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RECEIVED 30 March 2025

ACCEPTED 22 May 2025

PUBLISHED 03 July 2025

CITATION

Poggi A, Reggiani F, Azevedo HS,
Raffaghella L and Pereira RC (2025)
Medulloblastoma: biology
and immunotherapy.
Front. Immunol. 16:1602930.
doi: 10.3389/fimmu.2025.1602930

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Medulloblastoma: biology and immunotherapy

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Medulloblastoma is an aggressive central nervous system tumor affecting children more commonly between the ages of 5-9. It is usually localized in the cerebellum, leading to diffusion of tumor cells through the cerebrospinal fluid and metastases to other portions of the brain and spinal cord. Conventional treatment consists of surgical resection followed by adjuvant radiation and/or chemotherapy. The side effects of these therapies are critical to consider, especially given that patients are in a distinct stage of their lives. In addition, the overall survival is not satisfactory ranging from 50-90% depending on the type of medulloblastoma. The molecular characterization has broadly subdivided medulloblastoma into four subgroups, and more recently, the single-cell transcriptomics studies have further identified several other subgroups. Important advances have been reported on the cell origin, their plasticity, heterogeneity of genetic and epigenetic alteration, and interaction with the immune and stromal components of the tumor microenvironment. Research studies on these key points are essential to make advances in planning the application of conventional therapies together with immunotherapies. Herein, we discuss the main advances recently obtained on medulloblastoma biology and immunotherapies. Overall, the biological and molecular features of medulloblastoma are briefly summarized to understand the reason for the application of the old and new immunotherapies. Immunotherapies considered include the identification of potential medulloblastoma neoantigens and tumor-associated antigens to generate antigen-specific T lymphocytes. The main antigens expressed by medulloblastoma cells and/or by components of the tumor microenvironment will be considered as the molecular targets of antibodies, antibody derivatives, and chimeric antigen receptor effector cells to improve the conventional therapies. In the last portion of this review, the brief analysis of the activating and inhibiting receptors expressed by antitumor T, natural killer, and unconventional T cells can give new insights into the potential treatment of medulloblastoma.

KEYWORDS

medulloblastoma, adaptive and innate immune response, Tumor microenvironment, brain tumor, unconventional t cells, NK cells, immunotherapy

1 Introduction

Medulloblastoma (MB) is the most common embryonal malignant tumor of the central nervous system (CNS) in children, reaching about the 20% of all brain pediatric tumors. Large cohorts of patients underwent comprehensive omics analyses (genomic, transcriptional, proteomic, methylomic and epigenetic changes), resulting in the definition of four consensus molecular subgroups (1). These subgroups display differences in patient demographics, pathogenesis, prognosis and response to therapy. World Health Organization (WHO) criteria now integrate these molecular findings with the traditional histology classification (2). The four subgroups have been defined as Wingless and Int-1 (WNT)-activated, Sonic Hedgehog (SHH)-activated, Group 3 (non-WNT/non-SHH), and Group 4 (non-WNT/non-SHH), and they have been well characterized in other reviews and herein will be briefly considered thereafter (1, 2). The overall 5-year survival for MB can reach 70-85%, but the toxic effects of surgery, chemotherapy and radiation have long-term consequences for pediatric patients. It is necessary to identify more precision therapies to reduce the morbidity of treatments. The conventional treatments consisting of surgical resection, chemotherapy and radiation can lead to relevant drawbacks related to the age of the patient. Indeed, the major severe adverse effects comprise neuroendocrine dysfunction, growth alterations, infertility, neurocognitive disabilities and even secondary malignancies. The surgical intervention is the first line of treatment, and it tends to eliminate as much tumor mass as possible without causing more signs and symptoms. Following the surgery, the irradiation of the brain and spine with a proton beam is necessary as the MB tends to diffuse from the original site to the rest of the brain and spine. The reduction or omission of radiotherapy can result in an ineffective treatment, although this reduces the above-mentioned side effects (3, 4). Overall, the treatment will be chosen based on the subtype, its diffusion, patient response, associated side effects and quality of life, emphasizing the trade-off between survival and neurocognitive disabilities (5). Chemotherapy increases the survival of patients, but it is not effective in many cases as the 5-year overall survival (OS) can reach about 70% in patient in the high-risk group (6).

The immune system is involved in the control of tumor cell growth as it can sense the alterations present in tumor cells due to genetic mutations, which may lead to the expression of tumor neoantigens, tumor-associated antigens or stress molecules (7–11). By consequence, the immune system can react against tumors, both eliciting an adaptive and innate immune response (12–14). The recognition of tumor cells, can lead to their killing. The recent literature is full of reports that claimed the key role of immune system-mediated control of tumor cell growth (15–17). It has been reported in 1985 that lymphokine-activated killer (LAK) cells were efficient in some tumors, such as melanoma (18–20). This was one of the first clear experimental proofs of the concept that the immune system can check tumor growth. Since that discovery, the following clinical applications of infusing high doses of interleukin (IL)2 to treat human melanoma or renal cell carcinoma gave contradictory results in terms of effectiveness. More importantly, the

identification of life-threatening IL2-mediated side effects in humans such as vascular leakage syndrome (VLS) limited its clinical applications (21, 22). This stimulated researchers to better define the modalities of administration of IL2 and the use of IL2 derivatives instead of IL2 itself (23–25). Today, several clinical trials have shown that an immune response can be elicited by stimulating T lymphocytes, relieving the brake of autoreactivity using immune checkpoint inhibitors (ICI) and/or chimeric antigen receptor (CAR) engineered T or natural killer (NK) cells. The incoming approaches to block tumor cell growth and resistance will use a combo of targeted therapy together with conventional treatments (26–29). These treatments show important side effects such as cytokine release syndrome (CRS), immune effector cell-associated neurotoxicity syndrome (ICANS) for CAR-T cells or serious autoimmune reactions in the lungs, intestines, liver, hormone-making glands, kidneys, or other organs for ICI (30–34). However, the effective therapeutic responses observed with some types of tumors, such as hematological malignancies, melanoma and non-small cell lung carcinomas, justify the use of these antitumor biological drugs (35–37). Conceivably, these new therapeutic approaches can be applied to treat MB, limiting the toxic side effects of conventional therapies (38, 39). Herein, we will analyze in some detail the biological features of MB cells together with the molecular targets for triggering an efficient immune response, analyzing the rationale of using unconventional therapeutic approaches and suggesting new treatments for this pediatric tumor.

2 Epidemiology and biological features of medulloblastoma

Briefly, the epidemiology and biological features of MB will be considered to give an overall scenario in which the old and new immunotherapies can be applied. The main point to remark is that pediatric patients are primarily involved by this tumor. Pediatric patients have two relevant characteristics, among others, to be considered when immunotherapies are applied: 1- the immune response in these patients can be different from an adult; the immune system is developing; 2- the MB arises in the cerebellum in which are developing interactions with other portion of the brain to allow the coordination of the large majority of motor neuron functions and cognitive properties (40–42).

The designation “medulloblastoma” was first named in 1925 by neurosurgeons Bailey and Cushing, and reflects its anatomical origin in the posterior fossa (43). As the most frequent malignant tumor among pediatric CNS cancers, it accounts for approximately 60% of intracranial embryonal tumors. It arises in the cerebellum, a hindbrain structure responsible for motor coordination and learning, situated at the base of the brain near the fourth ventricle (44, 45). While most MBs occur sporadically, a subset is linked to genetic predisposition syndromes, notably within the SHH-activated subgroup (46, 47). These syndromes include Li-Fraumeni syndrome (TP53 mutations), Turcot syndrome (APC-associated polyposis), Fanconi anemia subtypes, and Gorlin

syndrome (nevroid basal-cell carcinoma syndrome) (46, 47). According to the CBTRUS report, MB is the most common malignant childhood CNS tumor, comprising the largest percentage of embryonal tumors (70.2%) and almost 20% of all pediatric brain tumors (48).

While primarily a childhood disease (approximately 10 cases per million children), it is rare in adults (approximately 0.54 cases per million). Pediatric and adult MBs exhibit distinct molecular profiles, with pediatric tumors typically harboring fewer genetic mutations in comparison with the adults (44, 49, 50). Notably, pediatric cases show a sex disparity; males are more likely to develop than females, suggesting biological sex as a potential risk factor. However, susceptibility also varies based on tumor histology and molecular subtypes, which makes it even more difficult to establish a predefined grade of risk factors (44–46).

Pediatric MB is often metastatic at the time of initial diagnosis, with a tendency to spread beyond the CNS via cerebrospinal fluid (CSF), lymphatic circulation, and the bloodstream. While external CNS metastasis is a rare feature in most brain tumors, it occurs more frequently in MB compared to other pediatric CNS tumors (44, 51, 52). External CNS metastases typically emerge later and are less commonly detected with metastasis, including bones, bone marrow and to a lesser extent, lymph nodes, liver and lungs (51, 52). This metastatic potential raises special concerns as it appears to be associated with a higher likelihood of progression to a specific subtype, indicating a poor prognosis. The aggressive nature of MB, coupled with its early metastatic behavior, underscores the challenges in managing this malignancy and highlights the need for early detection and targeted therapeutic strategies.

2.1 Classification and biology

Similar to other cancers, MB presents a high heterogeneity, both in terms of histology and molecular characteristics. Advances in tumor biology and genetics have led to a classification system that improves both diagnosis and treatment strategies. Originally, MB was categorized by histological properties into three main subtypes: classic (C), desmoplastic/nodular (DN), and large cell/anaplastic (LCA) depending on cellular phenotype (53–57).

The classic MB group represents the most common subtype, accounting for 66–72% of cases. Tumoral cells typically do not grow significantly in volume and rarely show structural alterations such as desmoplasia or nodules exhibiting a high nucleus-to-cytoplasm ratio, rounded nuclei, and significant mitotic and apoptotic activity. The DN group is much less frequent than the classic subtype, representing about 15% of all the cases. It is often associated with a favorable prognosis and a distinct molecular subtype (SHH), facilitating a quicker and more precise diagnosis. MB cells have the ability to deposit collagen in the pericellular space. Cytologically, these cells appear small and round, with characteristic arborizations and pericellular reticulin deposition. This subtype of MB tumors presents a specific variant - MB with Extensive Nodularity (EN) – that primarily occurs in newborns (50, 55). The LCA subtype accounts for approximately 15% of the cases

that have more aggressive behavior. It is characterized by nuclear pleomorphism, increased cell volume, and a tendency for cells to cluster. Within this subgroup, the large cell variant is particularly rare, occurring in only 2–4% of cases. Cells show high proliferative and apoptotic activity and are associated with a significantly poorer prognosis compared to other subtypes (54–57).

The 2021 fifth edition of the WHO Classification of CNS Tumors categorizes MBs into four distinct molecular subgroups: WNT-activated, SHH-activated with TP53 wild type, SHH-activated with TP53 mutation, and non-WNT/non-SHH (formerly classified as groups 3 and 4 (50, 58–60). (Figure 1). These subcategories are further segmented into more specific subtypes using advanced methylation profiling, which has identified multiple WNT-, SHH- and non-WNT/non-SHH-associated subtype. This molecular stratification has significantly enhanced the understanding of the biological diversity and clinical variability of MB, which enables more precise classification, prognosis and therapy (57–70). Information about epidemiological, clinical and molecular features of MB molecular subcategories is summarized in Table 1. It is to be noted that the identification of distinct methylation patterns and histological features continues to drive research into targeted therapies and personalized medicine treatment protocols, aiming to improve outcomes for patients with MB (61, 62). Furthermore, besides the sporadic medulloblastoma, this tumor can occur in association with cancer predisposition syndromes such as colon polyps in Turcot syndrome or basal-cell carcinomas in Gorlin syndrome [reviewed in (46, 47, 73, 74)]. This event should be considered in relation to each molecular subgroup and this knowledge can guide oncologist to perform the cancer surveillance to diagnose and treat early the medulloblastoma in collaboration with other specialists. This topic is of great relevance for MB as germline mutations are 5–6% and these genetic alteration affect specific molecular pathways leading to tumor development (75).

2.2 MB cell lines models

Like in other cancers, despite advancements in multimodal therapy, MB still remains a challenge due to its high heterogeneity and therapy resistance. To better understand biology and develop targeted therapies, researchers rely on well-characterized cell lines as essential tools for studying tumor pathogenesis, investigating signaling pathways, and screening therapeutic compounds (Figure 1). Several cell lines have been established, each representing different molecular subtypes and biological characteristics. Herein, we present a brief description of the most used cells, which include DAOY, D283-Med and D341-Med, UW228, ONS-76, HD-MB03 and D425-Med and D458-Med (76, 77) (Figure 1, Table 2).

Several others cell lines have been selected and described in the literature reviewed partly elsewhere (78, and listed in <https://www.cellosaurus.org/search?query=medulloblastoma+cell+line>). WNT and group 4 MB subtype cell lines are underrepresented compared to the frequency of these MB (78). For instance, the

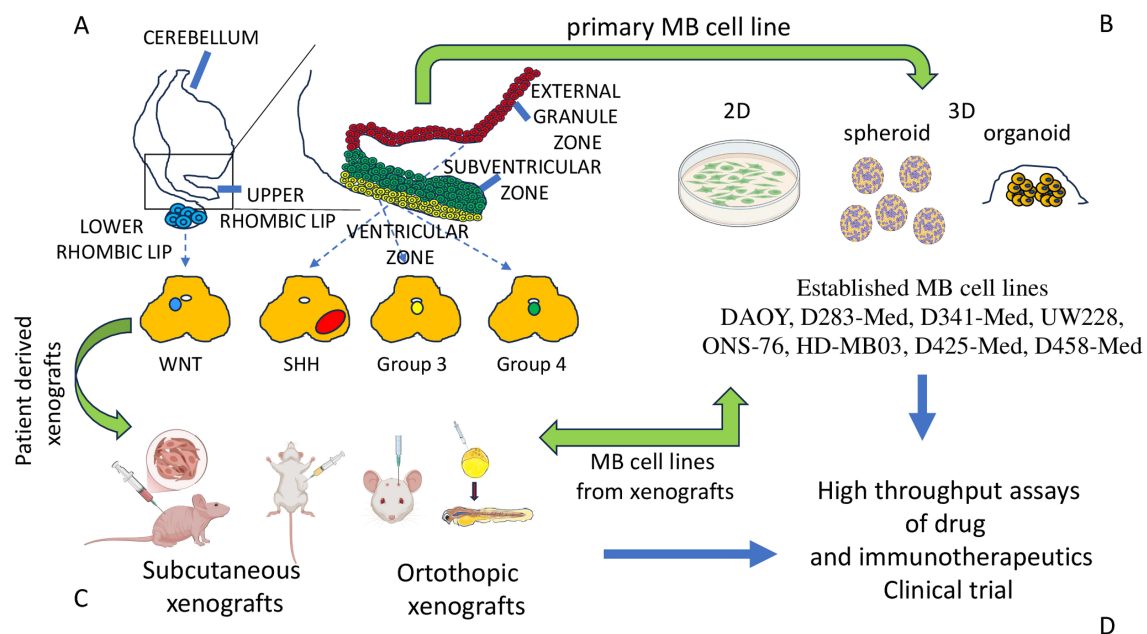


FIGURE 1

MB molecular subgroups and models for studying the biology of MB cells. (A) Four main subgroups of MB have been proposed on the basis of OMICS analysis. MB cells derive from different regions of the cerebellum, as shown. (B) The major models to study the biology of MB cells are represented by primary cell lines derived from tumor specimens of patients. Some of them can be stabilized during the culture, maintaining specific phenotypic and functional features leading to established cell lines (some of which have been listed). The primary and/or well-established cell lines can be cultured in conventional (inappropriately called 2D culture) or 3D conditions, such as spheroid or organoid. (C) Patients' tumor specimens or cell lines can be inoculated subcutaneously or orthotopically in small animals (mice/rats) with an impaired immune response to allow the engraftment of these xenografts. Also, MB cells from patients or cell lines can be inoculated in blastula of Zebrafish to generate orthotopic models of MB tumor. The animal models (subcutaneously or orthotopic models) are a key tool to study the growth and metastatic behavior of MB cells. From xenografts, cell lines can be obtained for *in vitro* studies. (D) Altogether, these research models are the basis for the selection of novel drugs and immunotherapeutic tools to be validated in clinical trials.

CHLA-01-MED and CHLA-01R-MED have been used to investigate features of primary and metastatic cells of group-4 MB (79) or the MED6 and MED5R of WNT group and MED1 of group 4 to evaluate the function of the MDR1 (ABCB1 molecule) to study drug-resistance mechanisms (80).

The most used and represented in literature is the DAOY cell line. This cell line has been derived from a desmoplastic tumor in a 4-year-old male and categorized within the SHH subgroup (81). It harbors a heterozygous *PTCH1* mutation (c.1312G>T; p.G438X), leading to constitutive SMO activation (54, 55, 76, 77). Genomically, it exhibits 9q loss (*PTCH1* locus) and 10q gain (*MYCN* locus), consistent with SHH-MB. DAOY retains wild-type *TP53*, differentiating it from *TP53*-mutant SHH MB. These adherent cells form neurospheres under serum-free conditions, expressing CSC markers such as CD133, nestin, and SOX2 (77). DAOY xenografts show desmoplastic histology and it expresses SHH targets (*GLI1*, *MYCN*) responding to SMO inhibitors like vismodegib (82). However, resistance via *SUFU* mutations or *G88LI2* amplification can emerge (70, 82, 83). DAOY also displays HDAC1 overexpression, and HDAC inhibitors reduce cell viability (84). Limitations include non-metastatic behavior in xenografts and genomic drift over time (78, 85). The D283-Med was established from a metastatic tumor obtained postmortem from pediatric patient while the D341-Med were from the primitive medulloblastoma tumor at craniectomy (71, 86). These cells

present *MYC* amplification and aggressive behavior (71, 78). Due to their non-adherent, suspension-like growth, these models are widely used to study metastasis and therapy resistance. Both exhibit *MYC* proto-oncogene amplification, a hallmark of Group 3 MB, correlating with their aggressive phenotype and poor prognosis (71, 87).

D283-Med presents isochromosome 17q (i17q), characteristic of Group 4, while D341-Med often has chromosome 8 gains. Their morphology mimics anchorage-independent growth, facilitating metastasis studies (71, 86). D341-Med xenografts show leptomeningeal spread; D283-Med is less metastatic but grows aggressively. Both express CSC markers (CD133, SOX2, nestin) and show intrinsic resistance to cisplatin and etoposide due to upregulated ABC transporters (e.g., ABCG2) and anti-apoptotic proteins (e.g., BCL2) (78, 86, 88–91). Notably, D341-Med responds to BET inhibitors that suppress *MYC* transcription (91). However, long-term culture can lead to genomic divergence, necessitating periodic validation. Nevertheless, these cell lines present some indicators of genomic drifts upon prolonged *in vitro* culture time, which can lead to unwanted clonal selection and genomic divergence from the initial primary tumor patients. This important fact raises the need to perform periodic molecular validations.

The UW228 cell line was derived from a MB recurrence (92). Unlike D283-Med and D341-Med, UW228 cells display moderate

TABLE 1 Main features of medulloblastoma tumor.

Characteristic	Medulloblastoma molecular subtype			
	WNT	SHH	Group 3	Group 4
Prevalence %	<5-10	20-30	20-25-40-50	35-40
Female/male ratio	1:1	1:1	2:1	2:1 3:1
Age	Children, teens	Infants, adults	Infants, children	Infants, children, adults
Histology	Classic	Nodular/Desmoplastic, Classic, LCA	Classic, LCA	Classic, LCA
Cells of origin	Lower rhombic	Cerebellar granule precursors of neuron	Hypothesis of neural stem cells	Upper rhombic leap progenitors
Metastatic strenght	low	low	high	high
Recurrence	rare	local	metastasis	metastasis
Prognosis	good	middle	poor	middle
Risk level	Low risk	TP53WT intermediate, TP53 mutant very high	Intermediate to high	intermediate
5-years survival %	95	75	50	75
Genetic alteration	WNT	SHH	Group 3	Group 4
Somatic mutation	CTNNB1, DDX3X, SMARCA4, TP53, CSNK2B, PIK3CA, KMTD2, CREBBP	PTCH1, SMO, TP53, SUFU, DDX3, TERT, IDH1, KMT2D, BCOR	SMARCA4, CTDNEP1, KMT2D, KBTBD4, MLL2	KDM6A, KMT2C, ZMYM3, KBTBD4, MLL3
Germline mutation	APC (<5%) CREBBP	PTCH1, TP53, SUFU		
Chromosome affected Copy number	Ch 6 loss monosomy	Ch 3 gain, Ch9p gain, Ch 9q loss, Ch 10q loss Ch 17p loss TERT, MYCN, GLI1/2	Ch 1 gain, Ch 5 loss, 10q loss, MYC, PVT1, OTX2, GF11/1B, GF11 overexpression	IsoCh 17 Ch X loss, Ch 17p loss PRDM6, SNCAIP, MYCN, CDK6, GF11 and GF11B

The table has been generated using information from references 1, 2, 67–72. Please note the high variability in the prevalence are reported in the references cited.

differentiation and exhibit a more adherent, epithelial-like morphology with moderate differentiation (78, 92). It carries inactivating *PTCH1* mutations and wild-type *TP53*. Genomic alterations include 9q loss, aligning it with SHH-MB. UW228 expresses intermediate neuronal markers (β 3-tubulin, MAP2), distinguishing it from less differentiated models. Cultured in monolayers, it supports studies on differentiation and SHH signaling (93, 94). UW228 shows partial sensitivity to SMO inhibitors and transient suppression of GLI1, with resistance mechanisms involving *SUFU* downregulation or *GLI2* amplification. Resistance to cisplatin and etoposide correlates with elevated ABCB1 expression. It is useful for modeling therapy-naïve SHH MB and differentiation therapy, though limited by low metastatic potential and genomic instability in long-term cultures. This cell line displays a partial sensitivity to SMO inhibitors, with reduced GLI1 expression and transient growth suppression. Nevertheless, some resistance arises via *SUFU* downregulation of *GLI2* amplification (78, 95, 96). Studies have shown that this cell line also shows medium resistance to cisplatin and etoposide. UW228 cell line presents low metastatic potential *in vivo*, which limits its utility for invasion and tumor dissemination studies. Like other cell lines, it presents some genomic instability over *in vitro* passages, which fosters low-passage genetic validation.

The ONS-76 cell line was established in 1989 from a 2-year-old Japanese patient with nodular/desmoplastic MB (98). ONS-76 cells display neuronal differentiation when exposed to retinoic acid, making this cell line a suitable model for studying differentiation therapy and SHH inhibitors (98). Integrated in the SHH molecular subgroup, ONS-76 has *PTCH1* mutations or deletions, disrupting its inhibitory function on SMO and promoting GLI-mediated transcription (98). These cells retain wild-type *TP53* and harbor *PTCH1* mutations, supporting SHH pathway activation. ONS-76 expresses neuronal markers (β 3-tubulin, GFAP) and undergoes differentiation upon retinoic acid exposure, marked by neurite outgrowth and NeuroD1 upregulation (98, 99). It grows as a 2D monolayer and can form desmoplastic xenografts. ONS-76 is sensitive to SMO inhibitors, and unlike DAOY, rarely develops GLI2-driven resistance, likely due to functional *TP53*. Retinoic acid reduces proliferation via HDAC inhibition (100). This cell line also shows modest cisplatin resistance. ONS-76 is ideal for SHH-targeted differentiation therapies, though differentiation capacity may decline with extended culturing.

The HD-MB03 cell line, established in 2014 from a MYC-amplified, represents a critical preclinical model for studying high-risk, metastatic MB biology (101). It exhibits *MYC* amplification, 1q/7 gains, and loss of 10q (*PTEN*) and 16q. These alterations

TABLE 2 Features of some MB cell lines.

Cell line	DAOY	UW228	ONS-76	HD-MB03	D425-Med	D341-Med	D283-Med	D458-Med
Subtype	SHH (DN)	SHH	SHH (DN)	Group 3	Group 3	Group 3	Group 3/4	Group3/4
Growth Type	Adherent and Suspension	Adherent (epithelial like)	Adherent (differentiation with RA)	Suspension	Suspension	Suspension	Suspension	Suspension
Key Alterations	PTCH1 mutation, 9q loss, 10q gain (MYCN), WT TP53	PTCH1 mutation, 9q loss, WT TP53	PTCH1 deletion/ mutation, WT TP53	MYC amp, 1q/7 gain, 10q (PTEN) & 16q loss	MYC amp, MYCN subclones, chr 7 gain, 10q loss (PTEN)	MYC and sometimes MYCN amplification, chr 8 gain	MYC amplification, i17q (Group 4)	MYC amp, i17q (Group 4), chr 8 amp
Stemness Markers	CD133, Nestin, SOX2	Moderate: β3-tubulin, MAP2	β3-tubulin, GFAP, NeuroD1 (with RA)	CD133, SOX2, Nestin	Not specified	CD133, Nestin, SOX2	CD133, Nestin, SOX2	Not specified
Drug Sensitivity	SMO inhibitor (vismodegib), HDAC inhibitor (panobinostat)	Partial SMO inhibitor response, ABCB1-mediated drug resistance	Vismodegib, RA modest cisplatin resistance	HDAC inhibitors (panobinostat), resistant to cisplatin/ vincristine	Not specified	Resistant to cisplatin/ etoposide, BET inhibitors (MYC-targeted)	Resistant to cisplatin/ etoposide, drug efflux via ABCG2	Not specified
Models Strengths	CSC dynamics, SHH pathway, plasticity	Differentiation studies, SHH pathway	Differentiation therapy model	High-risk, metastatic, CSC studies	Leptomeningeal metastasis model	Metastatic behavior, therapy resistance	Metastasis formation, drug resistance	Hematogenous metastasis (lung/liver)
Limitations	Low metastasis <i>in vivo</i> , genomic drift	Low metastasis, genomic instability	Differentiation potential lost in long-term culture	Genomic drift with time	Not specified	Genomic drift	Genomic drift, moderate metastasis	Not specified

ABCB1, ATP-binding cassette sub-family B member (P-glycoprotein); BET, bromodomain and extra-terminal domain; DN, desmoplastic/nodular; GFAP, glial fibrillary acid protein; HDAC, histone deacetylase inhibitor; MAP2, microtubule -associated protein 2; PTCH1, protein patched homolog 1; PTEN, phosphatase and tensin homolog; SOX2, sex determining region Y-box2; SMO, smoothened; RA, retinoic acid; SHH, sonic hedgehog protein; CSC, cancer stem cells; The features of the cell lines are described in detail in the site <https://www.cellosaurus.org/> and the references from 71, 76–105, and reviewed in ref. 78.

enhance proliferation and apoptotic resistance (56, 57). HD-MB03 forms floating spheroids and shows robust CSC marker expression (CD133, SOX2, nestin) (102). *In vivo*, it replicates leptomeningeal and spinal metastases. The cell line is intrinsically resistant to cisplatin and vincristine due to high ABC transporter and BCL2 expression. HDAC inhibitors such as panobinostat disrupt MYC-driven transcription and reduce tumor growth (91, 103). HD-MB03 serves as a strong platform for testing MYC and epigenetic-targeted therapies, though periodic molecular verification is necessary due to potential genomic evolution.

D425-Med and D458-Med were established in the late 1990s from high-risk, recurrent cases, both showing MYC amplification (104). Both have high proliferative and metastatic capacities. D425-Med shows 7q gain and 10q loss (PTEN), driving PI3K/AKT pathway activation, while D458-Med presents i17q and chromosome 8 amplification. Both grow as suspension spheroids and demonstrate strong *in vivo* metastatic capabilities. D425-Med shows leptomeningeal dissemination in orthotopic models; D458-Med metastasizes to lung and liver via systemic injection (104, 105). D425-Med may harbor MYCN amplification in subclones, enhancing proliferation. These models are vital for studying hematogenous dissemination and high-risk MB mechanisms.

3 Conventional and advanced models for studying MB biological properties

It is becoming evident that some features of tumor cells are better resembled *in vitro* using three-dimensional (3D) cultures instead of conventional cell cultures in flat-bottomed plates with adherent cells to plastic or matrix substrate (106–111). 3D cultures such as organoids and spheroids together with orthotopic animal models of MB can be useful to study the mechanisms of resistance to therapy as well as the interaction with other components of the Tumor microenvironment and immune system (Figure 1). The next chapters will deal with these up-to-date unconventional cultures and animal models in MB.

3.1 *In vitro* models: from conventional to 3D spheroid and organoid models

Like in other human tumors, traditional cultures of cell monolayer remain widely used in research, providing a controlled environment for rapid drug screening and genetic manipulation.

Still, *in vitro* culture conditions can influence both phenotype and cell signaling and drug sensitivity to a large extent. Simplistic conventional, also inappropriately called 2D, monolayer cultures with a single layer of cells attached to treated polystyrene have unnaturally low cell densities, lack cell-cell interaction or cell-peri and extracellular matrix interactions, and lack the exhibition of the brain tumor nutrient gradients or physiological levels of oxygen (112). For instance, it has been shown that some MB cell lines including DAOY (SHH), ONS-76 (SHH), D458 (Group-3), HD-MB03 (Group-3), CHLA-01-MED (Group-4) and CHLA-01R-MED (Group-4) showed the growth and metastatization features of MB tumors of each subgroup they belong only when they were cultured in a 3D hyaluronic acid hydrogels but not when cultured in conventional conditions as adherent cells (113). This would suggest that 3D cultures may be used to study MB behavior and drug sensitivity, mimicking better the *in vivo* physical conditions (113).

Ivanov et al. highlighted the importance of using different 3D culture systems with the relevant tissue architecture and phenotype as well as normal tissues and how the establishment of a collaborative online database linked to distinct cell banks would catalyze preclinical MB research (78, 112–114). The use of 3D spheroid models from cell lines and patient-derived xenografts (PDX) demonstrates having more representative resistance to conventional chemotherapies, e.g. etoposide and cisplatin, in comparison to conventional cultures, and the same 3D experiments were key to identifying hypoxia-induced genes that drive resistance. Brabetz and his co-authors generated PDX-derived 3D organoids retaining genetic and transcriptional heterogeneity of primary tumors that were strategic to demonstrate that group 3 organoids with *MYC* amplification exhibit invasive growth patterns depending on the 3D extracellular matrix (ECM) (such as collagen) and that venetoclax (a *BCL-2* inhibitor) synergizes with chemotherapy, overcoming apoptosis resistance (115, 116). The use of 3D culture assays has become central in research due to their ability to recapitulate key aspects of tumor biology, such as CSC niche enrichment, intra-tumoral heterogeneity preservation, and therapy resistance modeling. Using 3D models, Vinci et al. have identified and demonstrated *MYC*-dependent metabolic vulnerabilities in Group 3, including sensitivity to glutaminase inhibitors (117). In the past decade, many 3D bio-printed brain tumor models were developed for glioblastoma aiming for the recapitulation of TME and developing better drug screening platforms (118, 119). Still, to the best of our knowledge, there are no studies that developed 3D bio-printed constructs for other brain tumors such as MB. (Figure 1).

3.2 *In vivo* models

Similar to other brain malignancies, the study of MB frequently relies on established cell lines, from murine and/or human origin, and PDX models. Models have provided critical insights into tumor biology and therapeutic response. The heterogeneity of MB has been addressed *in vivo* through the development of genetically engineered mouse models (GEMMs), through the orthotopic

implantation of murine cerebellar progenitor cells and by PDX murine models. This section aims to provide a concise and short overview of the MBs *in vivo* models employed in research. It is not intended to serve as a comprehensive account of all available models, but rather to offer a general perspective on the principal systems currently utilized in the field. For more exhaustive information on *in vivo* models applied for MB research, readers are referred to the detailed reviews (120, 121).

GEMMs involve targeted modifications of the murine genome, such as gene knockout (deletion), knock-in (mutagenesis), or transgenic overexpression, enabling the study of tumor biology within an intact immune system and native tissue microenvironment. Unlike transplantation-based systems, GEMMs recapitulate spontaneous tumorigenesis, providing insights into the multistep progression from initiation to malignancy (120, 121). In research, GEMMs have been pivotal in confirming genetic drivers and elucidating the cellular origins of molecular subgroups, particularly SHH and WNT. Despite their high cost, technical complexity, and time-intensive nature, GEMMs remain among the most informative and widely utilized systems in cancer biology (120, 121). This model has been instrumental in validating oncogenic drivers and elucidating the cellular origins of distinct molecular subgroups, particularly SHH and WNT MB. For example, key studies on SHH MB utilized *PTCH1* heterozygous mice (*PTCH1*^{+/-}), which develop tumors at low penetrance (~20%) following loss of the wild-type *PTCH1* allele (122). Subsequent conditional or global knockout models have identified cooperative oncogenic alterations and solidified the granule cell progenitor (GCP) lineage as the cell of origin, with GCP identity being essential for SHH-driven tumorigenesis (123).

In contrast, WNT MB is proposed to arise from dorsal brainstem progenitors derived from the lower rhombic lip, rather than cerebellar compartments. Conditional knock-in models expressing a constitutively active β -catenin variant demonstrated that aberrant WNT pathway activation induces pathological cell accumulation in the brainstem but not the cerebellum, which can be aligned with putative extra-cerebellar origins (124). Additionally, transgenic mouse models overexpressing *NMYC* in cerebellar tissue have further revealed oncogenic versatility by inducing resembling MB in multiple subgroups, such as groups 3, 4, and SHH. This fact highlights the fact that the context-dependent effects of oncogene activation on pathogenesis.

PDX models have gained prominence in oncology research due to their capacity to closely mirror the biological and histopathological characteristics of the primary tumors from which they originate. In the context of MB, PDXs are typically generated by engrafting freshly resected tumor tissue either subcutaneously or within the cerebellar parenchyma-orthotopic engraftment into immunodeficient mice (125). The genetic diversity of recipient mice—covering inbred, outbred, or hybrid strains—does not preclude successful xenotransplantation. Nevertheless, the fact that these animals are immunosuppressed, a condition needed to allow tumor engraftment excluding the rejection in adult mice, represents a restraint in their use as immunotherapies testing (126). PDX models have been

successfully established from all major molecular MB subgroups and have demonstrated stability across multiple passages, although subclonal populations may undergo selection during serial transplantation. We highlight that most of the PDX models have been derived from high-risk cases, suggesting that tumors with more aggressive phenotypes are more amenable to engraft. Also, orthotopic implantation into the cerebellum has been associated with improved engraftment efficiency, particularly for tumors with lower proliferative potential, compared to subcutaneous approaches (127, 128). These types of *in vivo* models have been instrumental in validating key molecular drivers of pathogenesis and serve as versatile platforms for evaluating a range of therapeutic strategies for preclinical assessment of pharmacologic agents, dosing regimens, delivery routes, and combination treatments, but not so key for cell-based and immunotherapies due to animal immunodeficient environments. (Figure 1).

More recently, both MB cell lines and patient-derived cells (from SHH MB, SHH PDX, the Group 3 MB PDX cell line MB-LU-181) have been transplanted into blastula stage of zebrafish embryos leading to orthotopic MB growth (129). The localization to the hindbrain region of transplanted cells was increased by culturing MB cells in neural stem cell-like medium. This model could be used to test the efficacy of SMO inhibitor sonidegib and an active metabolite of cyclophosphamide (129). The model of zebrafish has been also used for group-3 MB cells (130) to identify the subgroup MB cellular origin (131), to mimic the SHH-MB with specific mutations (132), to generate transcription activator-like effector nucleases TALEN-mediated somatic gene inactivation of CDKN2A/B or RB1 tumor suppressor genes (133). Altogether, zebrafish models can well resemble the growth and the aggressiveness of MB in humans to analyze the effects on MB biology due to the genome editing and the presence of specific mutation and/or activation of oncogenes (130–134) (Figure 1).

4 MB immune response and immunotherapy

As defined by the National Cancer Institute, “immunotherapy is a type of cancer treatment that helps your immune system fight cancer.” The immune system interacts with autologous cells, and it is educated for not reacting with self but only with something that is sensed as “foreign” (7–11). MB cells are autologous cells and, by definition, should not have been recognized by the adaptive arm of the immune system (69). Indeed, T lymphocytes should be impaired to eliminate self-cells, as self-reacting T lymphocytes have been deleted during thymic central selection (135, 136). Mistakes in the mechanisms of deletion allow the insurgence of an autoimmune disease if the peripheral tolerance does not work too (135–137). On the other hand, innate cells such as NK cells do not react with self-cells as they bear inhibitory receptors that interacting with self-major histocompatibility (MHC) class I alleles can impair killing of autologous cells. The innate arm of the immune system can kill target cells when tumor cells do not

express MHC or when the activating signals are stronger than the inhibiting ones (138–142). The relevance of T lymphocyte response against MB can be exploited by two main tools: T cell-specific for associated peptide antigen or neoantigen expressed on MB cells presented by antigen-presenting cells (APC) or engineered chimeric antigen receptor (CAR) T cells recognizing surface receptors on MB cells (143). First, we will analyze the immune cells potentially involved in the response to MB namely: NK cells, lymphocytes subsets with functional properties between NK and classical $\alpha\beta$ TCR T cells and finally anti-MB specific $\alpha\beta$ TCR T cells. Second, the targeting of MB surface antigens with immunotherapeutic tools will be considered. Finally, we considered some potential molecular target expressed by the TME which can help to relieve the TME-mediated immunosuppression.

4.1 Innate immune response

It has been reported that NK cells can recognize and kill the MB cells (Figure 2) (160–164). The established cell lines DAOY and D283-Med and the primary cell line 1603-Med from an anaplastic MB can express different levels of ligands for the NK cell activating receptor NKG2D including MICA/B and ULBP3; further, they express nectin-2 and PVR, ligands for the DNAM1 activating receptor, low levels of LFA3/CD58 (a ligand of CD2 antigen express by most lymphocytes) and intercellular adhesion molecule (ICAM) 1, 2 and 3 ligands for the lymphocyte function associated antigen (LFA) 1. More relevantly, the use of specific mAb against NK cell activating receptors such as NKG2D, DNAM1, NKp30 and NKp46 could inhibit the NK cell-mediated cytotoxicity of these cell lines (161). The expression of ligands for NKG2D has been further confirmed both in immunohistochemistry of MB specimens and in cell lines, together with the relevance of NKG2D and HLA-class I molecules in NK cell-mediated recognition of the MB cell line DAOY (162). This finding indicates that several counter-ligands of NK cell-triggering molecules may be considered suitable targets for the elimination of MB cells in patients (162). The adoptive transfer of IL15-activated NK cells can induce a delay in the growth of the MB DAOY cell line in a subcutaneous xenograft mouse model (160), leading to an increase in the OS rate. The infiltration of these xenografts was characterized by NK cells showing expression of several activating receptors and bearing several markers of cytotoxic cells, including perforin, granzyme, tumor necrosis factor (TNF) α , and interferon (IFN) γ . It is well known that NK cells can recognize tumor cell targets independently by the recognition of HLA-I differently from T lymphocytes (141, 142). In this context, it is essential to be better able to define the role of HLA-I expressed on MB cells, as typically NK cells express potent inhibitory receptors for self-HLA-I, leading to blocking of autologous cell killing (141, 142). It has been reported in a series of 10 MB primary tumors the absence of reactivity with anti-HLA-class I antibodies in immunohistochemistry assays (165). This finding has been confirmed on a large series (n=106) of MB, but in addition, it has been shown that high levels of HLA-I are strongly expressed only in

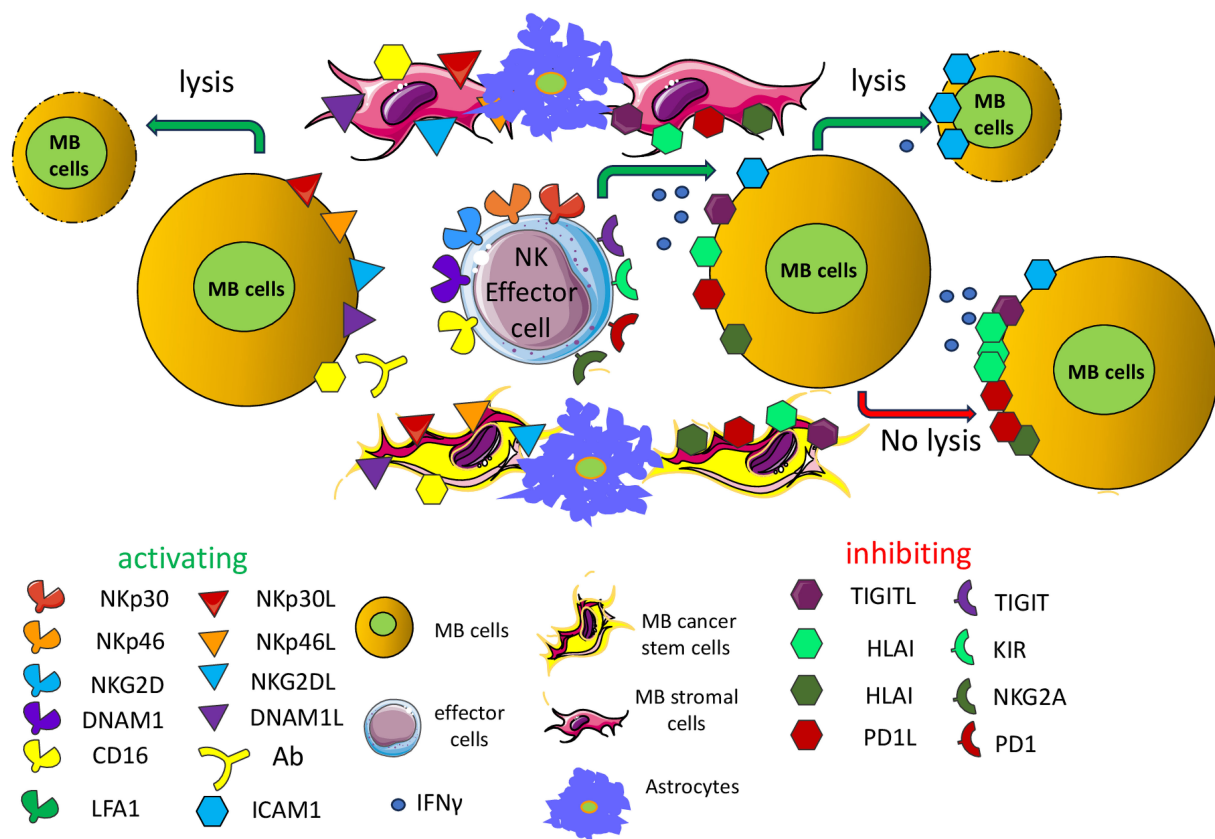


FIGURE 2

Activating and inhibiting receptors expressed on antitumor NK effector cells. Antitumor effector NK cells can express a plethora of receptors, whose engagement through the corresponding ligands expressed on MB cells can deliver an activating (green) or inhibiting signal (red). These ligands may be expressed also by other cellular components of the MB TME including stromal cells, cancer stem cells, endothelial cells and astrocytes. NKp30, NKp46, NKG2D, DNAM1 are some of the main activating receptors typically expressed on NK cells and subsets of T cells (effector cells). Some inhibiting receptors are represented by TIGIT (T cell immunoreceptor with Ig and ITIM domains), KIR (Killer Ig-like inhibitory receptor), NKG2A (killer cell lectin-like receptor subfamily C, member 1) and PD1 (programmed cell death receptor 1). The interaction of these receptors with the corresponding ligands on target cells can activate or inhibit the effector function of NK cells or subsets of T cells. The final outcome is related to the degree and/or affinity of each receptor/ligand interaction. The presence of the ligands on target cells regulates the fate of MB cells. The reported low expression of HLA-I on MB cells could limit the negative signal in self-NK cells of the killing of tumor cells, favoring the activation through the engagement of activating receptors. The production of IFN γ can lead to upregulation of HLA-I, PD1 and ICAM1 exerting opposing effects on NK cell-mediated killing. The TIGIT (inhibitory) and the DNAM1 (activating) receptors recognize the same surface ligands, CD115/PVR and/or CD112/nectin2. The expression on effector cells of the CD16/FC γ R1IIa can trigger cytotoxicity of target cells (antibody dependent cellular cytotoxicity, ADCC) in the presence of an antibody that links the CD16 on effector cells through its FC portion and makes a bridge with target cells by binding the antigen through its Fab component. CD16 is one of the main activating receptors of NK cells. Typically, the ADCC is mediated by antibodies of the γ 1 (IGG1) isotype but not by the γ 4 isotype (see Table 3). In this figure, activating and inhibiting ligands of MB cells are artificially shown on separate target cells, but this is an oversimplification. All these ligands may be present on the same MB cells. It is to note that the expression of the ligands for the various activating receptors is determined either by the reported expression on MB cells using 1- specific anti-ligand antibody; 2- with covering of the activating receptor with anti-receptor antibody leading to a reduction of cytotoxicity of target cells. Only the effects on MB cells are depicted but similar effects may be exerted on the other component of the TME such as stromal cells, astrocytes and MB cancer stem cells.

MB with evident anaplastic features in association with MYC expression (166). A role of HLA-I peptide complexes and the endoplasmic reticulum aminopeptidase (ERAP)1 in the regulation of MB cell killing has been reported (167). However, the increase of cell killing upon blocking of HLA-I recognition by NK inhibitory receptors was faint (161, 167). Furthermore, the degree of killing of the polyclonal NK cell populations against the same target (DAOY cell line) used in these reports is markedly different (161, 167). This variability would indicate a variable expression of the activating receptors expressed on NK cells. Thus, the killing of MB cells is the

result among positive and negative signals transduced by activating or inhibiting receptors upon binding with the corresponding ligands on MB cells (Figure 2). It is of note that an evident cytotoxic effect was also detected by injecting ^{19}F -labeled NK cells intratumorally or contralaterally to an orthotopic MB tumor in immunodeficient mice (168). Further, it is of note that NK cells are more infiltrated in patients with a better prognosis (169). Altogether, these findings further support the possibility of using adoptive NK cell transfer as a useful tool to eliminate MB cells and control tumor expansion.

TABLE 3 Monoclonal antibodies therapies targeted to some receptors expressed on MB cells and associated tumor microenvironment.

Target molecule	Antibody name	Type of antibody	Functional effect on target cells	Functional effect on immune cells	Tumor target	Reference
GD2	¹³¹ I-3F8	IGG3	Killing target cell	Not done	Medulloblastoma patients	(144)
B7H3	8H9 humanized	IGG1	Blocking cell proliferation	ADCC	Neuroblastoma LAN-1 cell line	(145)
B7H3	MGA271 Fully human	IGG1	Blocking cell proliferation	ADCC	ND Renal and bladder carcinoma	(146)
B7H3	MJ18 rat	IGG1	Blocking cell proliferation	CD8+cell infiltration and killing target cell	Pancreatic cancer	(147)
B7H3xCD3 nanorings	8H9 scFv	bispecific	Killing target cell Independent from HLA-I	Increase T lymphocytes memory phenotype	medulloblastoma	(148)
B7H3xCD16 Bike	8H9 scFv anti-CD16 scFv 3G8	bispecific	Killing target cell Altered glucose metabolism	ADCC NK cell killing	Non-small cell lung cancer	(149)
B7H3x4-1BB Bike	mAb B5 murine mAb ID8 murine	bispecific	Blocking cell proliferation	Costimulation Upregulation activation antigens, IFN γ release CD8	Murine models CT26 MC38 B16-F10	(150)
B7H3 duocarmycin ADC	PRCA157 mAb MGCO18	IGG1	Killing target cell	Not done	PDX of head and neck, prostate, breast carcinoma Toxicity tests Cynomolgus	(151)
B7H3 DNA Topoisomerase I ADC	DS-7300a	IGG1	Killing tumor cell	Not done	PDX small lung cancer, non-small cell lung cancer, head and neck. bladder	(152)
B7H3 Monomethyl auristatin (MMA) E- and pyrrolobenzodiazepine ADCs	m276 fully humanized	IGG1	MMAE kills target cell Pyrrolobenzodiazepine kills medulloblastoma and endothelial cells	Not done	Murine models B16, MC38, Py230), 4T1 DLD-1, HCT-116, KM12, KM12SM, MDA-231, HT29, DMS-273 SW620, UACC CB17 OVCAR3	(153)
B7H3-DM1 ADC	14A2	Not determined	Killing target cells	Not done	Craniopharyngioma organoids	(154)
EGFR	Cetuximab + neuromedin B receptor antagonist BIM-23127	IGG1	Inhibition DAOY proliferation	ADCC	Medulloblastoma models	(155)
HER2	Trastuzumab Deruxtecan	IGG1	Killing target cells	Not done	Reduction brain metastasis of refractory breast carcinoma	(156)
PDPN	NZ-1 NZ-1-(scdsFv)-PE38KDEL	IGG1k	Killing target cells	Not done	Glioblastoma	(157)
CD47	Hu5F9-G4	IGG4	Blocking interaction CD47 and SIRP1alpha Increase phagocytosis	No ADCC	Primary, metastatic, recurrent glioblastoma	(158)
VEGF-A	Bevacizumab combo with Temozolomide irinotecan	IGG1k	antiangiogenic	Not done	Medulloblastoma patients	(159)

GD2, disialoganglioside, B7H3, B7 homolog 3; DM1, mertansine/emtansine; EGFR, epidermal growth factor receptor; HER2, human epidermal growth factor receptor 2; PDPN, podoplanin; VEGF-A, vascular endothelial growth factor-A; ADC, antibody drug conjugate; ADCC, antibody dependent cellular cytotoxicity.

4.2 Effector lymphocyte with phenotypic and functional properties between NK and T cells and recognition of MB cells

The relevance of some unconventional T cells, such as invariant NK-like T cells, V δ 2 ($\gamma\delta$ TCR) T cells, CD1-restricted T cells, mucosal associated invariant T cells (MAIT) and other NK-like T cells, has not been studied in detail with MB cell lines and MB preclinical mouse models (65, 170–175). It has been reported that MB do not express CD1d, besides HLA-I, suggesting a molecular mechanism involved in MB escape from recognition by unconventional T cells (173). Furthermore, the molecular MB SHH subgroup can express elevated levels of CD1d mRNA compared to other MB subgroups (173). Importantly, CD1d-positive MB cells (DAOY and MED8A cell lines) presented glycolipid antigens (α -galactosylceramide) to NK T cells, inducing the production of cytokines such as IFN γ and IL4. Along this line, NKT cells induced remission of orthotopic injected MB xenografts of DAOY MB cells. In addition, the NKT cells in MB patients were present and functional, suggesting the possibility of using these NKT cells to kill MB cells at least in a subset of patients (173). More recently, it has been reported that $\gamma\delta$ T cells can infiltrate the MB, and in particular the group 4 MB, but there was not a significant correlation between the infiltration of $\gamma\delta$ T cells and patients OS. Furthermore, EphA2-expressing MB cells trigger V γ 9V δ 2T cell activation while amino bisphosphonates sensitize MB cells but not healthy neuronal cells to V γ 9V δ 2T cell lysis (175). This would indicate that $\gamma\delta$ T cells can be a useful tool to target MB cells. In this context, we reported that antibodies to tumor antigens conjugated with the aminobisphosphonate zoledronic acid can kill efficiently tumor and stromal cells (176). Thus, we can hypothesize that the use of antibodies to MB antigens conjugated with aminobisphosphonates can be effective in eliminating MB cells, as shown for colorectal carcinoma (176). Finally, it has been reported that six MB cell lines (DAOY, ONS-76, UW228, D341, D425 and D283) can be a good target for protein tyrosine kinase (PTK)7-targeted CAR $\gamma\delta$ T cells against MB (177). Altogether, these findings support the idea that besides conventional T cells and NK cells, other effector lymphocytes can be an immunotherapeutic tool to eliminate MB cells (Figure 3).

4.3 Tumor antigens of MB

The antigen-specific immune response could be considered one of the cellular-mediated mechanisms by which MB cells can be eliminated. Briefly, an adaptive immune response should be elicited against neoantigens and/or tumor-associated antigens (TAA) (178). The presence of neoantigens and TAA is also necessary for awaking exhausted tumor-infiltrating lymphocytes (TIL) upon treatment with immune checkpoint inhibitors (ICI) such as anti-CTLA4 or anti-PD1/PDL1 antibodies (179–183). Importantly, the possibility of evoking an adaptive immune response is one of the requisites to plan ICI therapy, and the identification of neoantigens or TAA together with antigen-specific TIL justify the cost of the treatment

and predict, at least in principle, the response (183). Conceivably, more are the neoantigens and TAA present together with their immunogenic potency; more probable is that the immune system will control the tumor growth. As reported above, the patients can be subdivided on the basis of subgroup-specific genetic alterations, and more recently it has been shown that it is possible to identify potential tumor antigens, possibly suggesting the development of antigen-directed cellular therapies for MB (184). In detail, it has been developed an algorithm predicting antigens able to trigger an immune response in the context of the patients' HLA-class I and class II. This point is relevant as it is well-known that the immune response needs both the help mediated by the recognition of CD4+ T cells of MHC-II-restricted peptides and the lytic activity of CD8+ T cells restricted to MHC-I antigens to eliminate tumor cells (185) (Figure 4). The antigens considered include neoantigens, TAA and fusion antigens (184). This pipeline has been named Open Reading frame Antigen aNalysis (ORAN) and it uses the gene expression data for identifying different classes of antigens (184). Importantly, the algorithm can predict putative peptide antigens that indeed trigger an efficient antitumor immune response after vaccination in preclinical glioblastoma models (186). It is of note that ORAN identified that only a subset of the genes mutated in MB could be immunogenic, and this subset was present in about 80% of patients; also, 44% of patients expressed three or more neoantigens. This would indicate that not all the MB patients may benefit from neoantigen-based immunotherapy. About 90% of MB expressed at least one TAA, and a quite high proportion of these patients expressed three TAA. Noteworthy, the TAA prediction showed a strong and better concordance with respect to neoantigens with proteomic data. The overall survival (OS) and the progression-free survival (PFS) of patients in the Group 3 of MB well correlate with the presence of MHC-I and MHC-II peptides of TAA. This work analyzed by RNA-seq 170 cases of MB of which 18 WNT, 46 SHH, 41 Group 3, and 65 Group 4, and it used a data set of microarray technology of 763 MB (64, 184). Moreover, the immune landscape and the pathways for antigen processing and presentation in tumor cells have been studied using up-to-date deconvolution computational methods. This analysis has given some insights on the possibility of identifying the so-called “recurring antigen”. It is conceivable that to design an MB vaccine potentially functional in any patient independently of the molecular subgroup and stage of development, a good immunogenic antigen should be identified. It is of note that MB expressed several private and immunogenic antigens. Except for the SHH-MB subgroup, several TAA were usually expressed. Furthermore, some cancer testis and neurodevelopmental antigens were expressed throughout all the subgroups. In detail, neoantigens from oncogenic driver mutations including *CTNNB1*, *DDX3X*, and *SMARCA4* and TAA such as *NEUROG1* and *PIK3R3* could be considered as potential therapeutic targets for immunotherapy. None of these identified potential targets have been validated in experiments showing that it is possible to trigger an immune response upon the use of a personalized vaccine. However, the same ORAN pipeline applied to the murine glioma GL261 cell line identified, after appropriate selection, 192 putative neoantigens and 37 TAA with a predictive

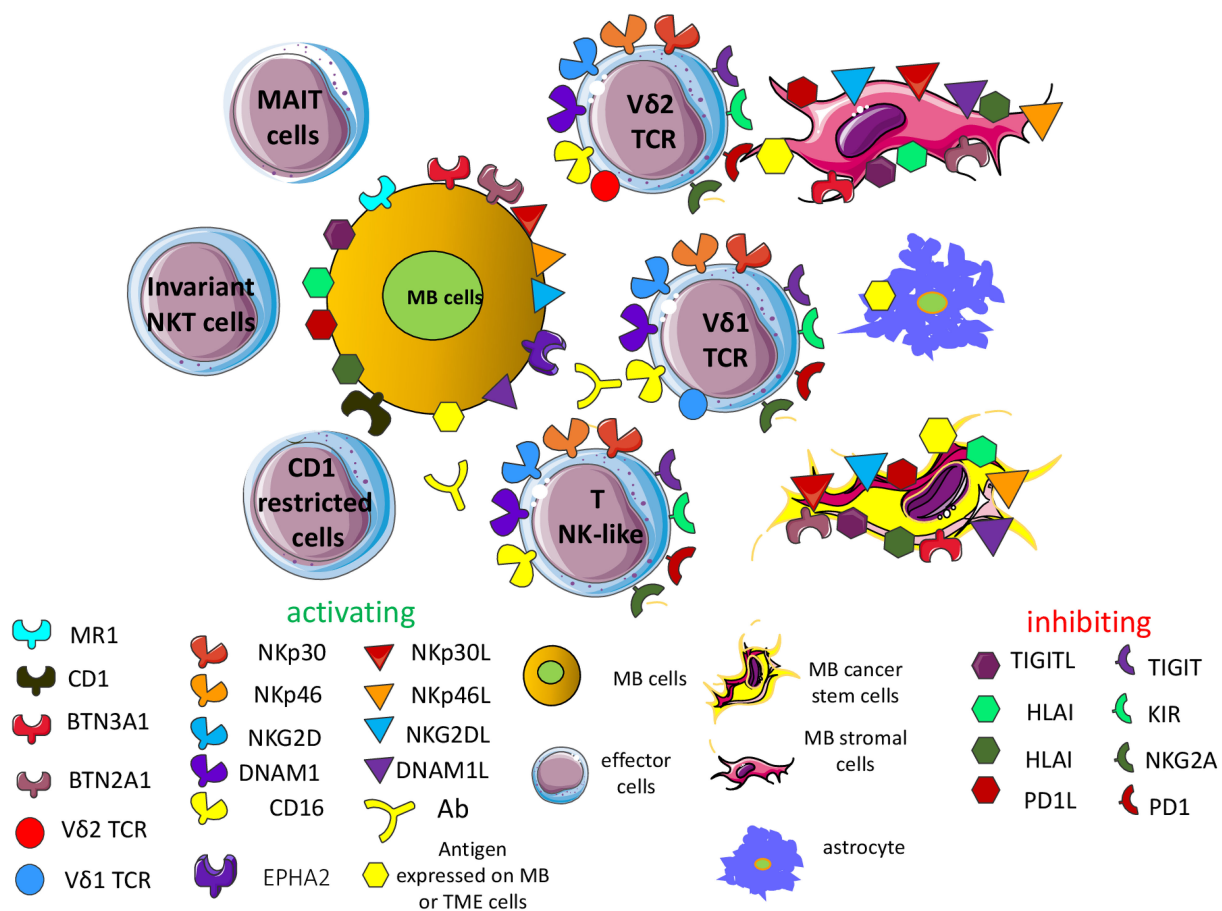


FIGURE 3

Additional effector cells targeting the MB cells. Several effector cell subsets can recognize the MB cells on the basis of the expression of some of the activating receptors expressed typically by NK cells. These subsets comprise invariant NK-like T cells, Vδ2 and Vδ1 (γδ TCR) T cells, CD1-restricted T cells, mucosal associated invariant T cells (MAIT) and other NK-like T cells. Typically, these cell subsets can show functional features of NK cells, such as killing of tumor target cells independently of the recognition of HLA-I antigens. Some of them express activating and inhibitory receptors like NK cells, and the final outcome of their engagement is similar to what is observed in NK cells. In detail, the Vδ2T cells can trigger TCR-mediated activation through the recognition of small phosphate antigens (pAg, such as isopentenyl pyrophosphate, IPP) presented by the butyrophilin members, including BTN3A1 and BTN2A1. pAg are derived from intermediates of the cholesterol synthesis of mevalonate pathway. Aminobisphosphonate such as zoledronic acid can increase the presentation of small pAg on tumor MB cells while Vδ1T cells can recognize the EPHA2 leading to the killing of MB. MAIT cells can recognize the major histocompatibility complex class I-related gene protein (MR1) presenting intermediates of riboflavin synthesis. On the other hand, CD1-restricted T cells can recognize lipid antigens instead of peptide antigens, like the majority of αβ TCR T cells. Lipid antigens can derive from endogenous or foreign origins. Overall, these cell subsets can recognize tumor cells if they express the corresponding counter-ligands (see also the [Supplementary Figures 1, 2](#)).

immunogenic effect (186). Importantly, it has been developed a gene enrichment platform for the production of tumor open reading frames that are unique (TOFU) specific for tumor antigens. This platform allowed the generation of mRNA by *in vitro* transcription for immunogenic tumor antigens without the need of providing large tissue samples from patients to obtain these mRNAs, overcoming this bottleneck to produce vaccines. Noteworthy, the TOFU mRNA vaccine was efficient to evoke an antitumor response in a murine model of glioma when loaded into dendritic cells in combination with immune-checkpoint inhibitors (ICI) and/or adoptive cell therapies (72, 187).

It is clear that these novel approaches in identifying the MB neoantigens and TAA support the idea that immunotherapy might be feasible and efficient. Previous observations in MB on the immunogenic property of the fusion proteins composed by the

enhancer of polycomb homolog 2 (EPC2) and GULP PTB domain containing engulfment adaptor 1 (GULP1) have shown that this protein can trigger the release of IFNγ by CD8⁺ T cells (188). Also, the finding that a CD8-specific T-cell response can be elicited in neoepitopes derived from Histidine Ammonia-Lyase (HAL), Neuraminidase 2 (NEU2), Proprotein Convertase Subtilisin (PCSK9), Programmed Cell Death 10 (PDCD10), Supravillin (SVIL) and tRNA Splicing Endonuclease Subunit 54 (TSEN54) variants is in line with the notion that specific T cell-mediated immunotherapy can be applied for MB (189). Finally, the proteogenomic approach allowed the identification of neoantigens from MB tumors with a low mutational burden and a limited amount of tissue (189). It is of note that T cells with different T cell antigen receptors (TCR) and producing several antitumor cytokines can be obtained using these neoantigens. This finding further

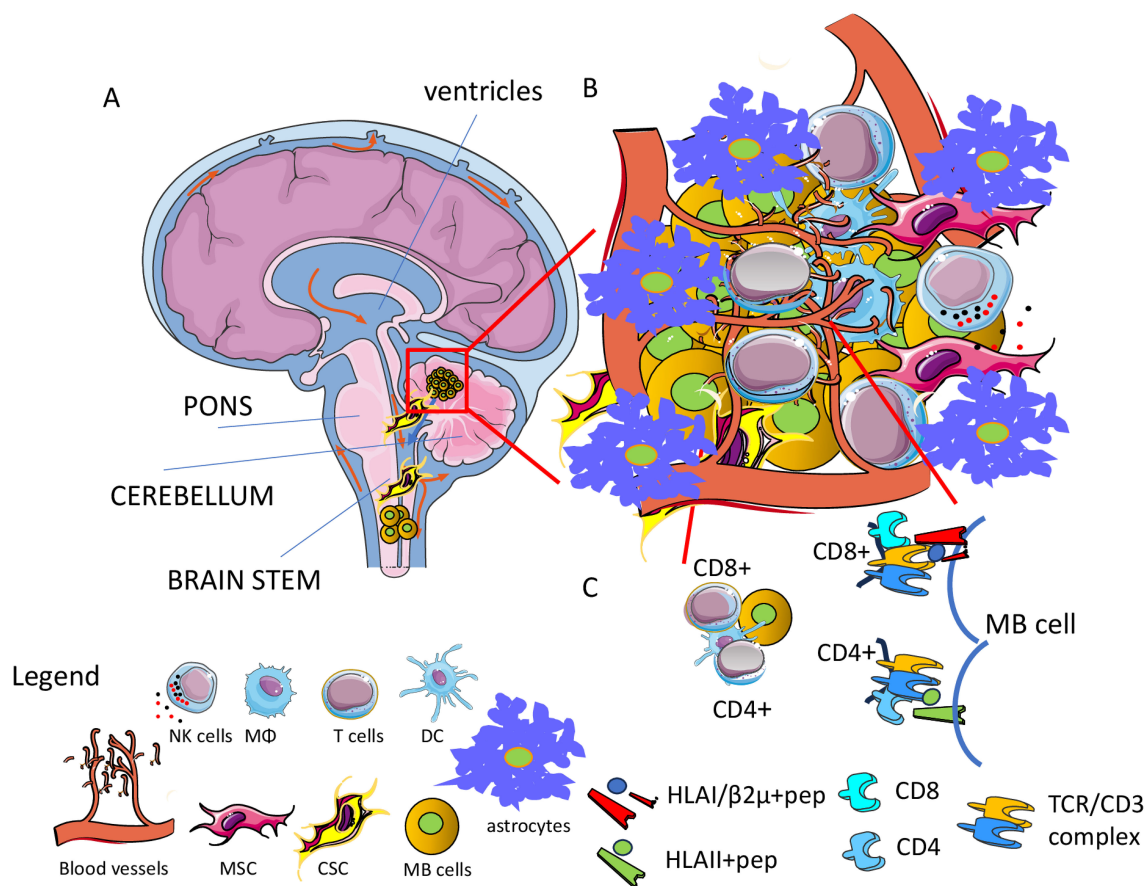


FIGURE 4

Tumor microenvironment (TME) in MB. **(A)** MB is a tumor usually localized in the cerebellum leading to metastasis in the spinal cord. **(B)** The TME is characterized by the presence of several cells of the immune system as macrophages (MΦ), dendritic cells (DC), and T cells together with mesenchymal stromal cells (MSC) and astrocytes (As). MB cancer stem cells (MB CSC) can be considered a major target of therapy to eradicate the tumor. Typically, the TME leads to the impairment of the immune response against tumor cells. Angiogenesis is an essential process that favors the growth of this tumor and possible spreading to other regions of the brain. The brain-blood barrier is a key anatomical and functional structure involved in the regulation of infiltration of antitumor effector cells, as well as tumor spreading and drug effectiveness. **(C)** Adaptive specific T cell immune response can be elicited against the MB cells and MB cell antigens potentially presented to either CD4⁺ or CD8⁺ T cells can be identified mainly by OMICS analysis.

supports the more recent publications (184, 186, 189) that it is possible to discover immunogenic-specific peptides that are the basis for generating appropriate anti-MB vaccines.

4.4 Targeting molecules expressed by MB: antibodies and CAR cells

Beside targeting tumor-specific antigens, immunotherapy can target receptors present on MB cells but widely expressed on other cell types (144, 190–207). The first point to consider for an efficient therapeutic effect without too many side effects is the expression of appropriate target molecules at the cell surface of MB cells (196–198). The ideal target should be expressed mainly, if not exclusively, on MB cells but not on healthy cells. The second critical point is the degree of the immune response; it should be enough strong to control and eliminate tumor cells, but sufficiently milder not to damage large amounts of healthy cells. This second point is much

more important, more relevant: it is the function of healthy cells as a potential target of therapy. Proved target receptors on MB cells include HER2, B7H3 (CD276), EphA2 and GD2 (144, 196, 197, 199–203). These antigens can be targeted mainly by two therapeutic tools: monoclonal antibodies (mAb) (Figure 5) and CAR cells (Figure 6).

Antibodies represent a key therapeutic tool for several kinds of cancers, including tumors of the nervous system (204, 205) and CAR cells could be considered one of the more recent tools developed from classical mAb (206–209). As for the native mAb, CAR shows a component of the engineered chimeric receptor that recognizes the target molecule at the cell surface of MB cells (202–206). The antigen recognition domain of the CAR molecule is typically derived from the variable regions of a mAb as a single-chain variable fragment (scFv) recognizing a tumor expressed antigen. The main difference consists in the molecular and cellular mechanisms of the therapeutic effects (210, 211). Antibodies can affect receptor-ligand interactions and signaling,

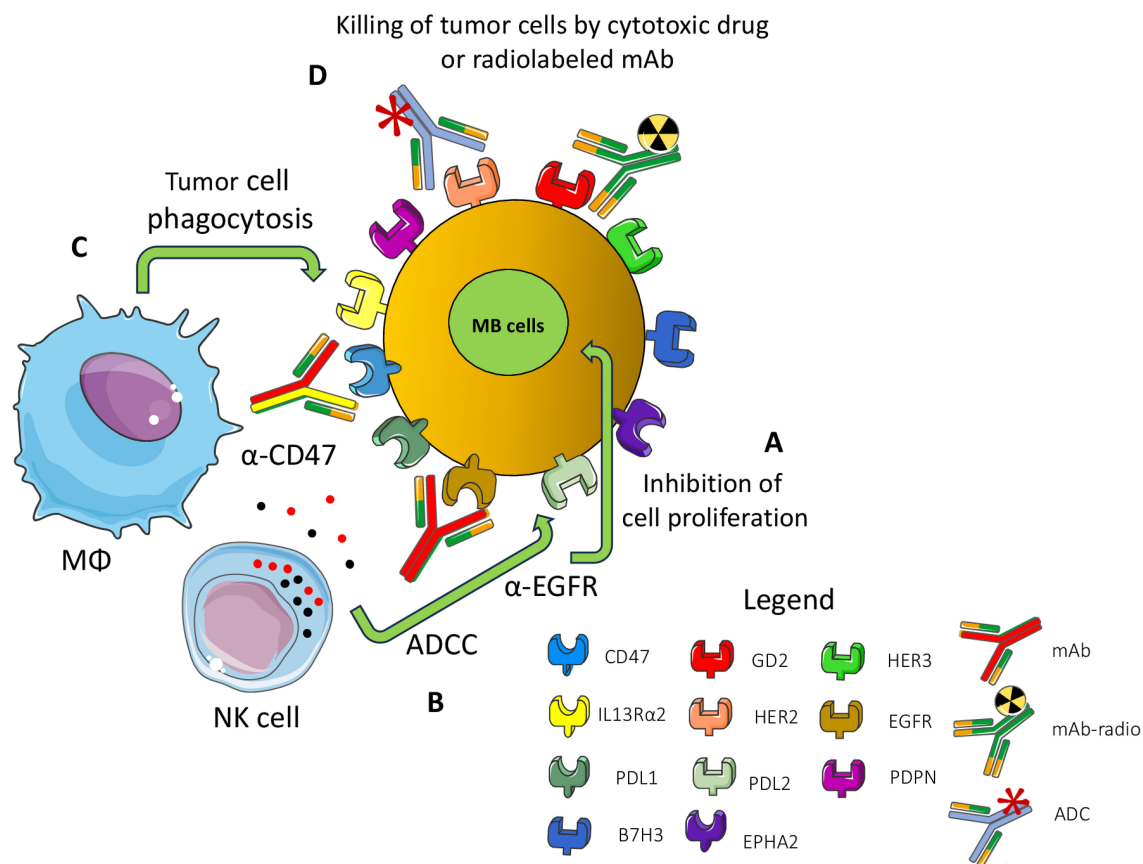


FIGURE 5

Surface MB receptors as targets for immunotherapy with antibodies. Several surface-expressed receptors of MB cells can be a target for therapy with monoclonal antibodies (mAb), radiolabeled-mAb (radio-mAb) or antibody drug conjugates with cytotoxic molecules (ADC). Some representative molecules are shown. EGFR, HER2 and HER3 are members of the epidermal growth factor receptor family; PDPN: podoplanin, PDL1/2: ligands for PD1, GD2: disialoganglioside, EPHA2 ephrin receptor A2, IL3 receptor alpha2. Not necessarily all these molecules are expressed at the cell surface of the same MB tumor cell. The antibodies directed against these molecules can inhibit (A) the proliferation of MB cells by blocking the binding with the natural ligand as growth factors (e.g., anti-EGFR Ab). (B) The Ab can trigger antibody-dependent cellular cytotoxicity (ADCC) by lymphocytes (such as anti-EGFR mAb), monocyte/macrophages and other innate cells or complement dependent cytotoxicity. (C) Antibodies can block the "do not eat me" signal (e.g., CD47) leading to phagocytosis or (D) they deliver cytotoxic drugs or radio-isotopes leading to the killing of tumor cells.

as well as trigger complement- and/or antibody-dependent cellular cytotoxicity (CDC and ADCC) and cytokine release (212). On the other hand, CAR cells, upon the interaction of the CAR molecule, deliver an activating signal that leads to the killing of the target cells (69). Also, CAR cells produce and/or release cytokines typical for the type of cell in which the CAR has been transduced (69). CAR molecules can be transduced into classical CD4⁺ or CD8⁺αβ T cells, γδ T cells, innate cells including NK cells, monocyte/macrophages (Mo/MΦ) and *in vitro* assays show that both mAb and CAR cells are efficient tools to eliminate MB cells (213–215). The main matter with both antibodies and CAR cells is the tumor localization of the antibody and the effector cells (216, 217). This could be considered the key factor that distinguishes the strong efficacy reported for CAR cells in hematological malignancies, compared to the disappointing results found for solid tumors (217–220).

4.4.1 Targeting of GD2 in MB

The GD2 is a disialoganglioside expressed during fetal development and by several tumors, but not in normal adult tissues

(197). Both mAb and CAR-T cells against GD2 are enrolling patients for the treatment of different brain tumors, including MB (196, 209, 220). Recently, it has been reported in an orthotopic MB murine model that CAR-GD2.CD28.4-1BBζ (CAR.GD2)-T construct, including the suicide gene inducible caspase-9 can control tumor growth and prolong the OS of treated mice. In addition, the use of the drug AP1903 induced the dimerization of caspase-9 leading to apoptosis of peripheral blood circulating and brain tumor-infiltrating CAR-T cells. These findings indicate the possibility to efficiently eliminate tumors and limit the side effects due to antitumor effector cells. Importantly, the *in vitro* pretreatment with tazemetostat, a first-class inhibitor of activating enhancer of zeste homolog 2 (EZH2), can upregulate the GD2 expression on GD2^{dim} MB cells (196). This upregulation of GD2 was sufficient to sensitize the MB cells to CAR-GD2 T cell-mediated cytotoxicity. A clinical trial of phase I/II is ongoing (NCT05298995) to determine the safety and effectiveness of CAR-GD2 T cell therapy in high-risk patients (196).

Furthermore, the preclinical evidence of treating a genetically engineered mouse model of MB with the ultra-high dose rate

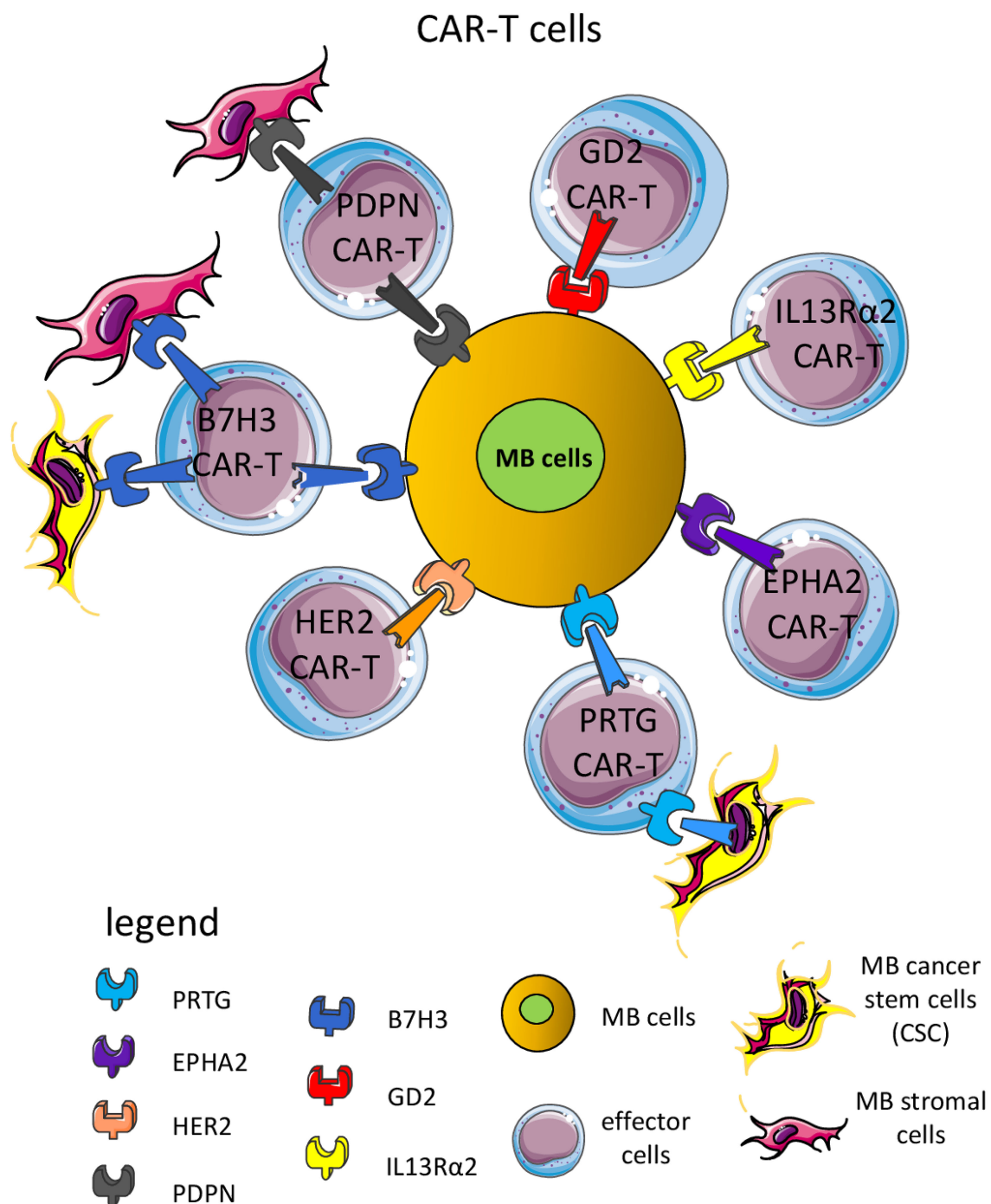


FIGURE 6

CAR-T cells are a suitable means to kill MB cells and other cellular components of the TME. Several CAR-T cells have been used in preclinical murine models and some clinical trials to target surface molecules expressed by MB cells, including B7H3, GD2 (disialoganglioside), EPHA2 (ephrin A2 receptor), HER2 (human epidermal growth receptor 2), PRTG (protogenin), PDPN (podoplanin) and IL13Rα2 (interleukin 13 receptor α2). Importantly, some CAR-T cells can recognize not only MB cells but also components of the TME, such as endothelial cells, stromal cells, and/or cancer stem cells (CSC). The majority of CAR-T cells can express CD4 or CD8 antigens. Usually, CD8⁺ CAR-T cells can recognize MB cells expressing the antigen recognized by the scFv portion leading to the signal transduction through the intracellular component of the CAR-T molecule. This elicited the release of perforin and granzyme and consequent killing of the MB cell. CAR-T can produce proinflammatory cytokines (IFNγ and TNFα) with potent antitumor effects.

radiotherapy (FLASH-radiotherapy (RT)) in association with CAR-GD2 T cells has demonstrated that the immunosuppressive MB TME can be reversed to a pro-inflammatory one giving optimal anti-MB responses (208). In detail, the FLASH radiation delivered in milliseconds can abrogate the oxidation of the lipids, reducing the activity of peroxisome proliferator-activated receptor- γ (PPAR γ). This is in contrast with the generation of reactive oxygen species triggered by the standard radiation, at a lower dose but for a longer time than FLASH-RT, leading to the PPAR γ activation and consequent lipid peroxidation. These events hit mainly the macrophages present in the TME, together with a reduction of arginase 1 expression. In summary, the FLASH radiation reprograms the macrophages from an immunosuppressive to a pro-inflammatory behavior favoring the CAR-GD2 T cell infiltration and MB cell killing (208). The fact that preclinical studies suggest that FLASH-RT induces fewer side effects on healthy tissues than conventional (CV) RT would suggest that FLASH-RT could modify the TME and eventually the clinical outcome of disease.

4.4.2 HER2, EPHA2 and IL13 receptor $\alpha 2$ as therapeutic targets of MB

The systematic characterization of the tumor of the CNS identified HER2, HER3, NECTIN4, TROP2, CLDN6, CLDN18.2, and CD276/B7H3 proteins as potential therapeutic targets (Figures 3, 4). Also, EPHA2 and IL13 receptor $\alpha 2$ are well expressed in some MB (221) and it has been reported that several cell lines as well as a consistent group of primary MB cells can express HER2 and other members of the EGFR family (156, 201, 207, 214, 215, 218, 221–233). Other molecular targets expressed by MB will be considered later in chapter 4 of this review in the context of the targeting of MB and TME. The HER2 and HER3 can be targeted by antibodies; antibody drug conjugates and CAR-T cells have been reported or present on the market (156, 229). It has been reported in different animal models that the treatment with the HER2-BBz-CAR T cells effectively clears MB tumors (227). Orthotopically implantation in the posterior fossa of NOD.Cg-Prkdc^{scid} Il2rg^{tm1Wjl}/SzJ (NOD scid gamma deficient, NSG) mice of the DAOY or D283-Med cells led to the generation of tumors. These tumors were efficiently treated with regional or intravenous HER2-BBz-CAR T cells.

This effect was further confirmed in non-human primates (Rhesus macaques, *Macaca mulatta*, NHP). It is to note that the dose necessary for regional delivery was a log lower for locoregional vs. intravenous delivery. Also, no systemic toxicity was detected in NHP after intraventricular delivery of autologous HER2-BBz-CAR T cells. It is of interest that the HER2-CAR-T cells contained the CD3 ζ and 4-1BB signaling motifs and showed robust anti-MB activity, indicating this kind of CAR-T cells can induce a complete and long-lasting regression of established tumors when administered regionally (227). This locoregional delivery of CAR-T cells with a medium-length CAR spacer increases the therapeutic efficacy of HER2-CAR T cells in an orthotopic xenograft model with the cell lines D283-Med and Med411FH, but not against the HER2-negative D431 cell line (234). Indeed, it appears that the cytotoxic

effect of HER2-CAR-T cells with a short spacer was not evident on HER2⁺ MB cell lines. This would indicate that the length of the spacer in the case of recognition of HER2 antigen on MB cell lines is a key point to consider. Furthermore, some patients suffering from other brain tumors (ependymoma or anaplastic astrocytoma, of the BarinChild-01 NCT03500991 clinical study) did not show dose-limiting toxicity after infusion of the HER2-CAR-T cells via CNS catheter into either the tumor cavity or the ventricular system.

This suggests the feasibility of repeated dosing regimens and well-tolerated of intra-CNS HER2-CAR-T cells delivery in young patients, supporting the notion that this way of administration of CAR-T cells could be adopted in MB as well. This idea is further supported by additional experimental evidence obtained using locoregional therapy with other CAR-T cell models (221). In fact, it has been validated the intrathecal delivery of a trivalent EPHA2, interleukin 13 receptor $\alpha 2$, besides HER2, CAR-T cell against primary, metastatic and recurrent group 3 MB xenografts in mouse models. Furthermore, these CAR-T cells alone or in coo with azacytidine is an efficient therapeutic regimen mouse model with multiple metastases of this highly aggressive MB, providing the rationale for application of the delivery of these types of CAR-T cells intracranially in humans (221). EPHA2, ephrin receptor A2, plays a key role in cancer development and its expression shows association with poor prognosis, elevated metastatic potential, and reduced survival of tumor patients (200). Also, the interleukin 13 receptor $\alpha 2$, the receptor for the anti-inflammatory cytokine IL13, is overexpressed in several brain tumors, playing a role in invasion and metastasis (235, 236). Overall, the three targets considered using the trivalent CAR-T cells were well expressed on the different stages of development of the MB of group 3. Conceivably, the definition of CAR-T cells to use for patient treatment should satisfy the condition of expression of the molecular targets through the primary, metastatic and recurrent MB. This condition would allow the response against the relapse of the MB.

4.4.3 B7H3 inhibitory immune receptor targeting MB

The B7 homolog 3 (CD276) is a transmembrane molecule expressed in several types of cancers where it functions as an immune checkpoint receptor, and it can be targeted efficiently by both CAR-T cells and mAb (237–241). In MB, it can be involved in the angiogenesis migration, invasion (see the 4.5.1 chapter of this review), as well as MB escape from the immune system (205, 242–246). The counter receptor expressed on immune cells of B7H3 has not been identified yet, but it is conceivable that activated CD4⁺ and CD8⁺ T cells express a receptor interacting with APC cells or tumor cells through B7H3 and this interaction leads to inhibition of lymphocyte functions such as tumor cell killing and cytokine production (247–249). Four putative candidates have been suggested, including the triggering receptor expressed on myeloid cells (TREM)-like transcript 2 (TLT-2), interleukin-20 receptor subunit α (IL20RA), phospholipase A2 receptor 1 (PLA2R1), angio-associated migratory cell protein (AAMP) and possibly other molecules (249–254).

4.5 Targeting the MB tumor microenvironment

The features of the tumor microenvironment (TME) are relevant for the growth and diffusion of several cancers, including MB (255). The knowledge of the features of MB during the interaction with the components of the TME could be considered as a therapeutic target in order to enhance the immune response to MB together with the impairment of the growth and spreading of the MB itself (255). This targeting can either reduce, or even convert, the immunosuppressive TME to immunostimulatory. The characteristics of the MB TME have been described in detail in several reviews elsewhere (255). Herein, we will point out in evidence the relevance of some components of the TME, such as the angiogenic factors, the role of podoplanin (PDPN) in MB spreading, “do not eat me” signals, and the interaction between the astrocytes and MB cells (Figure 5). This is to show some of the key cellular and molecular players against which it is conceivable to use old and new immunotherapeutic tools as antibodies or CAR-T cells to further improve the outcome of MB patients.

4.5.1 Angiogenesis and novel factors involved in the regulation of MB spreading

The TME is responsible for the presence of several growth factors that can allow the generation of new blood vessels, such as vascular endothelial growth factor (VEGF) that in turn are essential for the growth and possible spreading of cancer cells (237, 238). The limitation of tumor angiogenesis may help the efficacy of immunotherapy by limiting the growth of tumor cells. For example, the neo-angiogenesis in MB is associated with the most aggressive Group 3 (256). The level of mRNA coding for VEGF-A was markedly increased in this subgroup compared to the other molecular subgroups, correlating negatively with the OS of patients. Furthermore, in rat models, the increased vascularity was associated with less survival. Group 3 of MB is strongly associated with the amplification of MYC and this amplification is linked to the expression of VEGF-A in several solid tumors, including colon rectal carcinomas, breast cancer and gliomas (257–261). This is not surprising, as MYC is involved in the regulation of many genes (262). By consequence, the targeting of angiogenesis is possible by interfering with MYC transcription and molecular target of rapamycin (mTOR) translation with small molecule inhibitors demonstrating synergistic antitumoral effects against MYC-dependent driven MB both *in vitro* and *in vivo* models (263). Also, MB cells express several factors responsible for angiogenesis besides VEGFA, such as VEGFB, VEGFC, VEGF189, VEGF165, VEGF121, angiopoietin (Ang)1, Ang2, transforming growth factor (TGF) α , and basic fibroblast growth factor (bFGF) (264, 265).

The antiangiogenic therapy using the anti-VEGFA mAb bevacizumab did not lead to consistent improvements of OS and PFS, but increased its tolerability together with irinotecan (plus or not with temozolomide) (266). Similarly, the association of intravenous bevacizumab, intraventricular therapy and oral etoposide and cytarabine alternate to oral administration of thalidomide, fenofibrate, and celecoxib was well-tolerated and

better responses (although limited in time, about six months) have been detected (267). However, these results are far to be considered an advancement in treatment. It is evident that the complex interactions among these factors and the possibility that different portions of the tumor mass can express different factors do not allow a precise targeting of vascularization of the MB. Furthermore, the recent finding that the VEGFC may negatively regulate the metastatic properties of MB would suggest that not all the factors involved show a pro-tumor effect (102). In fact, VEGFC is involved in lymphangiogenesis, and it has been shown that VEGFC can decrease proliferation and migration of MB cells, inhibiting the formation of pseudo-vessels *in vitro*. Further, irradiation of resistant MB cells with strong expression of VEGFC inhibits the formation of vessel-like *in vitro*, and these irradiated cells generated smaller tumors in nude mice (98). In detail, bioinformatic analysis of several databases of MB suggested that the lower the level of VEGFC expression in the WNT MB group, the worse the outcome of patients. While it was detected the opposite analyzing the SHH, 3 and 4 subgroups in which high levels of VEGFC corresponded to a worse prognosis. This finding would suggest the dual function of VEGFC in MB. In addition, using some *in vitro* models of MB, some experimental evidence supports that the overexpression of VEGFC/VEGFC receptor axis is not only associated with lymphangiogenesis but exerts a beneficial effect in pediatric MB. This finding is based on the use of few cell lines compared to the large array of cell lines present, indicating that these results should be considered with attention (102). It has been shown that the irradiation of DAOY and HDMB03 cell lines increases their epithelial phenotype compared to mesenchymal phenotype, reducing the ability to disseminate like in glioblastoma (268). This would imply that VEGFC-reducing proliferation/migration of MB cells could keep the tumor in a condition more prone to being attacked by antitumor immune cells. This would be typical of MB of the WNT group, while in group 3 the excess of VEGFC would lead to a too strong generation of lymphatic vessels and consequently to metastasis. It is clear that these findings should be taken cautiously because they have been demonstrated with some MB cell lines. However, they can be the explanation of the role of the immune system in checking MB growth. Furthermore, it appears that the expression of VEGFC could be essential to favor immune response against MB at least in the early stages of development of the tumor. These findings would suggest that to plan efficient therapies for MB, combo therapies to TME and triggering of immune response are necessary. Also, it has been reported that B7H3 expression levels are relevant in promoting angiogenesis; this angiogenesis can be inhibited by miR-29 overexpression, leading to a downregulation of B7H3 (242). Thus, the targeting of B7H3 can hit MB cells and MB-associated angiogenesis.

4.5.2 Podoplanin as a potential target in MB

The role of PDPN in brain cancers has been reviewed recently (269). Briefly, it is expressed on several cell types of different embryonal origins, and it is involved in many processes related to brain system development and diseases. These processes, such as

thrombosis, lymphangiogenesis, angiogenesis and inflammation, play a key role in regulating the growth of any tumor, including the MB. PDPN can be considered a marker of neoplasia trending to generate metastases (270, 271). Indeed, it is associated with malignant progression leading to epithelial-mesenchymal transition (EMT) and consequent tumor tissue diffusion and metastasis (272–274). This has been demonstrated in a particular mouse model with breast carcinoma cells, or with the antibody NZ-1 anti-PDPN for lung metastatization of CHO-expressing human (h)PDPN, or using point-mutated hPDPN-expressing CHO cells. The PDPN is present on lymphatic endothelial cells in the lumen only in aggressive MB, while in the absence of metastasis this marker was diffusely expressed on the whole surface of endothelial cells. Importantly, PDPN is upregulated in several types of cells during inflammation, including epithelial cells, fibroblasts, fibroblast-like reticular cells, APC and T helper cells interacting with several potential ligands, among which C-type lectin domain family 1 member B (CLEC1B/CLEC-2), CD44 and galectin 8 (274–276).

It has been shown in mice xenografts that the MB cell lines D283-Med, D425-Med and DAOY were sensitive to a novel recombinant single-chain antibody variable region fragment (scFv) of NZ-11 (anti-PDPN antibody) fused to *Pseudomonas* exotoxin A with a C-terminal KDEL peptide (NZ-1-PE38KDEL). This immunotoxin was further stabilized with a disulfide bond to generate the NZ-1-(scdsFv)-PE38KDEL complex, displaying good stability at 37°C. This construct exerted a strong cytotoxic effect *in vitro* against MB xenograft cells; it delayed markedly the *in vivo* subcutaneous growth of the D283-Med xenograft and, more importantly, it increased by over 40% the OS when administered to mice with intracranial MB tumor xenograft. Also, CAR-T cells directed to PDPN by a construct composed of NZ-1-based single-chain variable fragments and CD28, 4-1BB and CD3 ζ intracellular domains have shown good efficiency against glioma cells both *in vitro* and *in vivo* glioma xenografts (277). Altogether, these findings suggest that PDPN could be considered a target in MB by using CAR-T cells, antibodies and lectins as shown for several types of cancers (257). Conceivably, the elimination of PDPN⁺ cells in the MB TME may reduce the immunosuppression exerted by different types of mesenchymal stromal cells allowing the triggering of NK or T cell responses as shown in other tumor models (278–281).

4.5.3 Do not eat me signaling and MB growth

Among the several surface receptors involved in the regulation of antitumor immunity, the relevance of CD47 is well-established (262). This is also true for the MB (282–285). The CD47, also known as integrin-associated protein (IAP), binds to thrombospondin 1 (TSP-1) and signal regulatory protein alpha (SIRP- α). The binding of CD47 with SIRP- α on macrophages gives a “don’t eat me” signal that can spare the expressing CD47 cells. Typically, CD47 can allow tumor cells to evade macrophage-mediated elimination; consequently, the use of specific anti-CD47 antibodies can restore this event, and this is the rationale for the anti-CD47 antibody used in tumor therapy (282). In the context of MB, it has been recently shown that the humanized anti-CD47

antibody termed Hu5F9-G4 shows therapeutic efficacy on orthotopic PDX models. In addition, the intracranial administration of Hu5F9-G4 can inhibit the dissemination of MB to leptomeninges while exerting minimal effects on healthy neural cells (158). In more detail, the MB of group 3 expresses well the CD47 at the cell surface (86–99% of cells (158); this expression was evident on primary and xenograft-derived cells. More importantly, the use *in vitro* and *in vivo* of Hu5F9-G4 led to strong phagocytosis.

Two primary and three MYC-amplified cell lines localized at the cerebellum and disseminated at leptomeninges have been treated with Hu5F9-G4 antibody (also named Magrolimab) intraperitoneally, and a reduction of tumor burden and increase of OS in mice have been detected. Importantly, MB tumors were consistently infiltrated with macrophages in animals treated with the antibody, while the diffusion to leptomeninges of MB cells was strongly reduced. The metastatic recurrence of MYC overexpressing MB is a major fatal event that can hit pediatric patients (286). Strikingly, the use of Hu5F9-G4 was able to clear spinal metastasis in this xenograft model using MYC-amplified MB cells, and this antibody targets CD15⁺ CSC. The administration into the blood of Hu5F9-G4 revealed that it can pass through the blood-brain endothelial barrier (BBB) apparently even when the tumor was not present. Overall, these findings strongly suggest that the targeting of CD47 on MB cells can affect the growth and diffusion of MB within the CNS. As for PDPN targeting, the relief of the inhibitory signal delivered through the CD47-SIRP- α binding on macrophages could generate a pro-inflammatory TME favoring the anti-MB immune response, as indicated for other tumor types (287, 288).

4.5.4 MB and astrocytes

Astrocytes are a star-shaped subtype of glial cells supporting the neuronal development, metabolism and function of the brain (289–292). They can express glial fibrillary acidic protein (GFAP) but also GFAP-negative astrocytes can be identified, indicating they are a quite heterogeneous cell population (290–294). Astrocytes can play a role in innate immunity in the brain by interacting with microglia and undergoing proliferation and activation induced by danger-associated molecular patterns and/or pathogen-associated molecular patterns (293–295). The molecular analysis of astrocytes at the single cell level has identified that several other subsets of this cell population can exist in specific brain regions (296, 297). In addition, it has been reported that astrocytes present in the cerebellum are different from those of other brain regions (274). Detailed reviews on the protein markers of astrocytes present in different regions of the CNS as well as during inflammation and diseases have been published (268, 297, 298). Overall, the astrocytes play a trophic role of MB TME as they secrete the ligand sonic hedgehog (SHH) and this factor triggers the expression of nestin in MB cells; the elimination of MB-associated astrocytes led to suppression of nestin expression, blocking the tumor growth (299). This finding supports the notion that astrocytes are essential for the expansion of MB of the SHH-subgroup (300–303). Furthermore, the secretion of the CCL2 chemokine by astrocytes is essential for the stemness of MB cells and the

generation of metastases throughout the CNS (304, 305). The impairment of astrocyte-mediated functions or their elimination by specific targeting may relieve the immune downregulation increasing the effect of immunotherapy.

5 Overall considerations and future/putative therapies and immunotherapies for MB

The MB is an aggressive tumor characterized by a relevant molecular and cellular heterogeneity. The advances in genomic and transcriptomic have identified several subtypes within the four subgroups (52, 56, 65, 71, 74, 79, 88, 306). This can give insights to modify the therapeutic approaches in favorable and very high-risk groups (58, 306–308). There is no doubt that the molecular classifications have improved the prediction of the clinical outcome, and they provide key markers for a therapy tailored to the risk group. Nevertheless, the old and new therapeutic approaches do not have a striking effect on the OS rate of MB patients, indicating that these attempts should be improved. Recently, some relevant advances in tumor therapy have been reached with the use of immune checkpoint inhibitors (ICI) (178–180, 309). To relieve of the brake of tumor immunity related to the exhaustion of antitumor-specific T lymphocytes should be considered as a requisite to justify therapy with ICI (178–180). The use of pembrolizumab, an anti-PD1 antibody, is under investigation in some clinical trials (NCT02359565 and NCT06514898) in recurrent groups 3 and 4, but results have not been published yet. One report on the treatment of MB considers as ICI the anti-PD1 antibody nivolumab with or without the anti-CTLA4 antibody ipilimumab in 15 patients each (310). An evident difference in OS was present between the MB treated with the combo of nivolumab and ipilimumab (85%) versus nivolumab (40%) only. Unfortunately, this difference was not present after 24 months, showing about 35% of OS for both treatments. This discrepancy could be justified with the subgroups of the MB treated, but this detail is not reported in the original article (310). Also, using nivolumab in a little series of CNS tumors (with just one MB), it appears that a difference in the median survival was associated with the expression of PDL1 and high tumor mutation burden (311). This finding would suggest that the high mutation burden possibly associated with the generation of neoantigens and TAA together with the expression of the ligand of PD1 on tumor cells are requisites to elicit a response in CNS tumors. Nevertheless, the results obtained are far from exciting. In this context, a key point is to define whether the MB expresses the ligands for IC molecules such as PDL1 and/or CD80/CD86 antigens. Indeed, it has been shown that the TME in MB shows a variable expression of ligands for IC (312–318). In detail, using K-means clustering, it has been shown that the immune cell infiltration was low in all the four groups of MB while the PD1/PDL1 expression was absent in the cohort analyzed of 19 MB (314). This finding is in agreement with other reports (38, 284, 317), but the PDL1 was markedly expressed in single WNT or SHH cases (312). This would suggest that the therapy with anti-classical IC

(CTLA4 and PD1) is not appropriate as there is no possibility to relieve the brake of the immune system. It is to be noted that several others IC receptors can be expressed by effector lymphocytes (142, 319–323) and further studies should show how much these additional IC are involved in the impairment of the MB recognition. On the other hand, favoring a pro-inflammatory MB TME, instead of an immunoregulatory one, can trigger the upregulation of ligands for classical IC molecules justifying their use for treatment (322, 323).

The definition of an atlas of naturally presented T cell antigens from 28 MB upon mapping of the HLA ligandome (WNT (n = 4); SHH (n = 9); Group 3 (n = 7); Group 4 (n = 8)) could help to the further characterization of how to target efficiently MB cells (318). It is determinant that the putative antigens show peptides presented in the context of both HLA-I and HLA-II. Indeed, a few candidate proteins: IGFBPL1, INSM1, and INSM2 satisfied this condition. The clinical relevance of the CD4⁺ T helper cells is considered a key point to elicit a durable and efficient anti-MB response. It is of note that the demonstration of the potential immunogenicity of these peptides has been obtained by tetramer staining (318) not by a functional assay. However, this atlas can facilitate the development of mRNA vaccines, primed DC with the peptides identified, and the possible adoptive transfer of antigen-specific T cells. Also, the targeting with the CT-179 drug of a basic helix-loop-helix (bHLH) transcription factor (TF) OLIG2 can suppress the recurrence of the MB of the SHH subgroup (324). Interestingly, OLIG2 promotes differentiation in oligodendrocyte lineage, but it favors the maintenance of an undifferentiated condition of neural stem cells and progenitor cells (325–328).

The block of the dimerization, phosphorylation and DNA binding of TF leads to prolonged survival of SHH-MB PDX and GEM models and potentiates radiotherapy (RT) *in vivo*. These findings could suggest the targeting of OLIG2⁺ CSCs that remain quiescent upon conventional therapy with a better clinical outcome for some patients. This would indicate that the targeting of appropriate TF regulating the fate of CSCs is a key tool to avoid resistance to therapy and relapse. The CT-179 increases the differentiation and upregulates the cyclin-dependent kinase (CDK) 4, favoring the proliferation. The further combination with the CDK4/6 inhibitor palbociclib prolongs the survival of the PDX and GEM models. Altogether, these findings indicate that the targeting of the TF in combination with other drugs can improve the clinical outcome. However, it should be considered the role of OLIG2 as a master regulator of oligodendrocyte lineage in early stages of differentiation as well as other components of the same family of TF (325). It is still undefined whether the blocking of one TF does not influence the others and how much the blocking of OLIG2 during the maturation of the CNS can affect its correct development. The treatment of a tumor during the development of the CNS is conceivably a challenging condition that is not so relevant if the tumor has been developed in an adult (325). Certainly, the targeting of OLIG2 is feasible for gliomas in adults, but it should be noted that the degree of expression can vary in glioblastomas and astrocytoma while it is usually well expressed in oligodendrogliomas (325). The use of OLIG2 as a target for MB

therapy should be considered cautiously as OLIG2 involved in the motor neuron fate and in neural repair as well (327, 328). It is conceivable that the combo of inhibitors of OLIG2 TF and immunotherapy can increase the efficiency of lymphocytes in eliminating CSC. This targeting has been demonstrated for the anti-MB protogenin (PRTG) CAR-T cells recognizing CSC of MB group 3 with a strong expression of PRTG (PRTG^{high}) and for the anti-PRTG blocking antibodies (329). Altogether, these findings suggest that the precise identification of the stem cells of MB could be the future target of the therapy. Another point to be discussed is the possible use of combination of immunotherapy and chemotherapy. It has been shown the association of chemotherapy and adoptive immunotherapy is more efficient in eliminating MB cells both *in vitro* and *in vivo* subcutaneously injected MB cell line DAOY (330). It is to be noted that in this experimental system the addition of LPS can further increase the anti-MB effect of the combo therapy, indicating that the activation of antigen presenting cells could be involved.

6 Conclusions

MB is an aggressive pediatric tumor, and although great advances have been reached, the present immunotherapeutic tools are not enough to modify the outcome of this disease. The detailed study of the expression of receptors on immune cells together with that of ligands on MB cells as well as other components of the TME could suggest the main molecules to be targeted by immunotherapy. MB groups show some relevant differences for the expression of ligands of several activating receptors of antitumor effector cells, immunecheckpoint molecules, MHC-I and II as well as inflammatory cytokines (Supplementary Figures 1, 2) (72, 331, 332). However, there is no a unique pattern of expression of these molecules for each subtype suggesting that each patient should be considered as a specific case. Also, there is a certain heterogeneity regarding the degree and type of immune cell infiltration (Supplementary Figure 3) (72) among the different groups of MB. This suggests that a tailored therapy is necessary on the basis of the results of molecular analysis. The identification of specific markers of MB stem cells could allow the study of the counter receptors expressed on immune cells to trigger the elimination of the source of the disease. Furthermore, the TME influencing MB and immune cells could influence the effectiveness of immunotherapy modifying the expression of the molecules involved in the recognition of MB cells by anti-tumor effector cells. Further studies focused on new effector cell subsets, novel antibodies and ADC, besides those listed in Table 3, and combination of drugs with immunotherapeutic tools may open new avenues to fight this tumor.

Author contributions

AP: Conceptualization, Funding acquisition, Resources, Supervision, Visualization, Writing – original draft, Writing – review & editing. FR: Writing – review & editing, Data curation, Methodology,

Software. HA: Writing – review & editing, Funding acquisition, Writing – original draft. LR: Data curation, Software, Visualization, Writing – review & editing. RP: Data curation, Methodology, Supervision, Writing – original draft, Writing – review & editing.

Funding

The author(s) declare that financial support was received for the research and/or publication of this article. This work was partially supported by the Associazione Italiana per la Ricerca sul Cancro (AIRC) IG 21648. RP and HA acknowledge the European Union's Horizon 2020 research and innovation program (MOBILISE project, 951723) and Fundação para a Ciência e a Tecnologia, I.P. (3D-BBB project, 2022.01690.PTDC).

Acknowledgments

Figures have been designed from the templates of Anatomy and Human Body and Cellular Biology series of Smart Servier Medical Art (<https://smart.servier.com/>). Some icons used in Figure 1 have been derived from Biorender.

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The author(s) declared that they were an editorial board member of Frontiers, at the time of submission. This had no impact on the peer review process and the final decision.

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Supplementary material

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fimmu.2025.1602930/full#supplementary-material>

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