and/or neurological symptoms) after 4–6 weeks from BrM cells systemic inoculation. An advantage of these models is that the incidence of extracranial multi-organ metastases is limited, which may be useful to evaluate brain-specific benefits of a given therapy. However, an important limitation of the need to use immune-compromised mice is that they cannot readily be used for evaluating immune-targeting therapies since these models do not present an intact immune system, which have been shown to contribute to many aspects of primary tumor growth and metastatic spread [124,277].

2.1.2. Of mouse origin (syngeneic models)

The inoculation of mouse cancer cell lines into immune-competent mice represent a growing interest among brain metastasis researchers since they allow to study this process in the context of an intact immune system, which is an emerging therapeutic target also in the context of secondary brain tumors [242]. In comparison to xenograft models, syngeneic models reach faster (2–3 weeks) the experimental endpoint after systemic BrM cell inoculation, which limits not only the time available to study the underlying biology of brain colonization but, more importantly, to assess the efficacy of therapeutic strategies. In general, syngeneic models tend to maintain high extracranial multi-organ metastasis in comparison with xenograft models, which have a moderate ability of multiorgan metastasis compared to the brain tropism [27,163,196,251]. One of the major advantages of syngeneic models is that they are compatible with genetically engineered mouse models (GEMM) that might be used to incorporate alterations in specific compartments of the microenvironment so they could be evaluated for their contribution to metastasis progression in the brain or elsewhere [250].

Numerous syngeneic BrM models are available [249] derived from lung cancer [41,62], breast cancer [41,132,224,225,284] and melanoma [64,242,284].

2.1.3. Spontaneous metastasis derived from BrM cell lines.

There are limited BrM cell lines from human [49,159,199] or mouse [62,107,221] origins that spontaneously metastasize in the brain. A spontaneous metastasis is considered when the cancer cell line is able to generate a metastasis not from circulation but when implanted orthotopically. Although working with these models is much more time consuming and requires bigger cohorts of mice, they are critical for

those approaches looking for preventing therapeutic strategies, for models that could incorporate therapies against the primary tumor that frequently coexists and might influence the development of metastases or for validation purposes of findings obtained with faster but less realistic models of metastasis.

2.2. Patient-derived xenografts (PDX).

Established cancer cell lines do not recapitulate the complexity of the original tumor from which they were derived since they acquire additional genomic alterations when cultured *in vitro* for extended periods of time [19]. Patient-derived xenografts (PDXs) are potential solutions to this limitation given that they are not cultured *in vitro* at all or only for a limited number of passages [34], during which they might be engineered with reporters. However, this strategy to model brain metastasis inevitably requires the implantation of the resected human metastasis into immune-deficient mice. Humanized models, which are generated after engrafting components of the immune system, are being used to mitigate this limitation although are still far from being a common practice in metastasis research [155,259].

PDX models have been established from systemic [64,122,226,238,290], local (intracranial injections) [226] [47,131,164,172], or orthotopic inoculations (mammary fat pad or subcutaneous injections) [115,139].

2.3. Genetically engineered mouse models (GEMM).

The majority of genetically engineered mouse models (GEMMs) of cancer obtained after deleting tumor suppressor genes or activating oncogenes are poorly metastatic. Even less GEMMs develop systemic disease that spread to the brain [44,104,147].

2.3.1. Melanoma

Two GEMM of melanoma have been reported to generate brain metastasis. The activation of the *Ret* oncogene induces skin tumors metastatic to liver, kidney, spleen, lungs, lymph nodes and brain [104]. Engineering the activation of AKT1 on a melanoma model was sufficient to drive spontaneous lung and brain metastases in an otherwise non-metastatic mouse model of melanoma [44].

2.3.2. Lung cancer

The somatic inactivation of *Trp53* and *Rb1* are the more frequent genomic alterations of small cell lung cancer. A GEMM incorporating them reproduced this cancer type

including its aggressive metastatic behaviour to bone, brain, adrenal gland, ovary, liver and brain [147].

2.4. *Ex vivo* and *in vitro* models.

Additional models of brain metastasis *in vitro* and *ex vivo* such as brain organotypic cultures [288], co-cultures between BrM cell lines and different components of the microenvironment and brain metastasis-derived organoids have been reported. These models are discussed in a specific chapter by Satchi-Fainaro *et al* (REF).

3. Targeting brain metastasis with small-molecule drugs and nanomedicines.

3.1. Small-molecule drugs tested in experimental brain metastasis.

A broad spectrum of systemic therapies has been tested in mouse models of brain metastasis, ranging from conventional chemotherapies to novel targeted agents (Figure 1).

Despite the general assumption of limited BBB penetration of commonly used chemotherapies [132,212], few agents are able to reach therapeutic levels in the brain and have been tested in experimental brain metastases from breast cancer [102,176]. Carboplatin, a platinum-based chemotherapy used to treat breast cancer, was tested alone or in combination with the PARP inhibitor ABT888 in four different models of triple-negative breast cancer (TNBC) brain metastasis with or without mutation in the *BRCA* gene [102]. Interventive treatment, started once metastasis were detectable by bioluminescence imaging (BLI), with carboplatin alone or in combination with ABT888 after intracranial implantation of cancer cells successfully delayed tumor growth and extended overall survival of animals in the *BRCA*-mutant models, while no effect was observed in the *BRCA*-wild type ones [102]. These results have led to a Phase II clinical trial of the same characteristics which is currently under evaluation (NCT02595905). On the other hand, temozolomide, an alkylating agent used for the treatment of primary brain tumors [235,236], completely prevented brain metastasis from MGMT-negative TNBC cells and extended overall survival of animals, when administered in a preventive setting after intracardiac inoculation of cancer cells [176]. Clinically, temozolomide has shown little or no efficacy in patients with active disease in the central nervous system (CNS) from breast cancer [35,228,245]. Notably, these clinical trials have included temozolomide therapy for established macrometastases,

thus the use of this therapy as a preventive strategy for these patients has just started to be explored clinically (NCT03190967).

Regarding targeted therapies, many BBB-permeable agents have been tested in preclinical brain metastasis models. HER2+ breast cancer brain metastasis has been heavily modelled experimentally due to the wide range of anti-HER2 therapies available for controlling systemic disease [5], with most of them also showing positive intracranial responses [256]. The use of the monoclonal antibody trastuzumab or its derivatives is the most popular anti-HER2 therapy [5,256] and will be discussed in detail in the next section. Other strategies to target this receptor in brain metastases from HER2+ breast cancer include the use of small molecule tyrosine kinase inhibitors (TKIs) such as lapatinib or neratinib [256]. In this regard, lapatinib delayed brain metastases outgrowth in a HER2-overexpressing breast cancer model when administered in a preventive scenario after intracardiac inoculation of cancer cells [87]. Other targets different from HER2 have also been studied in this subtype of breast cancer. Preventive treatment with buparlisib, a selective PI3K inhibitor, effectively decreased brain metastasis incidence in 50% when two HER2+ human breast cancer cell lines were implanted orthotopically (leading to spontaneous brain metastases) or injected intravenously [159]. Clinically, buparlisib, in combination with trastuzumab and capecitabine, yielded benefit in heavily pretreated, trastuzumab-resistant HER2+ breast cancer patients with progressive brain metastases [189]. Although enrollment in this cohort was low (9 patients), the fact that 78% achieved stable disease and all four patients with target lesions in the brain at baseline showed some degree of tumor shrinkage [189] is promising and warrants further investigation. Buparlisib has also been used to target brain metastases from TNBC [254]. In combination with the MEK inhibitor selumetinib, it extended animal survival in two out of four intracranial models of TNBC brain metastasis when administered following an interventive regimen [254]. In the same study, combination of selumetinib with the non-specific PDGFR inhibitor pazopanib showed similar effects [254]. Finally, other novel inhibitors have been shown to target TNBC brain metastases. Inhibition of PLK1, a protein with a significantly increased expression in brain metastases compared to extracranial metastases, prevented formation of large brain metastases in 62% and increased overall survival in 17% [200]. Treatment with the compound, called GSK461364A, was performed in a preventive set up after intracardiac inoculation of cancer cells [200].

Despite existing clinical studies reporting the intracranial benefit of different TKIs for patients with *EGFR*-mutant and *ALK*-translocated non-small cell lung cancer (NSCLC) [48,74,109,184,191,265], preclinical models of brain metastasis from this primary tumor including these therapies are scarce [249]. Osimertinib, the only FDA-approved third-generation EGFR-TKI against *EGFR* activating and T790M resistance mutations, induced sustained tumor regression BLI and increased overall survival in a lung cancer brain metastasis model harbouring an *EGFR* exon 19 deletion [17]. The inhibitor was administered at clinically relevant therapeutic doses when brain metastases were established following intracarotid inoculation of cancer cells [17]. In addition, case studies of two patients resistant to treatment with previous generations of EGFR-TKIs showed intracranial response to osimertinib in the AURA phase I/II trial (NCT01802632), suggesting that this inhibitor could potentially overcome reported resistance to first and second-generation TKIs [17]. YH25448, another third-generation EGFR-TKI with higher potency and selectivity, increased penetration to the BBB and improved toxicity profile compared to osimertinib, provoked tumor regression and increased survival in a lung cancer brain metastasis model harbouring the *EGFR* mutations L858R and T790M when implanted intracranially, as well as in a patient-derived xenograft with an *EGFR* exon 19 deletion [280]. YH25448 was superior to osimertinib and showed 30% more survival benefit [280] when administered at the same clinically relevant dose from the previous study [17]. Case studies of two patients with *EGFR*-mutant lung cancer who have progressed to previous EGFR-TKIs showed extracranial response to YH25448 and even 50% intracranial tumor reduction in one of them [280]. Finally, with regards to *ALK*-translocated lung cancer, the EML4-ALK variant 5a lung adenocarcinoma brain metastasis model was sensitive to both crizotinib and alectinib at the primary tumor site, but resistant to crizotinib and sensitive to alectinib in the brain [158], faithfully recapitulating clinical observations with this family of TKIs [48,74,109]. In another study, entrectinib, an orally bioavailable potent inhibitor of ALK, ROS1 and TRK family kinases, significantly reduced intracranially implanted tumors from EML4-ALK rearranged NSCLC and increased mice survival in more than 70% [10]. Treatment was started after tumors were detectable by MRI, and tumor growth was also monitored by the same technique [10].

Very few experimental models of melanoma brain metastasis that incorporate systemic therapies have been developed [249]. A recent study has reported a patient-derived melanoma brain metastasis model harbouring the mutation *BRAF*^{V600E} in

which treatment with the PI3K inhibitor buparlisib or the MEK inhibitor trametinib as monotherapies decreased tumor volume [2]. However, treatments were applied to established subcutaneous tumor masses generated from the patient-derived brain metastasis, which do not address potential limitations related to drug penetration in the brain [2]. Additionally, combined treatment with both inhibitors showed increased reduction of tumor volume compared to either monotherapy, suggesting a rational combination to overcome potential resistance to PI3K or MEK inhibition alone, which is often seen in melanoma patients [2].

In summary, there are extensive evidences that demonstrate that available small-molecule drugs could be used to treat brain metastases. The majority of these pre-clinical efforts have been developed in breast cancer brain metastasis, thus especial attention should be given to lung cancer and melanoma brain metastasis. Although several of these therapeutic interventions have been performed in a preventive setting, which has a more limited clinical interest, or by using intracranial implantation of tumor cells, which does not necessarily reproduce the step of colonization and involves additional influence on local inflammation [252], there are effective preclinical treatments involving interventive strategies applied to induced brain metastasis from systemic inoculation of cancer cells, which is the most accepted approach to model brain metastasis [252]. Notably, the majority of effective treatments correspond to drugs with known BBB-permeable profile, which suggests the importance of considering this aspect in spite of the possibility of a potential partial disruption of the BBB derived from the presence of a tumor.

3.2. Nanomedicines tested in experimental brain metastasis.

The physical barrier imposed by the BBB is a recurrent limitation in the delivery of drugs to the CNS [73]. Different strategies have been developed to increase access of drugs to the brain. Among them, the use of specific vector systems to deliver nanomaterials has shown superior efficacy and safety profile due to their biochemical properties [128]. Formulation of non-permeable drugs into nanomedicines leads to an improved brain penetration due to the enhanced permeability and retention (EPR) effect and an increase of their circulation time [25]. Moreover, brain targeting properties can be enhanced by the incorporation of targeting ligands, such as antibodies, endogenous proteins or peptides, on the surface of the nanoconjugate [50,177,192]. Once in the blood stream, serum proteins can bind to the nanoparticle

forming the so-called protein corona, which can reduce the targeting properties of the system [211]. The use of nanocarriers is not limited to the delivery of drugs. Several formulations have been proposed as diagnosis or theragnosis agents, for instance [262].

Several studies have used nanocarriers to deliver chemotherapies that do not or have limited penetration to the brain in their conventional formulation (Figure 1) [7,150,152,213]. Most of them have focused in experimental models of TNBC brain metastasis and have tested nanoconjugates based on chemotherapeutic agents that are FDA-approved (doxorubicin and paclitaxel) [7,150] or in clinical trials (irinotecan) [152]. PEGylated liposomal doxorubicin (Doxil®) showed both higher and longer accumulation in intracranially implanted tumors and extended overall survival in >20% compared to the group treated with unmodified doxorubicin, which did not give any benefit compared to untreated mice [7]. These preclinical results were translated into a clinical trial for this patient population that was terminated due to low recruitment (NCT00465673). Recently, modification of doxorubicin loaded liposomes and PLGA nanoparticles with an ApoE binding peptide has been reported as an efficient strategy to achieve brain targeting by controlling the protein corona of the nanosystem [287]. One of the most advanced targeting ligands is glutathione (GSH), an endogenous peptide with an specific transporter at the BBB/BTB [14]. It has been used to modify PEGylated liposomes resulting in improved brain uptake, as demonstrated for doxorubicin, methylprednisolone or an antibody fragment [76,206,208]. These formulations, named 2B3-101 for doxorubicin and 2B3-201 for methylprednisolone, are being evaluated in clinical trials (NCT01386580 and NCT02048358) for solid tumors (melanoma, breast and lung) and brain metastases and for multiple sclerosis, respectively [75,120]. Another example of nanoconjugated chemotherapy in TNBC brain metastasis is liposomal irinotecan (nal-IRI), for which positive results were shown when interventive treatment was given to brain metastases inoculated intracardially, with an increase in overall survival over 30% in mice treated with nal-IRI compared to irinotecan or vehicle-treated mice [152]. A Phase I study confirmed intracranial clinical benefit (partial response or stable disease) of this nanoconjugate in 6/10 patients with active CNS disease from breast cancer (NCT01770353). Currently, there is an actively recruiting Phase II clinical trial for progressing brain metastasis patients from breast cancer (NCT03328884). Another study has also shown improved survival of mice treated with an ultra-small hyaluronic acid (HA)

paclitaxel nanoconjugate compared to paclitaxel (>15%) or vehicle (>30%) with the same preclinical model in a preventive trial [150].

With regards to lung cancer brain metastasis, interventive treatment with the nanoconjugate PRINT-C2-docetaxel following intracranial implantation of the tumor increased animal survival in 35% compared to docetaxel and vehicle-treated animals [213].

Recently, AGuIX, a formulation consisting in a polysiloxane matrix and gadolinium chelate, has been evaluated as radiosensitizer. This nanoconjugate accumulates in the tumors due to the EPR effect and is characterised by its ultrasmall size, below 5 nm, that leads to renal clearance. In addition, it displays good properties as MRI positive contrast agent. Intravenous injection in mice bearing multiple melanoma brain metastases followed by irradiation lead to a 3-fold increase of the life span compared to those that were only irradiated [114]. These promising results has led to the evaluation of the formulation in a Phase 1b clinical trial to treat multiple brain metastases from lung, melanoma, breast and colon cancers (NCT02820454) [257]. Interestingly, MRI imaging showed accumulation of the nanoconjugate at the metastatic sites and not in healthy brain tissue suggesting that it bypassed the BBB/BTB [257].

Nanocarriers from several types, such as iron oxide nanoparticles, gold nanocrystals, protein-based nanoparticles or dendrimers are currently being evaluated to treat several CNS related disorders [161]. Ferumoxytol, ultrasmall superparamagnetic iron oxide nanoparticles, are investigated as contrast agent to detect neuroinflammation in multiple sclerosis (NCT02511028). CNM-Au8, an aqueous suspension of gold nanocrystals, has proved to be well-tolerated and safe in a dose-escalation Phase I clinical trial (NCT02755870) and recently got approval for five Phase 2 clinical trials to treat amyotrophic lateral sclerosis (NCT03536559, NCT03993171), Parkinson´s disease (NCT03815916) and multiple sclerosis (NCT03536559, NCT03993171). APH-1105, an intranasally administered nanoparticle that includes an alpha secretase modulator, will be evaluated in a Phase 2 clinical trial for the treatment of mild to moderate Alzheimer disease (NCT03806478) [51].

Nanocarriers have been applied to brain metastasis in still limited studies and some of them have shown positive results in early phase clinical trials. Given the number of existing drugs with limited BBB/BTB permeability but high anti-tumor efficacy and good profiles as drugs, more efforts to exploit promising nanomedicines should be taken,

specially out of positive experiences in other brain disorders where this type of compounds have been tested more extensively.

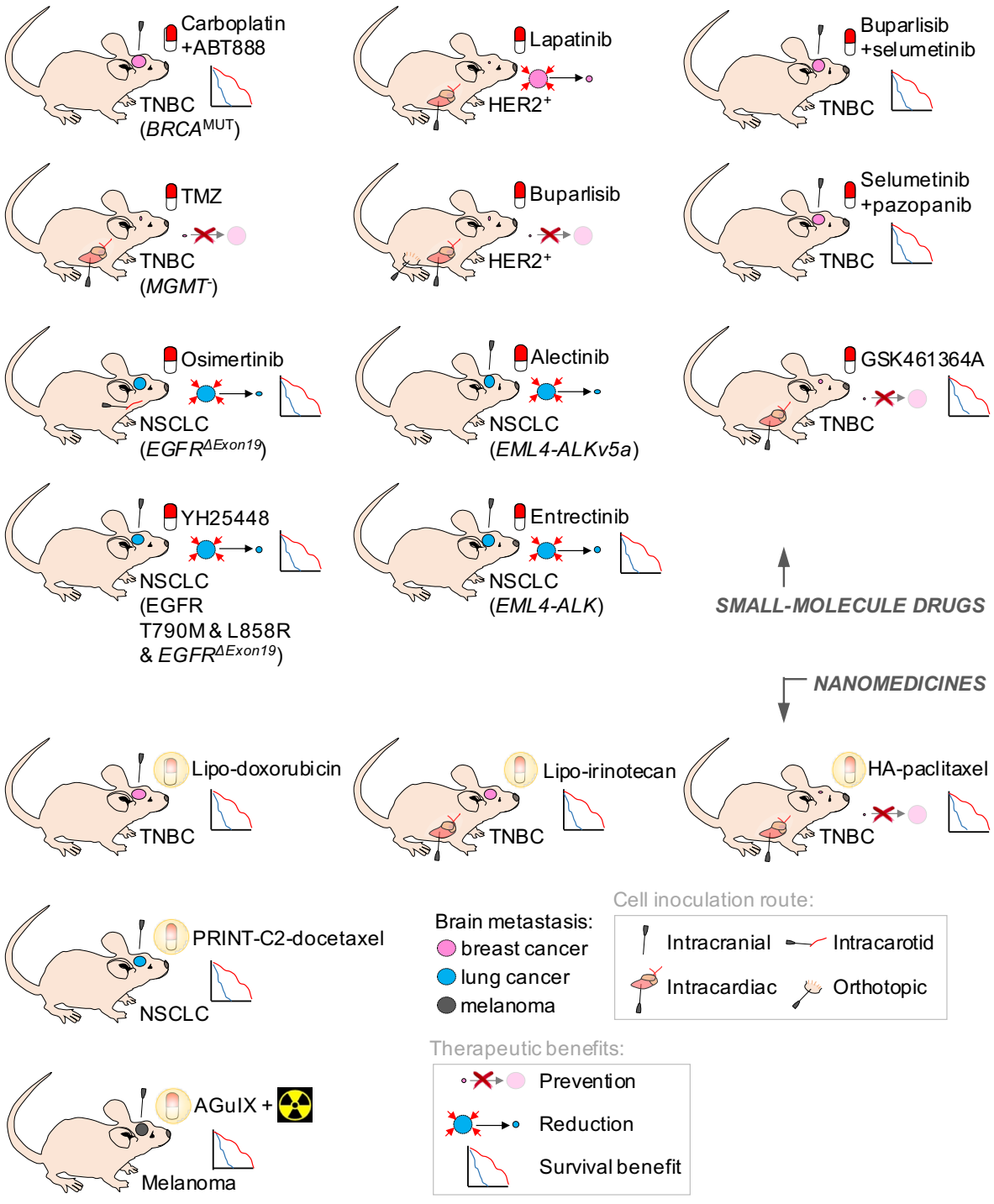

Figure 1. Experimental therapies tested in brain metastasis models. Mostly breast cancer and lung cancer brain metastasis models have been tested in the context of both small-molecules drugs and nanomedicines. The schema depicts not only the specific model used but also the benefits obtained.

4. Using the immune system to challenge brain metastasis.

The development of immunotherapy against cancer, mostly based on immune checkpoint blocking antibodies, is a major revolution in oncology that has recently started to be tested in patients with brain metastasis [28,84,85,113,133,140,243]. In spite of the positive results mainly related to a variable percentage of non-progressing brain metastases, many questions remain open: the local versus systemic source of the benefits derived from the use of blocking antibodies, the requirement of extracranial metastases to obtain therapeutic benefits locally, the biology of antigen presentation in the brain, the need to incorporate additional strategies to an organ with specific immune cell types and a limited access to systemic circulation. In this chapter we discuss the use of antibodies to treat brain disorders as a general update on this therapeutic strategy, which is currently the core of immunotherapies used in the clinic, we also discuss published immune-based strategies that have been applied to brain metastases and an update on the progress made characterizing the immune system in the brain.

4.1. Use of blocking antibodies for brain disorders.

The BBB prevents access to the brain of high molecular weight polar macromolecules. This includes most proteins and, of course, antibodies. At steady state, the relative concentrations of antibody in brain and blood are estimated to be 1:1000 [brain] / [blood] [179,190,203,232]. However, accurately determining the amount of a compound that is able to reach the cerebral parenchyma from the bloodstream is by no means an easy task. In the case of antibodies, this task is even more difficult as their high molecular weight does not allow the use of commonly used techniques for the study of transport to the brain of small-molecule drugs [170]. Pardridge has recently summarized which are the three main mistakes more often found when analyzing the bibliography in this field: i) use of antibody penetration of cerebrospinal fluid (CSF) as a surrogate marker of BBB transport; ii) failure to correct brain uptake measurement for the cerebral blood volume; and iii) administration of antibody in a setting that causes BBB leakiness or BBB disruption [181]. Very interesting, in this context, is the recent development of very large molecular weight cut-off dialysis membranes that is impoverishing the door to quantitative studies of antibody concentrations in the interstitial fluid of the brain using microdialysis techniques [38].

An exception to this poor CNS permeability are those antibodies capable of interacting with vascular endothelial receptors involved in processes of molecular transport to the

CNS [182]. In this case, the access of the antibody to the brain may become more effective. This concept is at the base of several strategies that use this type of antibodies as shuttles (or Trojan horses) for brain drug delivery. Antibodies against transferrin (TfR) or insulin receptors (InsR) have been the most used in this context [23,24,170,182,190]. However, the results published following this approach must be analyzed with great care since very frequently the data available do not allow to differentiate between transport to the brain as a whole (including blood vasculature) and transport to brain's parenchyma [165,170].

Multiple sclerosis is the only neurological disease in which monoclonal antibodies (mAbs) are extensively used for treatment, although the therapeutic effects are probably driven by a peripheral action [72,146]. Natalizumab, an adhesion molecule inhibitor, was approved in 2004 [275]. Alemtuzumab and ocrelizumab have followed, allowing to fight the evolution of the disease addressing a variety of therapeutic targets [258].

The search for a treatment for Alzheimer's disease has led to a tremendous amount of effort to find an anti-Aβ mAb with therapeutic efficacy. Several of these molecules have entered phase II and phase III clinical trials, but to date, none have reached the market [37,72,180,182]. The long list of molecules tested includes bapineuzumab [209], solenezumab [61], gantenerumab [98], crenezumab [4], ponezumab [33] and aducanumab [218]. The poor results obtained so far in all clinical trials with anti-Aβ antibodies could indicate that the therapeutic target is not adequate or could be justified by the fact that in the treated patients the disease was already too advanced [178]. However, an alternative explanation is the lack of use of delivery strategies efficient enough to ensure the BBB permeation of the antibodies [181]. Fortunately, new and promising approaches are continuously being developed in this direction. Kariolis *et al.* have recently reported a new system for CNS delivery of therapeutic proteins based on a human IgG1Fc fragment that has been engineered to bind to TfR and act as a BBB-shuttle [103]. The authors have applied this concept to deliver two anti-β-secretase-1 (BACE1) Fabs. They have also generated a bispecific antibody that targets both BACE1 and Tau that could be used to inhibit in a combined mode amyloids formation and Tau spreading. In a parallel study, Ulmma *et al.* report the use of the same strategy for the CNS delivery of iduronate-2-sulfatase, the lysosomal enzyme deficient in mucopolysaccharidosis type II [248]. Finally, it is important to pay

attention to the interesting work published in 2014 by Niewoehner *et al* [165]. In this study, the authors use a complete collection of biophysical and cell biology tools that allow them to demonstrate that the fusion of a single-chain Fab fragment of an anti-TfR mAb to one C-terminal end of the heavy chain of an anti-Aβ mAb (mAb31) allows transport to the brain parenchyma much more efficiently than when the same anti-Aβ antibody is, either, used alone or fused to two copies of Fab. In the later case, an important uptake occurs by the endothelial cells of the blood vessels of the brain, but the construct remains trapped in the endothelium without being able to access the parenchyma and, finally, is sorted to lysosomes. The authors report a 55-fold *in vivo* increase of β-amyloid target engagement in an Alzheimer's disease mouse model when using the monovalent binding mode to the TfR instead of the naked mAb31 antibody [165].

Bevacizumab was one of the first monoclonal antibodies to be used in the clinic. It acts against vascular endothelial growth factor (VEGF) and is, therefore, an antiangiogenic agent. In 2004 the FDA approved the use of bevacizumab in combination with standard chemotherapy for the treatment of metastatic colon cancer and some forms of metastatic lung cancer. It is well known that in cases of glioblastoma, tumor growth is accompanied by high neovascularization. This explains the efforts to use bevacizumab in this context [231]. The results obtained are, however, the subject of controversy. Thus, while bevacizumab was given fast track approval by the FDA as a single-agent treatment option for the treatment of glioblastoma, in Europe, the EMA has not authorized it because it considers that there is not enough evidence on its effectiveness [36,156,160]. Other mAbs that have been clinically tested to treat glioblastomas are anti-EGFR antibodies such as cetuximab and nimotuzumab, but the results obtained so far have not been sufficiently satisfactory [46,65].

Trastuzumab is an anti-HER2 receptor mAb extensively used in the clinic to treat breast cancer and stomach cancer [217]. The ensemble of mAbs used for the therapy of HER2-positive breast cancer include also other anti-HER2 antibodies (pertuzumab) as well as antibody-drug conjugates (ADC) such us trastuzumab emtansine and trastuzumab deruxtecan [148]. Trastuzumab and pertuzumab, either alone or combined with other therapeutic agents, can slow the growth of HER2-positive metastatic breast cancer and increase survival [151]. However, the use of trastuzumab for the treatment of CNS metastases from HER2-positive breast cancer is hampered

by the low BBB permeability of the antibody [171]. Several attempts to overcome this limitation has been reported these last years, including the use of angiopep-2, a BBB peptide-shuttle [202], a melanotransferrin conjugate [166], and a biparatopic anti-HER2 antibody-tubulysin conjugate [88]. Despite all these efforts, CNS metastasis from HER2-positive breast tumors continues to be a formidable clinical challenge. Trastuzumab is an excellent tool to combat primary tumors and to control extracranial relapse but, to date, has been shown to be of little use in preventing recurrence to the CNS.

It has long been known that cancer patients develop autoantibodies against self-antigens [52]. The production of these autoantibodies is part of the body's natural response to prevent tumor growth. Unfortunately, however, these autoantibodies against self-antigens are behind numerous cases of paraneoplastic syndrome, which is a cancer-derived autoimmune disorder [20]. For instance, 5% of patients with SCLC and up to 10% of patients with lymphoma of myeloma develop paraneoplastic syndromes [185]. Autoantibodies target proteins that are normally expressed in different organs including the brain [53]. Although neurological complications that are frequently part of the paraneoplastic syndrome could derive from antibodies targeting antigens present in brain cell types, more frequently the brain is damaged as a consequence of an uncontrolled response from the immune system [53]. Hyperactivated T cells derived from the systemic presence of auto-antibodies against immune checkpoints might be effective against cancer cells but at the same time target healthy organs including the brain with an intensity that could evolve in a life-threatening autoimmune encephalomyelitis [278].

The blockade of immune-checkpoints has emerged this last decade as one of the most promising strategies in cancer immunotherapy. Antibodies able to block a variety of checkpoint proteins have been approved for the treatment of several primary tumors. This list includes anti-CTLA4 antibodies (ipilimumab), anti-PD1 (pembrolizumab, nivolumab, and cemiplimab), and anti-PDL1 (atezolizumab, avelumab, and durvalumab) [82,141]. As seen in paraneoplastic neurological syndromes, there is an intrinsic risk derived from the use of this therapeutic strategy that requires close control of potential toxicities. Indeed, persistent severe neurological disorders and even fatal cases in patients treated with checkpoint inhibitors have been reported [125].

For many years it has been thought that immune-checkpoint inhibitors were not suitable for the treatment of brain metastasis. In contrast, some advances in

understanding the immunology of brain metastases are challenging this paradigm [59]. In fact, in multiple phase 2 clinical trials with checkpoint inhibitors, especially in patients with asymptomatic brain metastases resulting from melanoma or non-small-cell-lung cancer (NSCLC), clear intracranial activity and durable responses have been observed [85,113,243]. The checkpoint inhibitors immunotherapy is also beginning to give good results in some types of brain metastases in patients with breast cancer [110,157,204]. Indeed, one of the key factors for the success of this type of immunotherapy is the concentration of immune-checkpoint ligands, especially PD-L1, in the microenvironment of the tumor. Recent studies show that in some patients with brain metastasis these concentrations may be quite high [68,116,167]. Kudo *et al.* have recently reported the results from a direct comparative immune profiling of primary lung tumors and brain metastases from 39 patients with resected NSCLC. The data show clearly that the tumor microenvironment of brain metastases is, overall, more immunosuppressed than that of primary tumors. The same authors report, also, that in brain metastasis infiltration of tumor-associated macrophages (TAMs) is higher than in primary tumors and that this could be one of the prevalent mechanisms for the observed microenvironment immunosuppression [116]. All this allows us to be optimistic about the future of immunotherapy of brain metastases. However, given that these treatments may be accompanied by severe immune-related adverse effects [244], as important as the improvement of therapeutic agents will be the development of more sophisticated methods for detecting deleterious effects derived from these approaches. Additionally, more thorough characterization of the local amounts of therapeutic antibodies in the context of the BBB versus BTB will allow to clarify the local versus peripheral origin of therapeutic benefits.

4.2. Immunotherapies in experimental brain metastasis.

To improve the variable response rates among brain metastasis patients receiving immunotherapy, preclinical models compatible with the characterization of the crosstalk between metastatic cells and immune system are required. Specifically, models that incorporate different therapeutic pressures applied at different stages of the disease (stable versus active disease; local versus systemic disease) would be very informative. However, only few preclinical studies have been performed and thus, the specific biology of the brain responding to immune-based therapies is poorly understood.

The local immune microenvironment in brain metastasis is different from that in other brain tumors as demonstrated by the characterization of a distinct lymphocyte infiltration pattern and proximity between antigen presenting and T cells [112]. In addition, secondary brain tumors are frequently accompanied by extracranial systemic disease (i.e. the primary tumor and/or extracranial metastases) that also influences the immune system response [112]. Therefore, to study the efficacy of immunotherapy in brain tumors, the local and/or systemic nature of the specific malignancy should be considered.

Among immune-based strategies, the ones that have used immune checkpoint neutralizing antibodies were shown to be effective in models of melanoma and osteosarcoma brain metastasis [242,270]. Interestingly, the efficacy of anti-PD-1/anti-CTLA-4 combination in the melanoma model was shown to depend on the presence of extracranial disease since it favours CD8+ T cells being recruited to the brain. The mechanism behind it involves the induction of ICAM-1/VCAM-1 receptors in the immune cells, which allow them to recognize the corresponding ligands present in brain capillaries increasing their recruitment in this organ [242]. An alternative strategy was followed in an osteosarcoma model where radiotherapy was applied to the primary tumor. This strategy, which facilitates antigen presentation, induced a potent peripheral anti-tumor response when combined with anti-PD-1 antibodies. The treatment had the dual effect of increasing numbers of CD4+ and CD8+ T cells and decreasing myeloid-derived suppressor cells (MDSCs) [270], which was translated into a superior T cells infiltration and cytotoxic response with reduced numbers of regulatory T cells in the brain [270]. Although combination treatments with immune-checkpoints and radiation delayed brain metastasis progression, the assessed response is still mild, corresponding in the best-case scenario to what it would clinically score as a partial response. Both types of brain metastasis still maintained half of their size [242,270], which is not sufficient to increase survival rather than several days. Thus, a more potent immune system stimulation is needed to reduce metastatic brain tumors more dramatically. Potential reasons behind this need could be the limited permeability of antibodies to the brain but also the presence of local sources of immunosuppression. For instance, multiple experimental models of brain metastasis have shown PD-L1 expression downstream STAT3 positive reactive astrocytes [196], which could contribute to limit the anti-tumor local response when systemically activated T cells infiltrate the brain. Therefore, a potential combination between

inhibitors targeting the local immunosuppressive microenvironment combined with antibodies targeting immune checkpoints might increase response rates to immunotherapies in the brain.

Besides immune checkpoint blockade, additional strategies such as adoptive T cell transfer have been developed. For instance, intracranial or intraventricular delivery of HER2-Chimeric antigen receptor-engineered T cells (HER2-CAR T cells) generate a strong antitumor activity in a model developing multifocal brain metastases. The response reached complete tumor regression and subsequently extended survival when the 4-1 BB co-stimulatory domain was used [194]. Alternative strategies based on adoptively transferred T cells recognizing gp100 -a tumor-associated antigen present in melanoma brain metastasis- combined with a dendritic cell vaccination and systemic IL-2 treatment was also effective against established experimental metastatic tumors [198]. Treatment lead to an enhanced CD8+ T cell infiltration into the brain, a reduction in tumor size and extended mice survival in more than 50% [198].

Instead of infusing T cells previously activated or engineered exogenously, strategies to boost the immune response endogenously using vaccines have been tested. Vaccines based on subcutaneous implantation of irradiated B16 melanoma cells engineered to produce immune-stimulatory cytokines such as GM-CSF, IL-6, or IL-3 showed specific and potent immune response against melanoma brain tumors based on increased CD8+ T cells infiltration, but not CD4+ T cells or NK cells. The response was sufficient to increase mice survival in approximately one week in a model where control mice died at 24 days. This moderate benefit was rather significant in 15% of mice that survived more than 100 days. Interestingly, after tumor-rechallenge mice survived for 60 additional days, suggesting the development of T cell memory to metastatic tumor-derived antigens [214]. The benefits of brain metastasis vaccines have been further studied in more models [136]. Intracranial Injection of lyophilized vehicle cells engineered to produce IFNβ conferred protection against pre-existing brain metastases and prolonged 50% survival of mice as a result of specific CD8+ and CD4+ T cells responses [136]. Finally, mimicking the effect of a specific cellular based vaccine, CpG oligodeoxynucleotides (ODN) are alternative non-specific immunostimulatory molecules that have been used in mouse models of breast cancer brain metastasis as an adjuvant to immune-based therapies [272]. For instance, the

peritumoral injection of CpG ODN at the primary tumor in a model of orthotopic breast carcinoma with concurrent systemic disease, lead to an increased infiltration of innate and adaptive immune cells into the mouse brain as well as the IFN-γ activity. However, this strategy is milder in potency since, although the CD8+ T cells isolated from these mice showed cytotoxic ability in vitro, when adoptively transferred to mice affected by brain tumors, they only showed efficacy in incipient metastasis that are clinically not detectable [272].

Viruses have been used as vehicles in the context of brain metastasis. The local injection of retroviral replicating vectors encoding cytosine deaminase and 5-FC (Toca 511 and 5-FC), with anti-tumor effects through direct interference with metabolic pathways in cancer cells and also depleting immunosuppressive cells, induced systemic anti-tumor immune memory response that improved mice survival in a colorectal cancer brain metastasis model [274]. The effect of Toca 511 and 5-FC was shown to derive from reducing MDSCs in the brain and the spleen, but conveniently not in bone marrow, which might help to avoid frequent secondary effects seen with other immunotherapies [274]. Viral vectors have been also used to genetically modify dendritic cells (DC). DC infected with adenoviral vectors encoding tumor-specific antigens such as the melanoma-associated antigen MART-1 have been used to target melanoma brain metastasis [32]. Mice challenged intracranially with B16 cancer cells in DC-MART-1 immunized hosts were partially protected from brain tumor development since median survival was increased from 17 days to 23 days with 8.7% of mice showing complete response [32].

The still spared knowledge generated by these pioneer studies suggests that immune-based therapies might be effective against brain metastasis. These studies also suggest the need to explore potential combinations of inhibitors of the immunosuppressive brain microenvironment with checkpoint inhibitors. Additionally, better models of brain metastasis, such as those that spontaneously metastasize both in the brain and extracranially, should be incorporated in order to facilitate the translation of preclinical research into the clinic. Their use is critical to clarify, for instance, the divergent response between symptomatic and asymptomatic responses seen in patients [140] or the amount of the drug (i.e. blocking antibodies) getting into the brain compared to other organs affected with metastasis. Clarifying all these aspects, which consider the particularities of the brain, will help to design improved immune-based therapeutic strategies.

4.3. Emerging aspects of brain-specific immunity.

Microglia (MG) are the only leukocyte with access to the brain parenchyma in homeostatic conditions, yet the meninges surrounding the brain are enriched in a variety of immune cells that survey and regulate brain physiology [111]. While the inner meningeal layers drain CSF, the peripheral dura layer is populated by T and B cells, neutrophils and antigen-presenting cells (APCs: macrophages, monocytes and dendritic cells), among others [253]. The notion of the brain as an immune-privileged organ -due to its lack of lymphatic drainage- is now widely disputed [13,134,135], due to recent evidences showing that meningeal lymphatics constitute a drainage route for CNS and CSF-soluble molecules such us antigens, and for meningeal-associated immune cells.

Macrophages are the most abundant leukocyte in brain and other solid tumor-associated microenvironment [112,285]. Several studies over the last years have identified distinct phenotypes and functions in tumor-associated macrophages (TAMs) that arise from different developmental origins [43,71,289]. In brain metastasis and primary tumors, the pool of TAMs is composed by microglia (MG), which arise from yolk sac progenitors [80] and MoMacs, derived from circulating monocytes that locally differentiate into macrophages [91]. Importantly, both ontogeny distinct TAMs have shown to upregulate MHCII genes in multiple cancer types, including brain tumors [6,112] suggesting a potential contribution to antigen presentation to CD4+ T cells.

Perivascular macrophages (PVMs), another subset of phagocytes found in the brain, lie under the vessel basement membrane and astrocytes. While their function in steady state is mainly related to their role as ‘scavengers’ of blood-borne particles and the regulation of vascular permeability, their unique location allows them to sample blood and CSF draining antigens. PVMs in other tissues (LNs) are capable to present antigen-bound to their surface *in vivo* [101], but to date such studies are lacking in the brain and it is currently unknown whether these cells are capable to present antigens and induce T cell activation or any other tolerogenic functions. Importantly, PVMs are a prominent component of the BBB, and the permeability of mouse-brain derived endothelial cells is drastically reduced in co-cultures with macrophages [282], which could facilitate the entrance of disseminated cancer cells into the brain and give rise to metastasis, as shown in invasive breast tumors [90].

Classical DCs (or DC1) have the unique ability of capturing environmental- and cell-associated antigens, and process and present engulfed antigens to T lymphocytes. Through these processes, DC1 induce immunity to tumor-derived antigens that breach the tissues. While DC1 are not accessible to the brain parenchyma in steady state, their accessibility to the brain might not be required as long as they get soluble antigen to transport into the lymphatics. Indeed, in a series of elegant studies Louveau *et al.*, showed that T cells and DCs traffic from the meningeal spaces to deep cervical lymph nodes (dCLNs) and promote CD4 priming in a murine model of EAE [135].

Interestingly, meningeal lymphatic targeting by ectopic expression of VEGF-C is sufficient to promote immunosurveillance of glioblastoma tumors, which results in increased antigen drainage, T cell priming in the dCLNs and infiltration of T cells at the tumor site with no changes in either other myeloid cells, antigen-presentation machinery or BBB disruption [230]. In this way, meningeal lymphatics constitute a back entrance for the infiltration of peripheral immune cells to gain access to brain-derived antigens and to the brain parenchyma. While it is worth to point out that these studies were restricted to mice, the presence of lymphatics in the CNS and how to target them therapeutically has also been reported in humans [127]. However, there is still a lack of understanding in the ports of entry of the CNS lymphatics drainage system and whether mouse and human lymphatic systems can be directly compared, which is a fundamental notion when it comes to design targeted therapies to harness APCs and T cells in brain tumors.

Importantly, glioblastoma and other intracranial cancers are characterized by naïve T cell bone marrow sequestration [45] and concomitant lymphoid organ contraction, which can limit the ability of T cell DC1 priming in the lymph nodes. Hence, strategies that mobilize sequestered T cells in combination with mobilizing professional APCs, such as Flt3L, a cytokine used to mobilize DC progenitors [129,210], may results in a better scenario where anti-tumor immunity responses can be triggered.

5. Organ-specific properties that influence drug efficacy

5. 1. BBB/BTB-guided drug design.

The BBB regulates many different aspects contributing to maintain homeostasis in the central nervous system (CNS). Structurally, it comprises endothelial cells, pericytes and the astrocytic end feet [12]. The BBB is frequently impaired in the presence of

brain metastases, creating an altered but selective blood–tumor barrier (BTB) [79,132].

The BBB/BTB provides a unique challenge for researchers aiming to target brain tumors since it limits the access of therapeutic agents into this organ upon systemic delivery (Figure 2). Various strategies have been explored to circumvent this major obstacle including chemical barrier disruption, nanoparticle-delivery vehicles, focused ultrasound in combination with microbubbles, and viral vehicles among others [11,12,92].

The BBB/BTB accounts with a number of natural transport mechanisms. On the one side, small lipophilic compounds can cross by passive diffusion. On the other side, the presence of carriers and receptors enables the passage of hydrophilic drugs like sugars and amino acids or proteins. All these mechanisms have been widely studied to promote drug delivery to the brain [179].

Non-polar drugs have a higher probability of entering the brain as they can cross the lipid cell membrane. For this reason, molecular parameters such as polar surface area, logP (as a measure of drug lipophilicity), and the number of hydrogen donors have been traditionally used as criteria to predict CNS penetration [175]. Based on these premises, medicinal chemists have tried to increase the brain permeability of small molecules by chemical modifications, such as methylation or lipidation, to reduce the possibility of forming hydrogen bonds, increasing the lipophilicity of the drug [269]. For example, modification of paclitaxel with linoleic acid results in increased BBB/BTB penetration [105]. However, this approach faces some limitations since it would only be applicable to small molecules and it can increase the affinity of the drug for the efflux pumps present at the abluminal side of the endothelium [261].

Another strategy involves the modification of the natural ligand of carriers and receptors with the drug of interest [89,263]. The main limitation of this strategy is competition with the natural ligand. Also, modification of the substrate of natural carriers can result in a change of the transport mechanism. For instance, modification of peptides with glucose did not result in an enhanced uptake by the glucose transporter type I as expected [67].

BBB-shuttles represent a versatile, safe, and selective way of delivering therapeutics to the CNS. These compounds are brain-penetrating ligands, mainly antibodies or peptides that bind to the receptors expressed at the luminal side of the BBB and trigger transcytosis. As pointed out in section 4.1, several antibodies against receptors

expressed at the BBB, such as transferrin (TfR) and insulin receptor (InsR), have been developed, some of them reaching clinical trials, to treat mainly lysosomal storage diseases and Alzheimer [22,81,168,279]. For instance, 52 weeks of treatment for mucopolysaccharidosis type I patients with the fusion protein valanafusp alpha that comprises a mAb against the human InsR and α-L-iduronidase lead to stabilization of the disease both at somatic and cognitive levels, suggesting a good peripheral and CNS distribution of the drug (NCT03071341). One of the main parameters that may limit the delivery of a BBB-shuttle antibody through the BBB/BTB is its affinity for its receptor [165]. Very high affinities lead to entrapment into the capillaries and to the degradation of the receptor. On the contrary, very low affinities limit the access of the antibody to the brain parenchyma. One of the strategies to overcome this limitation is the use of bispecific antibodies. Antibodies with optimal receptor affinity have been proved as more efficient brain delivery agents [81].

More recently, the use of BBB-shuttle peptides has gained attention [99,170]. Peptides elicit low immunogenicity, their production is cost-effective and they display a medium affinity for their receptors, a highly desirable characteristic for a BBB-shuttle [170]. Currently, there are several strategies to overcome the limitations associated with the use of peptides in the clinic [266]. Thus, the possibility of incorporating non-natural amino acids reduces degradation by proteases. In addition, modification of various moieties such as polyethylene glycol limits their renal clearance increasing their circulation time.

To guarantee BBB/BTB targeting, BBB-shuttles peptides are obtained from the sequence of neurotropic biomolecules and from biopanning experiments by using phage display or chemical libraries against BBB/BTB related targets [170]. On the one hand, some virus, toxins or endogenous proteins have preferred brain distribution. To that end, analysis of their sequence has led to the discovery of several BBB-shuttles such as RVG29, derived from the glycoprotein of the neurotropic rabies virus [117]; MiniAp-4 or CDX derived from neurotoxins [169,283]; or ApoE peptides, derived from the endogenous protein [260]. On the other hand phage display against known BBB/BTB receptors, endothelial cell lines or even *in vivo* strategies has yielded peptides such as G23, obtained by phage display against the ganglioside GM1 [77]; Peptide-22 obtained by phage display against the extracellular domain of the LRP1 receptor [138]; or TGN obtained by *in vivo* phage display [126]. Several *in vitro* and *in vivo* models are used to evaluate the BBB/BTB targeting potential of these

compounds. *In vitro* models include the use of endothelial cell lines, of various origins, for uptake and transcytosis experiments [15]. *In vivo* models, both with healthy or disease models, imply the use of rodents, mostly, in combination with several non-invasive analytic techniques, like *in vivo* imaging or invasive techniques such as intracerebral microdialysis. In addition, samples from these studies can be evaluated *ex vivo* to obtain complementary information [170].

A clear example of the potential of BBB-shuttles peptides is Angiopep-2 [57]. This 19-mer peptide, designed from the alignment of the Kunitz-domains of human proteins and binds the LRP-1, has demonstrated an ability to increase the transport of a wide variety of cargos such as small molecules [154], nanoparticles [96] or mAb [202]. More importantly, a derivative bearing three paclitaxel units has been assessed in various clinical trials. Safety, tolerability and therapeutic benefit have been evaluated in patients with advanced solid tumors and brain metastases (NCT00539383) [119] and in patients with recurrent glioma (NCT00539744) [63]. In both studies, the compound was well tolerated reaching therapeutic concentrations at the tumor site. Of note, some of the patients experience the overall partial response with associated reduction of the size of the tumors. More recently, results from Phase II clinical trials (NCT01480583, NCT02048059) indicate again good safety, tolerability and therapeutic benefit in patients with leptomeningeal carcinomatosis and brain metastasis from breast cancer [118].

Peptides able to modulate the permeability of tight junctions have also drawn attention. Cereport, a bradykinin analogue that interacts with the G-coupled protein receptor for bradykinin, has been evaluated as an adjuvant of various anticancer drugs reaching Phase II (NCT00923117) before being discontinued due to low effectiveness [193]. Other peptides, inspired by natural proteins, have been used to increase the transport of drugs by transiently opening the BBB/BTB. ApoE-K16 co-administered with dabrafenib, a BRAF inhibitor, increase the life span in a model of human melanoma brain metastasis [1]. Moreover, cadherin-based peptides have been shown to modulate the adherent junctions tightness allowing for the brain delivery of several compounds such as small molecules [240] or mAb [247].

Despite the advances in the field, a better knowledge of the BBB/BTB is needed to improve the brain delivery of therapeutics. To be a target for brain delivery a given receptor should fulfil some requirements. Preferably, it should be overexpressed on brain endothelium, in comparison to other organs and to the brain parenchyma. In

addition, it should be able to undergo transcytosis. Several efforts have attempted to identify novel receptors of the BBB/BTB ranging from transcriptomic and proteomic analysis of isolated microvessels to combinatorial screening approaches [97,237,246,286,291]. In 2011, the group of Prof. Tesaraki used a quantitative proteomic approach in order to identify and quantify the carriers and receptors of brain capillary endothelial cells, both from human and mice. Several differences were found in the expression pattern. For instance, the efflux transporter BCRP was highly expressed in humans while the P-gp was more expressed in mice. Among the transcytosis receptors, the TfR was the most abundant being its expression in mice 5-fold higher than in humans. These differences in expression between species are important for the design of preclinical experiments to ensure correlation between preclinical and clinical data. More recently, Zuchero *et al.* identified [291] three receptors (basigin, glut1, and CD98hc) following an unbiased proteomic approach with higher expression levels than the ones corresponding to InsR or TfR, which are the reference receptors to engineer brain permeability. To prove if these newly identified receptors could serve as new targets for increasing access to the brain parenchyma they generated Abs against them. *In vivo* evaluation in mice demonstrated that the Ab targeting CD98 reached the brain parenchyma with a 5-fold increase when compared to the one targeting the transferrin receptor (80-fold when compared to a control IgG). Importantly, they demonstrated that the receptor homeostasis was not affected by the treatment with the antibody. Controversially, a recent transcriptomic assay has compared the expression pattern of human and murine brain microvasculature (BMV) cells showing that CD98 levels on human BMV are similar to those on the brain parenchyma while it is highly overexpressed on the murine one [286]. Combination of proteomic and transcriptomic assays of the BBB/BTB is of great interest to improve the knowledge about this heterogeneous barrier and to discover new targets for brain delivery of therapeutics. It will also help to understand the parameters that influence the permeability of the barrier enabling for better detection and treatment of brain tumors.

5. 2. The brain tumor-associated microenvironment as a target.

Many studies during recent years have focused on characterizing the impact of the brain microenvironment (BME) on the development of brain metastases [201]. Existing evidence support the modulation of the transcriptome, metabolome or proteome of

brain metastastic cells by the BME [18,183,225,267]. As a consequence, this tumor-stroma interaction might determine the presence or absence of a specific therapeutic target in the cancer cells and therefore influence drug efficacy (Figure 2).

On the other hand, the BME itself may constitute a target due to the pro-metastatic role that brain-resident cells can acquire during disease progression, typically immune cells that switch from an anti-metastatic state during early brain colonization to an immunosuppressive phenotype at advanced stages of the disease [196,225]. Gradual increase of cathepsin S expression in brain-resident macrophages from normal brain to early- and late-stage brain metastasis was reported [225]. VBY-999, an inhibitor of this breast-to-brain metastasis mediator, decreased brain metastasis burden in 65-77% as measured by BLI in a TNBC brain metastasis model [225]. In this assay, brain metastases were induced by intracardiac inoculation and the effect of the inhibitor was exclusively observed in the brain, and in preventive trials but not when metastases were established [225]. Another example of targeting the pro-tumoral BME in brain metastasis is the use of silibinin, a STAT3 inhibitor, to target a subpopulation of reactive astrocytes that benefit the growth of metastatic lesions by promoting an immunosuppressive environment [196]. Silibinin treatment both in preventive (intracardiac inoculation) and interventive (intracranial implantation) trials decreased brain metastasis burden in 56-62% in a melanoma brain metastasis mouse model [196]. Additionally, silibinin treatment in advanced lung cancer patients with brain metastases who failed previous lines of therapy induced 75% overall response rate in the brain, with 3/18 patients even showing complete response [196].

The influence of the BME in therapy response may also derive from its active role inducing resistance mechanisms to a given drug [41]. In this sense, different types of breast and lung brain metastatic cells were reported to engage with astrocytes through gap-junctions and activate a pro-inflammatory programme in this cell type, which signals back to the cancer cells and promote their growth and resistance to chemotherapy [41]. In this study, treatment with the gap-junction inhibitors meclofenamate and tonabersat in combination with the BBB-permeable chemotherapy carboplatin decreased brain metastasis burden by BLI, while carboplatin alone did not reduce metastatic growth significantly [41]. This chemoresistance was observed in a TNBC brain metastasis model, in which treatment was administered in an interventive set up following intracardiac inoculation of cancer cells [41]. As a result from this study, the use of meclofenamate in patients with

progressive brain metastasis from solid tumors is currently being evaluated in an ongoing clinical trial (NCT02429570).

5. 3. The plasticity of cancer cells is key to organ-adaptation.

Adaptation of cancer cells to the high selective pressure imposed by the brain implies rewiring of genomic, transcriptomic and metabolomic hallmarks, resulting in extremely dynamic tumors that are continuously evolving (Figure 2) [26].

At the genomic level, clinical sampling of tumors from the primary site, extracranial and brain metastases have consistently shown genomic divergence and branched evolution of brain colonizing clones, supporting an independent genomic remodelling of brain metastases [30,39,55,60,95,123,174,186,195,216]. In addition, genetic alterations associated with sensitivity to PI3K/AKT/mTOR, CDK, and HER2/EGFR inhibitors were identified in the brain metastases, suggesting potential differential response to targeted therapies particularly in the brain compared to the primary tumor [30,39,174,195]. Based on these findings, it will be of ultimate importance to exploit existing and novel preclinical models that reproduce therapeutically relevant alterations obtained from clinical sampling to explore the biology of CNS metastases and more importantly, to design rationale therapeutic approaches.

Transcriptomic analyses of brain metastatic cell lines from human origin xenografted in nude mice have allowed *in situ* characterization of how cancer cells grow in the hostile brain microenvironment following colonization [183,267]. Engraftment of multiple cell lines from four different primary tumor origins (melanoma, lung, breast and colon cancer) in the brain, skin and orthotopic sites allowed to investigate the influence of different organ microenvironments on the transcriptomic profile of the cancer cells [183]. Gene expression data revealed strong reprogramming of metastatic cells by the BME towards a neuronal phenotype, mimicking transcriptional programmes of neurogenesis [183]. These changes were driven by epigenetic modifications, mainly a shared DNA methylation profile between tumors engrafted in the brain compared to those from the orthotopic sites, which maintain methylation patterns from their respective cell lines [183]. Acquisition of neuronal-like programmes was also confirmed by another study, in which an RNA-seq approach that distinguishes between human and murine transcripts with higher sensitivity and accuracy was used [267]. In addition, not only the organ itself, but specific locations of the brain were shown to modulate the transcriptome of brain metastases *in situ* [267].

In contrast to spatially separated human brain metastases, which were genomically homogenous [30], the transcriptomic hallmarks between tumor cells from forebrain and hindbrain metastases were distinct [267]. Although some of the pathways induced in cancer cells by the BME were previously described, such as the WNT/TCF in a lung cancer brain metastasis model [163], both functional characterization and validation of these findings derived from experimental models [183,267] using human samples remains to be investigated.

While the genetic features underlying the metastatic process to the brain have been extensively investigated, the metabolic rewiring that might be associated to brain colonization has remained unexplored. Tumor cells that metastasize to the brain must reshape their metabolism to meet the energetic demands imposed by the novel microenvironment encountered. Concurrently, they must maintain the high metabolic activity that supports the rapid proliferation distinctive of malignant tissue. Since glucose is the main substrate utilized by the normal brain, brain metastatic cells rewire their metabolism to utilize alternative metabolic sources. For instance, brain-tropic breast cancer cell lines gain the capability for glucose-independent growth, not present in their non-metastatic counterparts. This switch involves the utilization of branched chain amino acids (BCAAs) and glutamine as alternative fuels in addition to upregulate gluconeogenesis via fructose 1,6-biphosphatase (FBP) activities [40]. Additionally, brain metastatic cells are also able to employ acetate as a substrate for acetyl-CoA formation via acyl-coenzyme A synthetase (ACSS2) [188], and therefore supply the tricarboxylic acids (TCA) cycle [144,187], similar to other metastatic tumors [219]. Mitochondrial oxidation has been revealed to be a key component of brain metastasis metabolism [142,144]. Indeed, RNA-seq analysis of melanoma brain metastasis compared to patient-matched extracranial metastases ranked oxidative phosphorylation the top upregulated gene network in brain metastases [69]. Morover, ^{13}C tracing analysis utilizing [U-^{13}C]-glucose conducted in mice harboring human melanoma cells intracranially and subcutaneously revealed an enhanced mitochondrial activity in view of the higher levels of m+3 TCA intermediates in the brain metastasis with similar levels for m+2 glycolytic metabolites [69]. Targeting the dependency on mitochondrial metabolism has been proved in BRAF-mutant melanoma brain metastases by treatment with β-sitosterol, which induces ROS formation and apoptosis *in vitro*. Furthermore, a mouse model of brain metastasis generated by intracardiac injection of DL2 cells in NOD/SCID mice experienced a

significantly increase in survival (80.2±4.8 days for treated versus 55.7±1 days for untreated) when a preventive treatment initiated 1 week before cancer cell inoculation was applied. β-sitosterol-treated mice displayed a reduction in the number of metastasis as determined by MRI as well as by bioluminescence [239]. Interestingly, breast cancer-derived brain metastases have also been reported to have lower antioxidant ability compared to those occurring in the lung, which was linked to an enriched NRF2 signature and active mitochondrial complex I in lung micrometastases compared to brain micrometastases and mammary tumors [18].

Metabolic adaptation to the new niche can be alternatively accomplished by modifying the substrate availability. This was observed in brain metastatic breast cancer cells releasing exosomes containing miR-122. Exosomes were taken up by astrocytes in the brain where the miRNA downregulated pyruvate kinase isozyme M2 (PKM2) and glucose transporter 1 (GLUT1), which are key enzymes for consumption of glucose. Consequently, the uptake of glucose by astrocytes was reduced and, consequently, its availability in the microenvironment increased, which promoted the survival of cancer cells colonizing the brain [70]. Alternatively, brain metastatic cells can reprogram their glucose metabolism to optimize its use for maximum proliferative capacity in the brain. For instance, the serine- and glycine-deprived environment that cancer cells encounter during brain colonization induced rewiring of glucose flux towards serine and glycine through D-phosphoglycerate dehydrogenase (PHGDH) providing intermediates for nucleotide synthesis, which allowed maintenance of cell proliferation. Treatment with PH-755, a second generation of PHGDH inhibitor, was used to study the consequences of inhibiting this metabolic adaptation of cancer cells to the brain. The prophylactic use of PH-755 (treating mice before systemic inoculation of cancer cells) resulted in 20-fold reduction of brain metastases, with half of the treated animals remaining metastasis-free, and increase the overall survival a median of 21.5 days more. Treatment of established metastases in a TNBC model and a renal cell carcinoma models also reduced the number of lesions as determined by histology leading to an increase in survival. [162].

In summary, mimicking the colonized organ (i.e. acquisition of neuronal phenotype, metabolic rewiring) is part of the process of adaptation that metastatic cells must accomplish. This process allows cancer cells to outcompete brain resident cells in the use of the limited resources while generating novel metabolic vulnerabilities that can be exploited by focusing on halting the specific benefits of such adapting behaviour in

cancer cells. These therapeutic strategies could be exploited in combination with more general anti-cancer approaches to achieve better responses when cancer has disseminated.

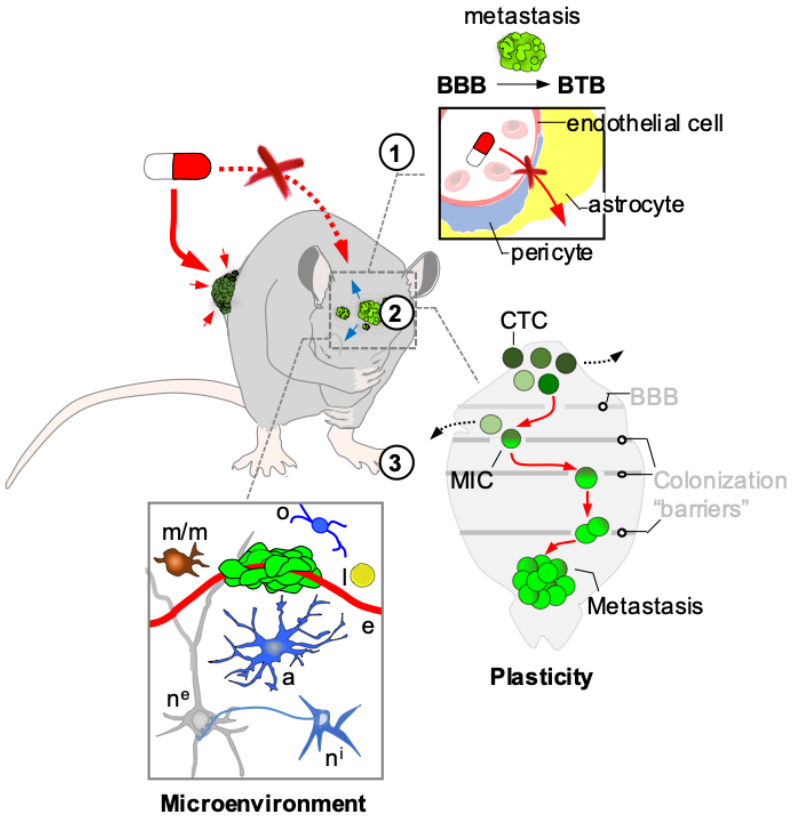

Figure 2. Organ-specific properties that influence drug efficacy. A given drug might or might not work equally in the orthotopic location, where the primary tumor is located (i.e. intradermal for a melanoma model), compared to the brain affected by metastases (light green). This differential therapeutic response could depend on the ability of a given drug to (**1**) access the blood-brain barrier (BBB) or the brain-tumor barrier (BTB), once the metastasis is established in the brain. Additionally, (**2**) once circulating-tumor cells (CTC) have reached the brain, only a few of them have the ability to cross the BBB. Out of them, only a minor percentage of metastasis initiating cells (MIC) will be able to adapt overcoming different barriers (i.e. microenvironment, metabolism, subtherapeutic drugs concentrations) to generate a macrometastases. Thus brain metastases could evolve differently to cancer cells located in the primary tumor. (**3**) The influence of the microenvironment involves interactions with many different cell types, resident or infiltrated from the periphery (a: astrocyte, e: endothelial cell, l: lymphocyte, o: oligodendrocyte, m/m: macrophage/microglia, n^e: excitatory neuron, n^i: inhibitory neuron).

6. The ideal brain metastasis model

Increasing efforts are taken in the preclinical arena to improve experimental models and their analysis so better therapeutic strategies could be identified. A major goal is to establish clinically compatible scenarios that could be applied as soon as possible

to patients so prevention rather than treatment of brain metastasis could be achieved. We discuss here emerging strategies (Figure 3).

6.1. Spontaneous metastasis: a key tool for preventive strategies

Although scarce (see section 2.3), genetically engineered mouse models (GEMM) that develop spontaneous tumors with the ability to evolve into a systemic disease are invaluable to explore various clinically relevant scenarios. The contribution of the pre-metastatic niche has been mostly studied using organotropic cell lines [94]. In contrast, the majority of spontaneous models of cancer are poorly metastatic, specially to the brain [44,86,104,147]. Additionally, the initiation of brain metastasis has been mostly examined in cancer-free mice after the inoculation of metastatic cells in circulation [252], in contrast to heavily treated cancer patients, which corresponds to one of the most frequent clinical presentations of secondary brain tumors. Finally, spontaneous models of metastasis are a key tool to evaluate the benefits of organ-specific therapeutic strategies and to optimize their use with more general anti-cancer approaches. However, the technical difficulties to perform these experiments (i.e. the need to incorporate surgery for the primary tumor), the significant time to detect brain metastasis in the very few models that develop them (i.e. approximately 6 months) and the amount of mice required to buffer the intrinsic variability of metastasis generation (i.e. approximately incidence of brain metastasis could vary from 20-50% of mice) make this approach very challenging [44,104,147,252].

6.2. Adding local and systemic therapies

Although brain metastasis could be the first manifestation in cancer patients, it is more frequently associated with advanced stages of systemic disease [241]. Consequently, patients with brain metastasis are frequently heavily pre-treated with systemic therapies. Indeed, this has been hypothesized to contribute to the ability of certain clones to emerge in the brain [95]. In addition, brain metastases could be treated locally either with surgery, whole brain radiotherapy (WBRT) or stereotactic radiosurgery (SRS), a more recent focal and more effective modality of radiation with less secondary effects. In spite of these considerations, the vast majority of publications on brain metastasis are based on treatment naïve experimental models. Indeed, no models exist to study local relapse after neurosurgery, a major clinical problem, and very limited studies have studied the influence of radiotherapy on the

biology of brain colonization to improve this therapeutic approach [143,220,229,276]. Ideally, in order to get as close as possible to the many different situations in which brain metastasis could arise in patients, incorporating different clinically relevant therapeutic modalities might provide faster avenues to translation.

6.3. Increasing genomic complexity

The genomic landscape of brain metastasis in patients contains additional complexities derived from its branched evolution [30,95]. This principle (i.e. that metastases have a more complex genome than primary tumors) is not specific to the brain but to metastases in general [21,95,197,207,281]. However, the degree of divergence from the primary tumor and extracranial metastasis as well as the similarity between metastasis in the brain has been reported to be higher [30]. Anyhow, reproducing the genomic complexity of brain metastasis in experimental models is a strategy that will allow to functionally evaluate additional organ-specific drivers, if exist [226]. Nowadays the landscape of available models is broad and includes not only organotropic cell lines but also PDX models [252] , which recapitulate the genomic complexity of specific patients. Additionally, CRISPR-Cas9 strategies [42] or approaches to induce genomic instability [16] could be applied in order to reproduce the levels of genomic complexity seen in patients without the caveat of banning the use of immune competent hosts.

6.4. Liquid biopsies

The best approach to challenge systemic cancer, including metastasis in the brain, is early diagnosis. Early stages of metastasis in the brain (and elsewhere) respond better to therapy both in experimental models and in patients [8,233,234]. Thus, strategies to detect metastatic deposits in the brain or, even better, biomarkers that could reliably identify primary tumors with high risk to metastasize in the brain, is a major goal for researchers and clinicians interested in this progression of cancer. At the moment, clinical trials have demonstrated that liquid biopsies, mostly from the cerebrospinal fluid (CSF), are compatible with the analysis of the evolution of clinically-detectable established brain metastasis in response to treatment [223]. However, improved technologies applied to liquid biopsies will surely get to a point where small metastasis could be detected and its evolution followed over time to decide when and what treatment should be applied. At the moment liquid biopsies could help to decide

whether actionable genomic alterations could be specifically identified in the brain, independently of its presence or absence in the primary tumor, which could facilitate to initiate organ-specific treatments [108,223].

6.5. Improving non-invasive imaging

Traditionally, the assessment of a brain malignancy is performed by MRI to obtain the location and size of the tumor mass. *In vivo* molecular imaging is usually conducted through PET (Positron Emission Tomography) using the glucose analog ^{18}F-FDG (Fluorodeoxyglucose), although other alternatives do exist [66,83,255,273]. However, this approach presents some restrictions in light of the intrinsically high glucose uptake of the brain [227], and the information retrieved, which indicates the uptake of the substrate only, without providing any additional insight about its subsequent metabolism. Alternatively, chemical composition of malignant tissue can be obtained non-invasively through MRS (Magnetic Resonance Spectroscopy) and chemical exchange saturation transfer (CEST) [58], which display a static landscape of the tumor metabolism. In order to explore metabolic fluxes, ^{13}C MRS, wherein the tumor is infused with a ^{13}C-labeled substrate has to be performed. However, this technique is limited by its low sensitivity, although new signal processing procedures have been recently implemented to increase the signal-to-noise ratio for ^{13}C MRSI (Magnetic Resonance Spectroscopy Imaging) [31]. In the recent years, alternatives for improved molecular imaging have been developed, such as hyperpolarized ^{13}C MRS. This method increases the sensitivity of ^{13}C detection through MRS by 10,000-fold [9] that results in quantification of *in situ* metabolic flux. Hyperpolarized ^{13}C MRS outperforms FDG-PET in some tumors [93] while also avoiding the use of ionizing radiation. As the most aggressive forms of cancer have high glycolytic activity and rapid pyruvate-to-lactate conversion [56], the most widely used ^{13}C tracer is ^{13}C-pyruvate. This methodology has been applied in investigations of CNS malignancies and in patients with brain metastasis [149]. Moreover, ongoing clinical trials are also utilizing the pyruvate-to-lactate conversion through hyperpolarized ^{13}C-pyruvate as a reporter of response to treatment in intracranial metastasis (NCT03324360). Despite of the notable abilities of hyperpolarized MRS, the high costs of the equipment currently prevent wide clinical implementation. An alternative approach for imaging the dynamics of tumor metabolism *in situ* was recently reported in animal models of brain malignancies. By monitoring the loss-of-signal in the ^{1}H MRS spectrum after injection

of a deuterated substrate, glycolytic flux and acetate consumption can be investigated using standard MRS approaches [205]. Moreover, deuterated probes have been revealed to be safe in humans and deuterium-based metabolic imaging of brain tumor patients have been recently reported [54].

Molecular imaging must occupy a major position in the assessment of brain metastasis considering its remarkable abilities to inform *in situ* about the functioning of biochemical pathways associated to the disease and the development of new imaging strategies with increasing sensitivity. The possibility to incorporate some of these technologies to mouse models of brain metastasis will help to apply additional interventions mimicking the disease in patients.

6.6. Getting to know the immune system

The rapid development of immunotherapies that have been applied more recently to brain metastasis with positive results have challenged the prevailing dogmas regarding the use of antibodies to treat brain disorders and the limited contribution of the immune system in the brain. However, given the limited knowledge on the biology of response (or lack of it) to immune checkpoint blockade in general and in particular in the brain and the still primitive interpretation of the crosstalk between the brain and local and peripheral components of the immune system, it is too naïve to assume that this therapy is a major achievement against brain metastasis. For instance, the importance of corticoids, broadly used in brain metastasis, on the variable response of patients with secondary brain tumors is not well-defined [133], the influence of extracranial disease to enhance positive response to immunotherapy locally in the brain [242], the local versus systemic action of immune checkpoint blockade on T cells [250], the lack of characterization of circulating lymphocytes among responders and non-responders [215], the contribution of resident brain cells to influence local responses to immunotherapy [196], the differences with other brain tumors (i.e. glioblastoma) that do not show positive responses [215] are all open questions of major importance to understand basic aspects of local immunology and its role in the therapeutic response to immune checkpoint blockade. Indeed, research on these aspects will be useful to design immune based therapies to other highly prevalent and incurable brain disorders [130,222].

6.7. The ideal model versus the real disease in patients.

The huge variability in prognosis and survival that could be seen among patients affected with brain metastasis suggests that it is not a single disease. The presence or absence of systemic disease extracranially, the presence of single or multiple metastasis, the amount of lines of therapy previous to the diagnosis of the brain metastasis, the age and general status of the patient are all major contributors to the clinical behaviour of metastatic cells in the brain [3,241]. Experimental models should evolve in a direction that could reproduce the complexity of the many faces of the human disease in order to study not just a single situation (i.e. aggressive disease, treatment naïve, cancer free previous to the presence of metastasis, limited genetic complexity, immunosuppressed hosts) but the many clinically-relevant status that patients with brain metastasis have to face including the prevalent neurocognitive impairment, which remains completely uncharacterized experimentally [153].

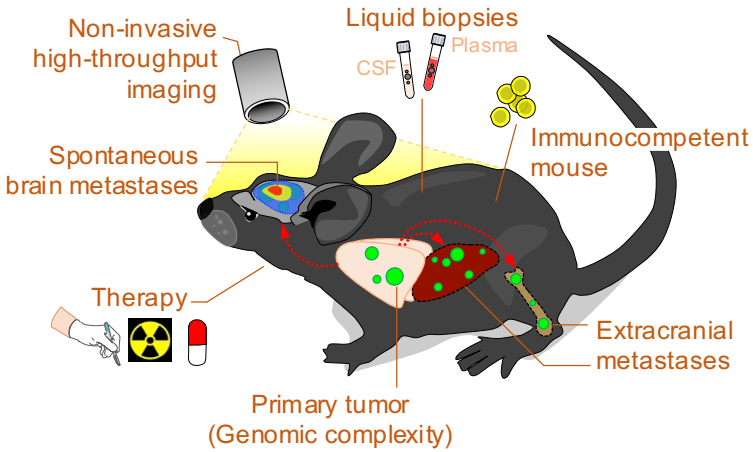

Figure 3. The ideal brain metastasis model. Potential strategies to improve existing experimental models of brain metastasis aiming to recapitulate all the stages and pathophysiology of brain metastatic disease in patients.

Concluding remarks

Models of brain metastasis are abundant and well-characterized however still limited as models since they are mostly able to reproduce induced metastasis rather than spontaneous and are frequently studied in a naïve situation rather than in a situation involving any treatment, as it is frequently the case in patients. Knowledge generated with available brain metastasis models have been translated to clinical trials and contributed to the modest but real improvement of patients with brain metastasis. This is a strong evidence that experimental approaches using experimental *in vivo* tools

are a valid strategy to exploit cellular and molecular mechanisms of this progression of cancer. Continuing with the efforts already invested in mouse models of metastasis is an absolute need to decipher basic aspects and vulnerabilities of systemic disease. Combining such models with 3D strategies and patient-derived material in clinically-relevant contexts will continue to generate highly needed therapeutic opportunities to this unmet clinical need.

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