



Review Article

Comparison between glioblastoma and brain metastases: How epigenetic remodeling can enhance immunotherapy

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ABSTRACT

Glioblastoma (GBM) is the most common, aggressive and lethal primary tumor of Central Nervous System (CNS). Despite the standard of care and emerging treatment strategies, patient prognosis remains dismal. Over the past decade, immunotherapy has significantly improved outcomes in several tumors, and the clinical success observed in brain metastases (BM) from extracranial tumors has provided insights into CNS tumor immunobiology. However, immunotherapeutic approaches in GBM have so far yielded limited clinical benefit, underscoring the need to better understand the unique features of its tumor microenvironment (TME). This review compares the immunobiology of GBM and BM to elucidate potential mechanisms underlying immunotherapy failure in GBM. In addition, it explores the potential role of epigenetic drugs in reprogramming the GBM TME toward a more inflamed phenotype, thereby potentially enhancing immunogenicity and improving responsiveness to immunotherapy. Such strategies may offer a promising avenue to harness the immune system and inhibit GBM progression.

1. Introduction

Glioblastoma (GBM) is the most common, aggressive and lethal primary tumor of Central Nervous System (CNS). It accounts for approximately 50% of all primary brain tumors, with an annual incidence of ~3.20 per 100,000 individuals in the United States. The median age at diagnosis is 65 years, and the median Overall Survival (mOS) remains approximately 15 months, with a 5-year survival rate below 5% [1]. According to the most recent 2021 World Health Organization (WHO) Classification of Tumors of the CNS (2021 CNS WHO), GBM is defined as a grade IV, isocitrate dehydrogenase (IDH) wild-type glioma within the category of “adult-type diffuse gliomas” [2]. The cellular origin of GBM remains incompletely understood. One prevailing hypothesis suggests the involvement of neural stem cells (NSC) [3], a population of multipotent cells essential for the development of the nervous system (NS). NSC possess the ability to self-renew and differentiate into diverse neural cell types and are characterized by the expression of stemness-related markers such as CD133, Nestin, and SOX2. These cells reside in specialized neurogenic niches, including the subventricular zone (SVZ) of the lateral ventricles. It has been proposed that cancer stem cells (CSC), which share key features with NSC, originate from these populations and function as tumor-initiating cells responsible for tumor formation, hierarchical organization, maintenance, progression, and

recurrence, thereby acting as a reservoir for disease persistence [4,5]. Moreover, tumor heterogeneity and cellular plasticity are important features of GBM and are strongly associated with its unclear origin and therapeutic resistance [6–8]. The current first-line therapy or standard of care (SOC) for GBM consist of maximal safe surgical resection followed by standard or hypofractionated brain radiotherapy (RTx) combined with chemotherapy (CTx) based on the alkylating agent temozolamide (TMZ), administered according to the Stupp protocol, which has remained largely unchanged for over two decades [9]. Despite this aggressive multimodal approach, more than 90% of patients experience tumor recurrence, and prognosis remains poor [10]. Second-line therapies have demonstrated limited efficacy, and outcomes in recurrent GBM (rGBM) remain dismal [11,12]. Although numerous therapeutic strategies have been investigated in both preclinical and clinical settings, clinical benefit has remained limited [13–16]. This is particularly relevant in light of recent discoveries demonstrating the existence of a CNS lymphatic system in mice, known as meningeal lymphatic vessels, that enable macromolecules and immune cells present in the cerebrospinal fluid (CSF) and meninges to drain into cervical lymph nodes, thereby connecting the CNS to the peripheral immune system [17–19]. Moreover, tumor-derived antigens and antigen-presenting cells (APC) can reach cervical lymph nodes and initiate antitumor immune responses, provided that meningeal lymphatic

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structures remain functional [20,21]. Additional evidence indicates that cytokines present in the CSF can stimulate immune cell proliferation originating from skull bone marrow, which is directly accessible through cranial channels [22]. Collectively, these findings suggest that the CNS is immunologically connected to both peripheral immune compartments and locally resident immune cells, including skull bone marrow-derived cells [23]. In addition, the blood–brain barrier (BBB) is largely compromised in CNS tumors due to tumor infiltration and aberrant hypoxia-driven neoangiogenesis, resulting in the formation of a partially permeable blood–tumor barrier (BTB). This altered barrier may facilitate both immune cell infiltration and drug delivery into the tumor mass [24]. These insights have provided a strong rationale for harnessing the immune system against CNS tumors through immunotherapy [25]. Indeed, immunotherapeutic strategies have achieved significant clinical success in the treatment of a subset of brain metastases (BM) derived from extracranial tumors characterized by an inflamed tumor microenvironment (TME) [26]. In contrast, immunotherapy has thus far failed to produce meaningful clinical benefit in GBM [27], which remains a highly lethal disease in urgent need of novel therapeutic approaches. In this review, the immunobiology of GBM and a subset of “hot”, inflamed BM will be compared to elucidate the mechanisms underlying the differential response to immunotherapy. Furthermore, the potential role of epigenetic drugs in remodeling the GBM TME toward a more inflamed phenotype, similar to that observed in a subset of BM, particularly in melanoma-derived BM, will be explored as a potential new strategy to sensitize GBM to immunotherapy. In this context, DNA hypomethylating agents (DHA) will be discussed as candidate compounds for combination with immunotherapy, supported by encouraging evidence from pre-clinical models. Overall, this hypothesis provides a rationale for combinatorial therapeutic strategies aimed at harnessing the immune system against this highly aggressive and lethal tumor.

2. The controversial efficacy of immune checkpoint inhibitors in brain tumors

BM derived from extracranial tumors occur in up to 50% of cancer patients and are associated with a dismal prognosis, with a mOS of ~6 months [28]. Historically, therapeutic options for BM have been largely palliative, while CTx has been infrequently administered because of its limited ability to effectively cross the BBB. Consequently, the management of BM has long remained clinically challenging due to the poor efficacy of conventional therapeutic approaches. Moreover, patients with active BM were systematically excluded from pivotal immunotherapy clinical trials, including those investigating immune checkpoint inhibitors (ICI). However, the recent findings in the understanding of CNS immunobiology, together with the recognition of a compromised BTB, the potential presence of an inflamed TME, and the influence of the intrinsic characteristics of the primary tumor on tumor immunogenicity, have provided new insights into the biology of these lesions. These advances paved the way for clinical trials assessing ICI in patients with active BM derived from extracranial tumors, particularly melanoma and non-small cell lung cancer (NSCLC) (NCT02320058, NCT02374242, NCT02869789), characterized by combination strategies based on anti-cytotoxic T-lymphocyte antigen 4 (CTLA-4) antibody ipilimumab and anti-programmed cell death protein 1 (PD-1) antibody nivolumab [29–31]. These studies demonstrated significant clinical activity in this hard-to-treat patient population. In particular, the “NIBIT-M2” clinical trial (NCT02460068) reported prolonged OS while preserving health-related quality of life (HRQoL) in patients with melanoma-derived BM [32] treated with the combination of ipilimumab and nivolumab. These results generated considerable interest in extending immunotherapy to GBM. Consequently, several clinical trials have investigated the efficacy of immunotherapy in GBM, failing to improve patient outcome. For example, nivolumab failed to improve OS compared with bevacizumab, a second-line anti-vascular endothelial growth factor A (VEGF-A) therapy targeting neoangiogenesis, in the “CheckMate 143” clinical trial

(NCT02017717) involving patients with rGBM. These results highlighted the limited efficacy of ICI monotherapy and underscored the profoundly immunosuppressive nature of the rGBM TME, characterized by low immune infiltration together with extensive immune dysfunction and T cell exhaustion. [33]. Similar results emerged from the “CheckMate 548” clinical trial (NCT02667587), which evaluated nivolumab in combination with SOC in newly diagnosed GBM patients with methylated O6-methylguanine-DNA methyltransferase (MGMT) promoter status [34]. MGMT is a DNA repair enzyme involved in maintaining genomic stability and can counteract the cytotoxic effects of TMZ when expressed, thereby influencing responsiveness to CTx. This study failed to demonstrate improvements in either OS or progression-free survival (PFS). In addition, the trial emphasized both the immunosuppressive nature of the primary GBM TME and the potential negative impact of TMZ-induced lymphopenia on the efficacy of nivolumab. Likewise, the “CheckMate 498” clinical trial (NCT02617589) evaluated nivolumab combined with RTx in newly diagnosed GBM patients with unmethylated MGMT promoter status, compared to only surgery and RTx. In this setting, TMZ was omitted not only because MGMT expression reduces its therapeutic efficacy, but also to avoid treatment-related lymphopenia that could impair antitumor immune response. Nevertheless, the study failed to demonstrate an OS benefit, suggesting that overcoming CTx-induced immunosuppression is insufficient to restore ICI efficacy in the absence of a pre-existing inflamed TME [35]. Collectively, these pivotal clinical trials failed to meet their primary endpoints, largely because of the profoundly immunosuppressive microenvironment characteristic of GBM, as discussed later in this review. This immunologic context likely limits the efficacy of ICI despite successful results observed in other CNS tumors such as BM. Currently, several ongoing clinical trials are investigating novel ICI targeting both canonical and non-canonical immune checkpoint (IC) molecules, including LAG-3 (lymphocyte-activation gene 3), TIM-3 (T-cell immunoglobulin and mucin-domain containing-3), and TIGIT (T cell immunoreceptor with Ig and ITIM domains). In parallel, innovative therapeutic strategies based on neoadjuvant immunotherapy, in which nivolumab is administered before surgical resection, are under investigation and have demonstrated promising effects on TME reprogramming (NCT02550249) [36,37].

Overall, only modest clinical benefits have been achieved thus far with ICI, as well as with other immunotherapeutic approaches such as cancer vaccines, adoptive T cell therapy, and viral therapy. Nevertheless, the scope of this review is not to provide a systematic overview of immunotherapy-based clinical trials, as this topic has already been extensively reviewed elsewhere [38–43]. Instead, elucidating the mechanisms regulating the complex interplay between the brain, the immune system, and tumor cells may help to explain the failure of immunotherapy in GBM. These insights may, in turn, enable the identification of novel combinatorial strategies capable of overcoming the immune resistance mechanisms characteristic of this tumor.

3. The immunobiology of GBM compared to BM

GBM is a highly aggressive primary brain tumor that is classified as a “cold”, non-inflamed tumor, due to the limited inflammatory infiltrate within its immunosuppressive TME [44–46]. It expresses low levels of HLA class I molecules (HLA-I) and lacks HLA class II molecules (HLA-II) expression, resulting in poor immunogenicity [47]. In addition, GBM is characterized by a low tumor mutational burden (TMB), with a median of ~1.5 mutations per megabase, further contributing to its weak immunogenic profile. However, in hypermutated rGBM following temozolomide (TMZ) treatment, TMB may increase to ~10 mutations per megabase as a consequence of alterations in molecular pathways involved in genomic stability, particularly the DNA mismatch repair (MMR) system. Except for this latter condition, these characteristics collectively establish immunosuppression as a major hallmark of GBM and contribute to resistance to current therapies, particularly

immunotherapies [48]. This sharply contrasts with other CNS tumors, especially BM originating from extracranial tumors [49–51]. Although BM share the same anatomical niche as GBM, their immune landscape differs substantially. Indeed, several subsets of BM are considered “hot”, inflamed tumors due to the presence of an active TME enriched with dense immune infiltrates, particularly in melanoma-derived BM. Moreover, these subsets generally express appreciable levels of HLA-I and HLA-II, although these levels may be lower than those observed in the corresponding primary tumors [52]. Furthermore, they display a higher TMB than both their primary tumors and GBM, with a median of at least 10 mutations per megabase. Collectively, these characteristics render these BM more immunogenic and more heavily infiltrated by immune cells, especially CD8+ T cells, thereby increasing their susceptibility to immunotherapy [53]. Nevertheless, these concepts do not apply uniformly across all BM, as substantial differences exist among tumor histotypes. These differences are influenced by the intrinsic characteristics of the primary tumor, the composition of the immune infiltrate, and the marked intratumoral and intertumoral heterogeneity that characterize BM. In the following sections, a detailed comparison of the immune landscape of GBM and a subset of “hot”, inflamed BM will be presented, with the aim of better elucidating their differential responses to immunotherapy (Fig. 1).

3.1. Lymphoid cells

In general, GBM is characterized by a markedly lower degree of immune cell infiltration within the tumor mass compared with BM. Tumor-infiltrating lymphocytes (TIL) are scarce and are predominantly represented by regulatory T lymphocytes (Treg) CD4+ CD25+ FoxP3+, which release immunosuppressive cytokines within the TME, including transforming growth factor beta (TGF- β) and interleukin-10 (IL-10). These cytokines suppress the activity of CD4+ T helper cells and CD8+ T cytotoxic cells when present. Notably, B cells and natural killer (NK) cells are poorly represented. Treg also express CTLA-4, which interacts

with CD80 and CD86 molecules on resident and migratory APC, thereby impairing their ability to activate T cells [54,55]. Furthermore, the immunosuppressive TME contributes to the downregulation of HLA-II expression on APC, when present, thus compromising antigen presentation. As a consequence, immune effector cells remain in an anergic or exhausted state and are unable to effectively counteract tumor cells, expressing multiple IC molecules, including CTLA-4, PD-1, LAG-3, TIM-3, TIGIT, VISTA (V-domain Ig suppressor of T cell activation), CD160 antigen, CD39 antigen, and CD244 antigen [56]. Importantly, the highly dynamic expression of programmed death-ligand 1 (PD-L1), detected in approximately 37% of GBM samples, further contributes to suppression of the immune infiltrate [57]. However, the levels of soluble PD-L1 (sPD-L1) in peripheral blood do not correlate with PD-L1 expression on tumor cells. Interestingly, elevated sPD-L1 levels have been associated with improved survival compared with other primary and secondary brain tumors [58].

GBM cells also express tumor necrosis factor receptor superfamily member 14 (TNFRSF14), which contributes to T cell suppression [57,59], as well as Fas ligand (FasL), a transmembrane protein capable of inducing apoptosis upon interaction with Fas receptor (FasR) expressed on T cells [60].

Finally, patients with GBM frequently exhibit systemic lymphopenia characterized by sequestration of T cells within the bone marrow. This phenomenon is associated with internalization of sphingosine-1-phosphate receptor 1 (S1PR1), a receptor involved in T cell egress from lymphoid organs following interaction with its ligand sphingosine-1-phosphate (S1P). Nevertheless, the precise relationship between GBM and S1PR1 internalization remains incompletely understood [61].

In contrast to GBM, BM are characterized by substantial accumulation of immune cells, which are not sequestered within the bone marrow. BM contain approximately twice the number of TIL observed in GBM, mainly localized at the tumor border and within the stromal compartment, and their presence correlates with improved prognosis. In particular, CD8+ T cells and, to a lesser extent, CD4+ T cells are highly

Comparison of the immune landscape of GBM and BM

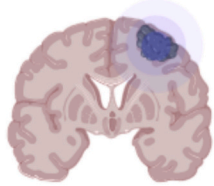
Glioblastoma		Brain Metastases	
 Cold Tumor		 Hot Tumor	
↓ HLA-I, HLA-II	↑ PD-1, CTLA-4	↑ HLA-I, HLA-II	↑ PD-1, CTLA-4
↓ TMB	↓ LAG-3, TIM-3, TIGIT	↑ TMB	↑ LAG-3, TIM-3, TIGIT
↓ CD8+ T cells	↑ ↓ PD-L1	↑ CD8+ T cells	↑ PD-L1
↓ CD4+ T cells	↑ sPD-L1	↑ CD4+ T cells	↓ sPD-L1
↑ Tregs		↑ ↓ Tregs	
↓ B cells	↑ Anti-inflammatory cytokines	↓ B cells	↑ ↓ Anti-inflammatory cytokines
↓ NK	↑ ↓ Pro-inflammatory cytokines	↓ NK	↑ Pro-inflammatory cytokines
↑ TAM		↓ TAM	
↑ MDSC	↑ Chemokines	↑ ↓ MDSC	↑ Chemokines
↑ ↓ TAN		↑ TAN	
= Astrocytes	↑ ARG1, IDO-1	= Astrocytes	↑ ↓ ARG1, IDO-1
= Pericytes	↑ NO, ROS	= Pericytes	↑ NO, ROS
↑ upregulated; ↓ downregulated; ↑ ↓ variable; = unchanged.			

Fig. 1. Comparison of the immune landscape of GBM and BM. GBM is characterized by a “cold” TME in contrast to the “hot”, inflamed TME observed in a subset of BM. Specifically, GBM exhibits downregulation of pro-inflammatory mediators and enrichment of anti-inflammatory and immunosuppressive components. Additional details regarding the involved immune cell populations, immune-related genes, molecular pathways, and their regulatory mechanisms are discussed in the main text. Created in [BioRender.com](https://www.biorender.com).

abundant in melanoma-derived BM [62,63].

Similar to GBM, B cells and NK cells are less represented. The anti-tumoral role of CD4⁺ T cells in BM remains less understood than that of CD8⁺ T cells. However, a large proportion of these lymphocytes are either anergic or represented by Tregs, which suppress effector T cell function through the production of TGF- β , IL-10, and IL-35, thereby promoting an exhausted phenotype [64]. Consistently, several IC molecules are highly expressed on effector T cells. In particular, PD-1 expression is detected in more than 60% of TIL, while alternative IC molecules, including LAG-3, TIM-3, and TIGIT, are more highly expressed compared with GBM, making them attractive targets for immunotherapy [65]. Immune infiltrates are further suppressed by PD-L1, which is expressed in more than 50% of tumor cells, whereas sPD-L1 levels are lower than those observed in GBM, despite being associated with responses to ICI [66,67].

3.2. Myeloid cells

GBM recruits immunosuppressive myeloid cell populations through the release of C-C motif chemokine ligand 2 (CCL2) and other chemokines, including resident and infiltrating tumor-associated macrophages (TAM) and myeloid-derived suppressor cells (MDSC), thereby further strengthening the highly suppressive TME. Additional cytokines secreted by GBM include colony-stimulating factor 1 (CSF1 or M-CSF), which promotes macrophage polarization toward the M2 phenotype [68], and CSF2 (GM-CSF), which induces MDSC activation [69]. Another important factor expressed by GBM is fibrinogen-like protein 2 (FGL2), a secreted protein that enhances the immunosuppressive properties of the TME by promoting M2 polarization of TAM and increasing Treg abundance [70]. GBM also releases extracellular vesicles (EV) containing immunosuppressive molecules capable of modulating target cells and inducing, for example, M2 polarization of TAM [71,72].

Peripheral monocytes infiltrating the brain parenchyma differentiate into macrophages, which are subsequently driven toward an anti-inflammatory M2 phenotype by the TME. These cells, referred to as TAM, have attracted considerable interest in recent years because of their established roles in tumorigenesis and tumor progression, as well as their differential composition among GBM subtypes [73,74]. TAM preferentially localize within the tumor core and contribute to several protumoral mechanisms, including support of CSC, promotion of genomic instability, induction of epithelial–mesenchymal transition (EMT), and suppression of adaptive immunity through the expression of IC ligands that inhibit effector T cells. Similarly, resident microglia recruited to the tumor site, predominantly at the tumor periphery, are polarized toward the M2 phenotype under the influence of the TME, thereby promoting immunosuppression and tumor progression [75,76]. In general, tumor-infiltrating microglia are considered part of the TAM population, which is often more abundant in primary tumors than in recurrent lesions [77].

In GBM, TAM may constitute up to 50% of the immune infiltrate. However, these cells exhibit impaired APC functions because of the downregulation of co-stimulatory and HLA molecules, which compromises antigen presentation. Moreover, TAM are spatially separated from T cells, reflecting both the scarcity and heterogeneous distribution of lymphocytes within the tumor mass. Notably, when CD40 is expressed, macrophages appear capable of interacting with T cells and promoting their activation. Importantly, macrophages located in peri-necrotic and necrotic regions display activation of the IL-10 signaling pathway, further supporting their immunosuppressive functions [78].

MDSC also play a crucial role in maintaining the immunosuppressive TME. For example, these cells produce nitric oxide (NO) and reactive oxygen species (ROS), which deplete amino acids essential for effector T cell function and induce molecular damage, respectively, ultimately leading to T cell apoptosis. Both TAM and MDSC additionally secrete arginase 1 (ARG1), which contributes to T cell suppression through depletion of L-arginine [79].

Neutrophils represent another characteristic immune cell population in GBM and are referred to as tumor-associated neutrophils (TAN). However, their precise role remains incompletely understood. After rapidly infiltrating the tumor from the periphery in response to GBM, neutrophils are reprogrammed by tumor cells. Similarly, TAN recruited by TAM-derived C-X-C motif chemokine ligand 8 (CXCL8) acquire immunosuppressive functions. Consequently, neutrophils contribute to remodeling of the TME through the release of IL-1 β and IL-6, as well as the expression of PD-L1 [80,81]. Importantly, patients with GBM typically exhibit an elevated neutrophil-to-lymphocyte ratio (NLR), which has been associated with poor prognosis [82].

BM are also capable of recruiting immunosuppressive myeloid cell populations, including TAM and MDSC, which together account for approximately 33% of the tumor infiltrate. Although these populations are less abundant than in GBM, they display a more inflammatory phenotype, together with increased neutrophil infiltration [63,83]. Common chemokines released by BM include CCL2, CCL5, CCL9, CCL18, and CSF1, all of which contribute to recruitment of these cell populations to the tumor site [84]. Within this context, TAM are polarized toward the M2 phenotype under TME pressure and release CCL8, CCL13, CCL17, and CCL18, while also promoting extracellular matrix (ECM) remodeling and neoangiogenesis through secretion of matrix metalloproteinase-2 (MMP2), MMP9, and VEGF-A [85]. Nevertheless, TAM in BM appear to retain a certain degree of plasticity, shifting between M1 and M2 phenotypes depending on local TME conditions. Moreover, TAM and MDSC produce NO and ROS, thereby impairing effector T cell function and contributing to the establishment of an immunosuppressive TME [84]. However, unlike their counterparts in GBM, these myeloid populations preserve the ability to process and present antigens through upregulation of CD80. Importantly, TAM, MDSC, and T cells are found in closer spatial proximity in BM, facilitating T cell activation and partially mitigating the immunosuppressive environment [78].

Resident microglia in BM express IL-6, CXCL10, and CXCL8, thereby suppressing effector T cells and recruiting neutrophils [86]. Neutrophils, in turn, express integrin subunit alpha 3 (ITGA3), CD177, adenosine A2a receptor (ADORA2A), and MET proto-oncogene, receptor tyrosine kinase (MET) and release CXCL17, promoting tumor neoangiogenesis and suppression of effector T cells [62]. Compared with GBM, neutrophils are more abundant in BM and exhibit a more pro-inflammatory phenotype. Nevertheless, similarly to GBM, BM are also associated with elevated NLR values [81].

3.3. Astrocytes

GBM-associated astrocytes release EV in response to brain lesions in order to recruit immune cells from the periphery, which subsequently undergo immune modulation depending on the local microenvironment [87]. Through the secretion of anti-inflammatory cytokines, including TGF β , IL10, and colony-stimulating factor 3 (CSF 3 or G-CSF), astrocytes, in cooperation with microglia, contribute to the establishment of an immunosuppressive environment [88]. Moreover, astrocytic expression of FasL in response to neuroinflammation induces apoptosis on effector T cells [89]. Finally, astrocytes undergo a process known as reactive gliosis, leading to the formation of a functional barrier referred to as the “glial scar,” which contributes to the protection of GBM from both immune surveillance and therapeutic interventions [90,91].

Astrocytes also play a critical role in BM progression after being reprogrammed by tumor cells, although their role during the initial stages of micrometastasis formation remains incompletely understood [92]. Once BM are established, tumor cells can reprogram astrocytes through MET expression and the formation of functional gap junctions that enable transfer of cyclic GMP-AMP (cGAMP). This process stimulates the production of the MET ligand, IL-1 β , and tumor necrosis factor alpha (TNF- α), which subsequently activate NF- κ B signaling in tumor cells, thereby promoting tumor proliferation [88]. BM cells can

additionally release multiple cytokines that further modulate astrocyte function. For instance, lung cancer-derived BM secrete IL-8, macrophage migration inhibitory factor (MIF), and plasminogen activator inhibitor-1 (PAI-1), inducing a protumorigenic astrocytic phenotype [93]. These molecules stimulate astrocytes to release IL-6, IL-1 β , and TNF- α , which contribute to immune modulation and tumor dissemination.

Similarly, activation of the NOD-like receptor protein 3 (NLRP3) inflammasome and the release of IL-1 β and brain-derived neurotrophic factor (BDNF) by astrocytes have been associated with breast cancer-derived BM [94]. Likewise, melanoma-derived BM reprogram astrocytes to express IL-23, which promotes MMP2 release and ECM remodeling, thereby enhancing tumor invasiveness [95]. In addition, reprogrammed astrocytes can release EV containing microRNA targeting phosphatase and tensin homolog (PTEN) in tumor cells, further promoting tumorigenesis and enhancing CCL2 expression [96].

3.4. Pericytes

Pericytes play a pivotal role in the immunosuppressive TME of GBM. These cells, which are normally involved in maintaining the structural integrity BBB, are reprogrammed by GBM cells following tumor establishment through direct cellular interactions and subsequently exploited in a process known as vascular co-option, whereby tumor cells utilize pre-existing vasculature to infiltrate the brain parenchyma. As a result of this reprogramming, pericytes express immunosuppressive mediators, including TGF- β , IL-10, IL-6, and PD-L1, thereby contributing to immune suppression. In addition, cytoskeletal alterations impair their ability to function as APC for T cell priming [97].

Pericytes are also involved in BM progression. The extent of pericyte coverage surrounding blood vessels has been shown to negatively correlate with immune cell infiltration. Although BM are characterized by larger and more prominent vasculature compared with GBM, these vessels permit greater immune cell infiltration. Notably, single-cell RNA sequencing analyses have demonstrated that pericytes in lung cancer-derived BM display molecular features similar to those observed in GBM, whereas pericytes from melanoma- and breast cancer-derived BM more closely resemble those of the corresponding primary tumors, potentially contributing to a greater responsiveness to immunotherapies [98].

3.5. Immunological dysfunction metabolism-dependent

Anti-inflammatory mediators released by GBM cells, including TGF- β , IL-10, IL-6, and prostaglandin E (PGE), are able to induce several metabolic alterations within the TME [99,100]. Hypoxia represents a hallmark feature of GBM and arises from rapid tumor growth that is not adequately compensated by oxygen supply. Under these conditions, hypoxia-inducible factor 1 α (HIF-1 α) is upregulated, promoting glucose uptake and glycolytic metabolism in tumor cells, CSC, and TAM, ultimately leading to glucose deprivation within the TME. Because glucose is essential for the survival, activation, and proliferation of T cells, this metabolic competition contributes significantly to immune suppression [101]. This process of aerobic glycolysis, known as the “Warburg effect,” results in excessive lactate production, which further acidifies the microenvironment. Such acidic conditions impair effector T cell function while simultaneously favoring Treg activation and infiltration [102]. A similar metabolic profile has also been observed in BM, where glucose represents the primary energy source. However, BM cells can additionally activate alternative metabolic pathways, including fatty acid and glutamine metabolism, to sustain tumor growth and survival [103].

Both GBM and BM express indoleamine 2,3-dioxygenase 1 (IDO1), an enzyme involved in tryptophan metabolism that promotes Treg expansion and suppresses effector T cell activity through tryptophan depletion and the accumulation of toxic metabolites within the TME [104,105]. Currently, IDO1 is considered an IC molecule and therefore

represents a potential therapeutic target [106].

Another distinctive feature of the GBM microenvironment is the constitutive activation of signal transducer and activator of transcription 3 (STAT3) across nearly all cellular components of the tumor mass through multiple signaling pathways. STAT3 is a transcription factor whose persistent activation has been associated with several malignancies and poor clinical prognosis. Consequently, STAT3 inhibition has become the focus of numerous preclinical investigations. Hypoxia, IL-10, and IL-6 contribute to STAT3 activation in GBM cells, particularly in CSC. *Ex vivo* studies have demonstrated constitutive STAT3 activation in CSC isolated from GBM specimens, resulting in increased Treg expansion and further reinforcement of the immunosuppressive microenvironment [107,108].

Similarly, BM cells induce and maintain STAT3 activation within the TME, particularly in astrocytes. Activated astrocytes are able to suppress CD8 $^{+}$ T cell function by promoting the expression of CTLA-4 and PD-1 on their surface while simultaneously favoring the recruitment and accumulation of TAM [109].

3.6. Immunological dysfunction treatment-dependent

Finally, immunological dysfunctions are commonly observed in patients with GBM following administration of the SOC. In particular, RTx and TMZ treatment in newly diagnosed GBM patients may further exacerbate systemic immunosuppression by inducing a reduction in CD4 $^{+}$ T cell counts [110]. The immunological effects of TMZ remain a matter of debate. Indeed, accumulating evidence suggests that TMZ may exert detrimental effects on immune cells depending on both the administered dose and the route of administration [111]. Furthermore, corticosteroids administered to control cerebral edema in patients with GBM contribute additional immunosuppressive effects [112], thereby further complicating an already profoundly immunosuppressive clinical context.

In contrast, the use of corticosteroids in symptomatic patients with BM remains controversial. Some patients exhibit suboptimal responses to immunotherapy, potentially because of the size of the metastatic lesions and the protective role of peritumoral edema rather than corticosteroid administration itself. Notably, the number of TIL has been shown to inversely correlate with the extent of edema. Nevertheless, corticosteroid administration has been associated with reduced OS in a dose-dependent manner and should therefore be avoided whenever possible, even though TIL numbers may remain unchanged. Additional studies are required to clarify the mechanisms through which corticosteroids negatively affect BM treatment outcomes [113].

4. Novel therapeutic strategies: epigenetic drugs

Targeting a single hallmark of GBM has thus far proven ineffective, and immunotherapy alone appears to present significant limitations. Consequently, combinatorial therapeutic approaches aimed at overcoming immune resistance have emerged as a promising strategy for GBM treatment. The development of novel combination therapies is therefore essential to maximize clinical benefit. In this context, epigenetic therapy has gained increasing attention as an emerging strategy capable of remodeling the tumor epigenome while simultaneously priming the immune system. This approach is currently an area of active clinical investigation across multiple tumor histotypes [114–116], particularly given that epigenetic dysregulation is now considered a fundamental hallmark of cancer that contributes to genomic instability [117]. Several tumor suppressor genes (TSG) are epigenetically silenced in GBM through promoter DNA methylation [118–120], thereby enabling uncontrolled tumor proliferation. At the same time, oncogenes associated with neoangiogenesis, EMT, cellular plasticity, and stemness may become activated, further enhancing tumor progression. Moreover, epigenetic mechanisms can also promote resistance through upregulation of IC molecules, which suppress T cell activity and enable tumor

cells to evade immune surveillance, as previously described for PD-L1 expression [99]. Epigenetic alterations contribute to GBM resistance to both immune surveillance and conventional therapies through the downregulation of immune-related genes involved in antigen processing and presentation machinery, ultimately impairing immune recognition and elimination of tumor cells. Furthermore, tumor antigens themselves may undergo epigenetic silencing, thereby facilitating immune evasion [121,122]. Importantly, epigenetic signatures in tumor cells are increasingly recognized as promising predictors of clinical outcome in patients with GBM [123]. Epigenetic dysregulation also affects immune cell function through alterations in the expression of genes involved in T cell differentiation and activity. These modifications impair the capacity of effector T cells, particularly CD8⁺ T cells, to recognize and eliminate tumor cells effectively [124].

For these reasons, epigenetic drugs have recently been investigated in preclinical setting as potential strategies to reverse the immunosuppressive TME and overcome the resistance mechanisms acquired by GBM, with the final aim of restoring effective antitumor immunity [125]. Most importantly, by disrupting oncogenic signaling pathways, epigenetic therapies may sensitize GBM cells, particularly CSC, to the activity of other therapeutic agents, such as immunotherapy [126]. This hypothesis has provided the rationale for combining epigenetic drugs with additional treatment modalities in order to overcome the limitations of monotherapies. The following sections of this review will focus on the preclinical potential and limitations of DHA in remodeling the GBM TME toward a more inflamed phenotype resembling that observed in BM, which is generally more responsive to immunotherapy, thereby providing the rationale for a combinatorial therapeutic strategy for translation into clinical setting.

5. DNA hypomethylating agents

DHA are an important class of epigenetic drugs that have been extensively investigated in preclinical cancer models [127]. Previous studies have demonstrated the efficacy of these agents, particularly 5-azacytidine (AZA), its deoxy analog decitabine (DAC) and the next generation prodrug guadecitabine (SGI-110), in promoting epigenetic remodeling of tumor cells across different histological origins [128,129]. These compounds, classified as nucleoside analogs due to their structural similarity to cytidine nucleosides, interfere with DNA methylation during the S phase of the cell cycle, following incorporation into DNA, thereby inhibiting DNA methyltransferase (DNMT) enzymes [130]. DNMT1, the primary maintenance methyltransferase responsible for methylation of the newly synthesized DNA strand, represents the main target of these inhibitors. Its inhibition leads to global DNA hypomethylation, resulting in the re-expression of TSG and, in some contexts, oncogenes. At higher concentrations, DNMT1 inhibition may also induce apoptosis through replication fork arrest and genomic instability. DNMT3A and DNMT3B, which mediate *de novo* DNA methylation and are primarily active during development, may also contribute to tumorigenesis through aberrant methylation programs and partial functional redundancy with DNMT1, further highlighting their relevance as therapeutic targets. For these reasons, DNMT inhibition represents a promising antitumor strategy. Given their clinical efficacy, DHA have already been approved for the treatment of hematological malignancies [131] and are currently under investigation in solid tumors. The global hypomethylation induced by these agents can upregulate or induce the expression of multiple immune-related genes in tumor cells, including HLA-I, co-stimulatory molecules, and tumor-associated antigens (TAA), such as cancer-testis antigens (CTA) [132,133]. This results in enhanced tumor immunogenicity, increased immune cell infiltration, and the conversion of the TME toward a more inflamed phenotype. Consequently, tumor cells become more readily recognizable by the immune system, supporting the rationale for their use in solid tumors. Moreover, preclinical comparisons among different epigenetic agents have identified DHA as among the most promising

immunomodulatory compounds, either as monotherapy or in combination with other epigenetic drugs [134–136]. These findings support the development of combinatorial therapeutic strategies, particularly in combination with immunotherapy [137–139].

6. DHA to remodel the GBM microenvironment

DHA have been investigated in several preclinical GBM studies, with particular emphasis on their effects on CSC. Unlike AZA, DAC and SGI-110 are incorporated exclusively into DNA and have therefore been more extensively investigated in this context. DAC has been evaluated in preclinical settings to enhance the immunogenicity of GBM and render it more susceptible to immune-mediated eradication through the upregulation of multiple TSG, immune-related genes, and TAA, which are typically epigenetically silenced. Importantly, this compound was historically reported to cross the BBB, achieving appreciable concentrations within the brain, thereby attracting interest for GBM treatment [140]. However, more recent human pharmacokinetic studies suggest that DAC may exhibit limited BBB penetration, resulting in lower CNS exposure and thus warranting further investigation [141]. Several authors have analysed the expression of possible TAA in established and primary GBM cell lines that could enhance CD8⁺ T cells recognition. They observed that CTA such as MAGE-A1, MAGE-A3, and NY-ESO-1, are not consistently expressed in GBM. However, their expression can be induced by DAC treatment. These CTA can be processed and presented on the tumor cell surface *via* HLA-I and recognized by antigen-specific CD8⁺ T cells *in vitro* and *in vivo* [142–145]. Ma et al. further demonstrated increased expression of tumor-specific antigens (TSA) following DAC treatment, supporting the concept of DHA as immunomodulatory agents capable of enhancing tumor immunogenicity [146]. Similarly, Riccadonna et al. showed that DAC increases the expression of immune-related molecules on tumor cells, including MHC class I molecules (MHC-I), intercellular adhesion molecule 1 (ICAM-1), FasR, and PD-1. This was associated with enhanced CD8⁺ T cell-mediated cytotoxicity through improved antigen processing and presentation [147]. Konkankit et al. reported that DAC induces the expression of Th1-type pro-inflammatory cytokines, including IFN- γ and TNF- α [148], while Low et al. demonstrated restoration of the stimulator of interferon genes (STING) pathway, a key component of innate immunity involved in type I interferon and pro-inflammatory cytokine production [149,150]. These findings were further supported by integrated transcriptomic, proteomic, and HLA peptidomic analyses of GBM cell lines following DAC treatment. However, it should be noted that HLA-restricted peptides represent only a small fraction of the changes detected at the transcriptomic and proteomic levels, highlighting a discrepancy between multi-omics datasets [151–153]. Finally, single-cell RNA sequencing confirmed the upregulation of immunogenic signatures in specific tumor subpopulations following DAC exposure, as well as enhanced CD8⁺ T cell-mediated killing of immunogenic tumor cells [154,155]. More recently, the immunomodulatory effects of SGI-110 in primary human GBM cell lines have been demonstrated, yielding an immune phenotype comparable to that observed in inflamed melanoma-derived BM. In particular, this hypomethylating agent was able to induce a more immunologically permissive and ICI-responsive state in GBM cells, enhancing both innate and adaptive immune pathways while simultaneously inhibiting genes involved in cell proliferation and metabolism, thereby shifting tumor cells toward an immunotherapy-responsive phenotype [156]. These preclinical data have shown that DHA may remodel the GBM TME toward a more inflamed subtype resembling that observed in a subset of BM, thereby providing a therapeutic hypothesis for innovative epigenetic-based therapeutic strategies in GBM that could be translated into the clinical setting (Fig. 2).

7. DHA limitations in GBM

These preclinical studies have demonstrated the potential of DHA to

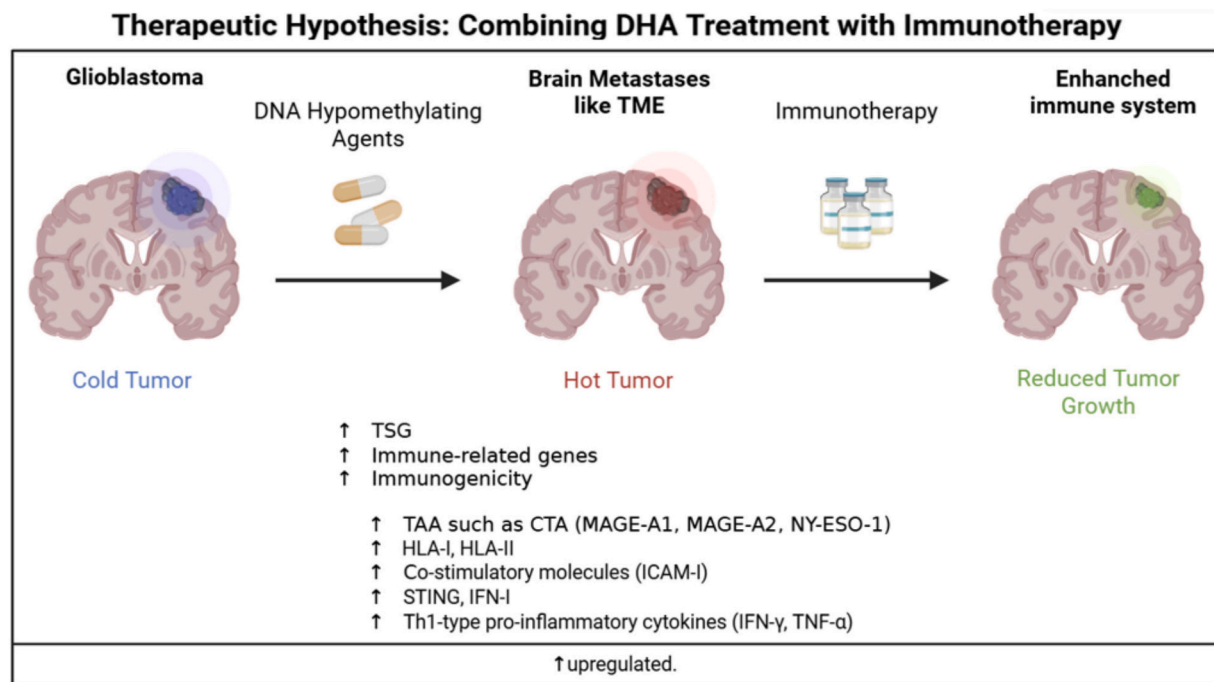


Fig. 2. DHA may remodel the GBM TME toward an inflamed phenotype resembling that observed in a subset of BM. By increasing the expression of tumor suppressor genes and immune-related genes, DHA may enhance GBM immunogenicity through activation of multiple immune-associated pathways. This may shift GBM toward a more inflamed state, increasing its susceptibility to immunotherapy, as observed in a subset of inflamed BM, and thereby supporting a combinatorial therapeutic hypothesis. Additional details regarding the immune-related genes and molecular pathways involved are discussed in the main text. Created in BioRender.com.

remodel the GBM TME through the induction of TSG and immune-related genes, thereby enhancing the cytotoxic activity of CD8⁺ T cells and promoting immune-mediated control of tumor growth. Nevertheless, the non-selective nature of these agents raises important safety concerns that may limit their clinical translation. Systemic administration may result in CNS cytotoxicity, genomic instability, and widespread DNA hypomethylation, potentially leading to the activation of oncogenes associated with cellular proliferation, neoangiogenesis, EMT, cellular plasticity, stemness, and immune evasion mechanisms. These effects may be particularly pronounced at high concentrations and following prolonged treatment exposure [157,158]. For example, PD-L1, which is already moderately expressed in GBM [58], may be further upregulated following DHA treatment through the activation of interferon pathway. Its dynamic and inducible expression is related to adaptive immune resistance. For this reason, PD-L1 has not been established as a prognostic marker in GBM and does not reliably predict response to immunotherapy. Nevertheless, its induction as well as that of other IC, may primarily reflect the broad immunomodulatory effects of DHA to sensitize GBM to immunotherapy.

However, differential gene expression analyses performed on primary human GBM cell lines have suggested that DHA treatment downregulates genes involved in cellular proliferation, cell-cycle regulation, lipid metabolism, and cellular respiration, while upregulating genes associated with opposing biological functions. In addition, components of the Hippo signaling pathway, which is implicated in glioma progression, were found to be inhibited following treatment [156]. For example, DHA treatment has been shown to downregulate the expression of ER Lipid Raft Associated 2 (ERLIN2) and Transmembrane Protein 41B (TMEM41B), two genes implicated in tumor progression and metastatic dissemination [159,160]. Conversely, DHA induces the upregulation of Zinc Finger and BTB Domain Containing 7 A (ZBTB7A), a gene associated with reduced migratory and invasive capacities in GBM cells [161]. Collectively, these preclinical findings highlight the potential of DHA to counteract tumor aggressiveness and progression despite their global hypomethylating activity. Nevertheless, the balance between

these opposing effects remains unclear, given the remarkable adaptive plasticity of GBM cells, particularly CSC, which may ultimately maintain or re-establish oncogenic pathway activation over time. Consequently, long-term studies are required to evaluate the durability and therapeutic stability of DHA-mediated remodeling of the GBM TME, as well as its potential to bridge the translational gap between preclinical findings and clinical application. Moreover, knowledge regarding the BBB permeability of DHA drugs remains limited, as comprehensive pharmacokinetic studies are still lacking. In general, compromised BTB may enhance drugs concentration within the CNS. However, the pharmacokinetic profiles of DHA are likely to be highly drug-specific, dose-dependent, and strongly influenced by the TME, in addition to the degree of BBB/BTB integrity. Most importantly, these agents alone appear insufficient to fully counteract the highly immunosuppressive GBM TME *in vivo*. To date, clinical trials investigating DHA, either alone or in combination strategies, have primarily focused on IDH-mutant gliomas (NCT03922555, NCT03666559, NCT03684811), with encouraging preliminary results. This is likely attributable to the biological features of IDH-mutant gliomas, which are characterized by widespread DNA hypermethylation and a more epigenetically maintained stem-like state compared with IDH wild-type gliomas. These alterations arise from inhibition of cellular demethylases by the oncometabolite 2-hydroxyglutamate (2-HG), resulting in the glioma CpG island methylator phenotype (G-CIMP), a truly hypermethylated tumor subtype that may be particularly susceptible to DHA activity, even if these agents may achieve relatively low CNS concentrations. Therefore, given the intrinsic biological differences between IDH-mutant and IDH wild-type gliomas, as well as the apparently greater responsiveness of IDH-mutant tumors to DHA-mediated epigenetic remodeling, alternative strategies may be required to maximize the therapeutic potential of DHA in the IDH wild-type setting. In this context, the ability of DHA to promote a more inflamed TME resembling that observed in a subset of BM, may represent a promising strategy to enhance tumor immunogenicity. Such immune reprogramming could increase the susceptibility of IDH wild-type GBM to immunotherapeutic interventions, providing a rationale for

combining DHA-based epigenetic therapy with immunotherapy to overcome the profoundly immunosuppressive microenvironment and improve disease control in this highly lethal malignancy.

8. Discussion and conclusion

Glioblastoma remains the most common, aggressive, and lethal primary tumor of CNS, with a dismal prognosis despite the current SOC and the development of novel therapeutic strategies. Several biological features contribute to its therapeutic resistance, including a highly immunosuppressive “cold” TME, low TMB, marked intratumoral heterogeneity, cellular plasticity, invasive growth, and the presumed protective role of the BBB [44–47]. Concurrently, advances in the understanding of CNS immunobiology and the immune landscape of brain tumors have provided important insights into the biology of BM derived from extracranial tumors. Notably, a subset of BM exhibits a more inflamed and immunogenic phenotype, often reflecting the intrinsic immunological characteristics of the primary tumor, as exemplified by melanoma-derived BM. This favorable immune landscape has supported the successful implementation of ICI in patients with “hot”, inflamed BM, demonstrating that effective antitumor immune responses can be elicited even within the CNS [26,29–32]. However, these observations cannot be generalized to all BM, as substantial differences exist among tumor histotypes influenced by the intrinsic characteristics of the primary tumor, the composition of the immune infiltrate, and the marked intratumoral and intertumoral heterogeneity. Nevertheless, the clinical success of ICI in this subset of inflamed BM has generated considerable interest in extending immunotherapeutic approaches to GBM. However, immunotherapy alone has thus far provided limited clinical benefit, primarily due to the profoundly immunosuppressive TME, low immune cell infiltration, and restricted tumor immunogenicity [48]. Furthermore, the intrinsic heterogeneity and plasticity of GBM enable the activation of multiple resistance mechanisms, making single-target therapeutic approaches largely ineffective. Consequently, future treatment strategies will likely require rational combination therapies capable of simultaneously targeting multiple tumor hallmarks and enhancing the initiation and maintenance of effective antitumor immune responses.

Among emerging therapeutic approaches, epigenetic therapies have attracted considerable attention. Epigenetic dysregulation is recognized as a fundamental hallmark of cancer [117], and several epigenetic drugs have already been approved for hematologic and solid malignancies. Among these, DHA have shown remarkable clinical success in hematologic cancers [127,131] and are increasingly being investigated for their immunomodulatory properties in solid tumors. These agents appear to remodel the tumor epigenetic landscape, restoring the expression of TSG and immune-related genes involved in antigen processing and presentation, including HLA class I molecules, co-stimulatory molecules, TAA, with particular attention to CTA. In parallel, DHA may modulate immune cell function and partially reverse immune exhaustion, thereby enhancing antitumor immunity [132–136]. In GBM, preclinical studies have suggested that DHA may remodel the TME toward a more inflamed phenotype resembling that observed in a subset of BM. Notably, SGI-110 has recently been shown to induce profound epigenetic remodeling in primary human GBM cell lines, promoting a more immunogenic tumor phenotype. Comparative analyses between GBM and melanoma-derived BM further demonstrated that SGI-110 treatment can shift the GBM immune landscape toward a more inflamed state resembling that of immunotherapy-responsive BM [156]. These preclinical findings show promise for the development of innovative epigenetic-based therapeutic strategies. However, as monotherapies, DNA appear insufficient to achieve durable tumor control, supporting the hypothesis of their use in combination with immunotherapy [137–139].

Importantly, the ability of DHA to enhance immunotherapy has already been demonstrated in both preclinical and clinical settings of other different solid tumors. The phase Ib “NIBIT-M4” clinical trial

(NCT02608437), which combined SGI-110 with ipilimumab in patients with advanced melanoma, showed increased tumor antigenicity, enhanced expression of TAA and endogenous retroviruses (ERV), TME reprogramming, and greater CD8⁺ T cell infiltration [162,163]. Building on these encouraging results, several clinical trials are currently investigating DHA–ICI combinations across different solid tumor histotypes (NCT02998567, NCT02901899), including the phase II “NIBIT-ML1” study (NCT04250246), which combines SGI-110 with ipilimumab and nivolumab in patients with advanced melanoma and lung cancer [164–167]. Collectively, these studies suggest that epigenetic therapies can enhance tumor immunogenicity by reversing epigenetic mechanisms that suppress immune responses and impair immune cell function. Therefore, the combination of DHA and immunotherapy may represent a promising strategy for transforming immunologically “cold” GBM into a more inflamed and treatment-responsive tumor, potentially reproducing in GBM the clinical benefits already observed in “hot”, inflamed BM, paving the way for future clinical investigations.

However, no clinical trials have yet evaluated their efficacy in GBM patients, since the non-selective nature of DHA raises important safety concerns in this peculiar tumor. In fact, systemic administration may result in CNS cytotoxicity, genomic instability, and widespread DNA hypomethylation, potentially leading to the activation of oncogenes, particularly at high concentrations and following prolonged treatment exposure. In contrast, IDH-mutant gliomas, which are truly hyper-methylated tumor subtypes, appear to be particularly susceptible to DHA activity, leading to the start of some clinical trials with encouraging preliminary results. Consequently, long-term studies are required to evaluate the safety, durability and therapeutic stability of DHA-mediated remodeling of the GBM TME, as well as its potential to bridge the translational gap between preclinical findings and clinical application.

In this context, the ideal candidates for DHA-based therapeutic strategies are likely to be patients who retain the characteristic immunosuppressive TME of GBM and may derive the greatest benefit from epigenetic immune reprogramming. Particular attention should be given to MGMT promoter methylation status, which may represent a clinically relevant stratification biomarker, as MGMT-methylated tumors could exhibit enhanced susceptibility to epigenetic modulation and immune activation. To maximize therapeutic efficacy, sustained epigenetic reprogramming should be achieved through prolonged low-dose immune-priming regimens designed to maintain tumor immunogenicity while minimizing the risk of treatment-induced tumor escape. Conversely, excessive DHA exposure may increase the likelihood of genomic instability, toxicity, and adaptive resistance mechanisms. In this setting, oral DHA formulations may be particularly attractive, as they enable continuous low-dose administration and prolonged epigenetic exposure, thereby promoting durable immune remodeling while limiting cytotoxic effects. Following epigenetic treatment, patients should undergo longitudinal molecular monitoring to evaluate treatment-induced changes in tumor biology. Global DNA methylation status could be assessed through circulating tumor DNA (ctDNA) and circulating tumor cells (CTC), while transcriptomic analyses of exosomes and CTC could provide insight into the expression of immune-related biomarkers, including HLA molecules, co-stimulatory molecules, ERV, and signatures associated with interferon and immune signaling pathways. Such an integrated molecular and immunological profiling strategy may facilitate patient stratification and identify those most likely to benefit from subsequent immunotherapeutic interventions aimed at overcoming immune suppression and controlling GBM progression.

Consequently, this therapeutic model, based on the combination of DHA-mediated epigenetic reprogramming and immunotherapy, warrants further investigation as a potential strategy to overcome immune suppression and improve disease control in this highly lethal malignancy.

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Data availability

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