Expert Opinion on Therapeutic Targets

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REVIEW

Glioblastoma multiforme: novel therapeutic targets

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ABSTRACT

Introduction: The increasingly detailed genetic characterization of glioblastoma (GBM) has failed to translate into meaningful breakthroughs in treatment. This is likely to be attributed to molecular heterogeneity of GBM. However, the understanding of the tumor microenvironment in GBM has become more refined and has revealed a wealth of therapeutic targets that may enable the disruption of angiogenesis or immunosuppression.

Areas covered: This review discusses the selective targeting of tumor-intrinsic pathways, therapies that target the GBM tumor microenvironment and relevant preclinical studies and their limitations. Relevant literature was derived from a PubMed search encompassing studies from 1989 to 2020.

Expert opinion: Despite appropriate target engagement, attempts to directly inhibit oncogenic pathways in GBM have yielded little success. This is likely attributed to the molecular heterogeneity of GBM and the presence of redundant signaling that allow for accumulation of adaptive mutations and development of drug resistance. Subsequently, there has been a shift toward therapies modulating the pro-angiogenic, immunosuppressive tumor microenvironment in GBM. The non-transformed cells in the microenvironment which includes endothelial cells, myeloid cells, and T cells, are presumably genetically stable, less susceptible to heterogeneity, and easier to target. This approach offers the highest potential for a therapeutic breakthrough in GBM.

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Glioblastoma; oncolytic virus; personalized therapy; signaling pathways; immunotherapy; tumor vaccines; therapeutic targets; oncogenic pathways; tumor microenvironment; tumor associated microglia and macrophages

1. Introduction

Glioblastoma (GBM) is the most common malignant primary brain tumor. After diagnosis, median overall survival (OS) is 15 months despite the current standard of care with surgical resection followed by adjuvant chemoradiation [1,2]. Early clinical trials have primarily focused on delineating the optimal dose and combination of chemotherapy and radiotherapy in conjunction with surgery; however, there has been little progress beyond the 'Stupp protocol' [3]. Indeed, in the past 25 years, only 1 out of 11 phase III clinical trials for GBM showed an increase in overall survival [4]. A broader survey of 44 phase III clinical trials from 1966 to 2004 found only a 7 month improvement in overall survival (from 8 to 15 months) in the experimental groups [5]. This review will compare the two main approaches for targeting GBM – reversing tumorigenesis versus revitalization of the microenvironment – and highlight key preclinical findings. We searched pubmed.gov for the following search terms with various combinations: GBM, therapeutic targets, pathways, immunotherapy, macrophages, personalized medicine, vaccines, oncolytic viruses. We reviewed relevant papers from 1989 to 2020.

2. Selective targeting of oncogenic pathways

Detailed molecular characterization of GBMs identified common mutations in genes such as epidermal growth factor receptor (*EGFR*), tumor protein 53 (*TP53*), isocitrate dehydrogenase 1 (*IDH1*), neurofibromin 1 (*NF1*), and phosphatase and tensin homolog (*PTEN*) that define classical, mesenchymal, and proneural subtypes [6,7]. While pathways affected by these mutations have been targeted therapeutically, these attempts have met with little success.

2.1. EGFR

Recent sequencing data have shown that 57% of GBM show evidence of gain of function mutation and/or focal amplification of *EGFR* [8]. EGFR is activated by ligands such as EGF, transforming growth factor alpha, heparin-binding EGF-like growth factor, amphiregulin, epiregulin, betacellulin, and epigen [9]. Ligand binding induces receptor dimerization and autophosphorylation by the intracellular tyrosine kinase domain, resulting in recruitment of effector proteins and activation of downstream signaling cascades including phosphoinositide 3-kinase, mitogen-activated protein kinase, and signal transducer and activator of transcription 3 (STAT3) pathways. The most frequently occurring EGFR mutation in GBM, EGFRvIII, contains a deletion within the extracellular domain of the receptor that renders it constitutively active. Importantly, single cell sequencing studies showed that wild-type and mutant forms of EGFR are almost mutually exclusive, with only 1-2% of cells co-expressing wild type EGFR and EGFRvIII [10].

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Article Highlights

- GBM is a molecularly heterogeneous disease with signaling pathway alterations that vary at a single-cell level. This likely accounts for the clinical failures of therapeutic agents directed against single oncogenic pathways, such as peptide vaccine against EGFRvIII.
- The GBM tumor microenvironment is pro-angiogenic. While the role for the FDA approved VEGF inhibitor, bevacizumab, is still being defined, inhibition of integrin, Notch, and Wnt pathways are additionally being explored for anti-angiogenic effects in GBM.
- Immunosuppressive TAMs in GBM offer a wide range of therapeutic targets that affect their migration (e.g. LOX), polarization (e.g. STAT3), and phagocytic capacity. (e.g. CD47).
- Checkpoint inhibition in GBM has met with little success so far, but combination therapy with STING agonists, FGL2 inhibitors, or oncolytic viruses may boost T cell infiltration into GBM and render checkpoint inhibitors more effective.
- Ultimately, targeting the tumor microenvironment in GBM may offer the best chance for a therapeutic breakthrough.

This box summarizes key points contained in the article.

Small molecule inhibitors targeting the tyrosine kinase domain of EGFR have demonstrated efficacy in non-small cell lung cancer. However, clinical trials evaluating these agents in GBM showed disappointing results [11–13]. While tyrosine kinase inhibitors were capable of engaging its target, as evidenced by EGFR dephosphorylation in tumors of treated patients, its downstream signaling pathways were unaffected, suggesting that they are regulated by redundant mechanisms [14]. Indeed, EGFR pathway alterations have been shown to be associated with alterations in other receptor tyrosine kinases, providing an escape route from anti-EGFR therapeutic targeting [15–17]. Similarly, a peptide vaccine against EGFRvIII which showed initial promise in phase I and II trials ultimately demonstrated no survival benefit in a phase III randomized controlled trial [18–20]. Interestingly, 82% of vaccinated patients demonstrated loss of EGFRvIII expression upon GBM recurrence, suggesting a shift toward non-EGFR dependent oncogenic pathways following antigen loss [21].

2.2. *IDH1*

IDH1 is an enzyme that catalyzes the oxidative decarboxylation of isocitrate to alpha-ketoglutarate as part of the Krebs cycle. *IDH1* mutations, most commonly R132 H, are genetic markers of secondary and proneural GBMs. They confer a gain of function in glioma cells, resulting in accumulation of an oncometabolite D-2-hydroxyglutarate (2-HG), which inhibits a key enzyme involved in histone modification and DNA methylation, leading to de-differentiation of GBM cells [22,23]. Consistent with this mechanism of action, IDH1 R132 H inhibitor reduced 2-HG production in *IDH1* mutant glioma lines and impaired growth of *IDH1* mutant glioma xenografts by promoting astrocytic differentiation, forming the basis for ongoing clinical trials [24]. *IDH1* mutations also contain immunogenic epitopes that elicit specific CD4 and humoral responses, and preliminary results of phase I study of an anti- IDH R132 H peptide showed safety and sufficient immunogenicity [25,26].

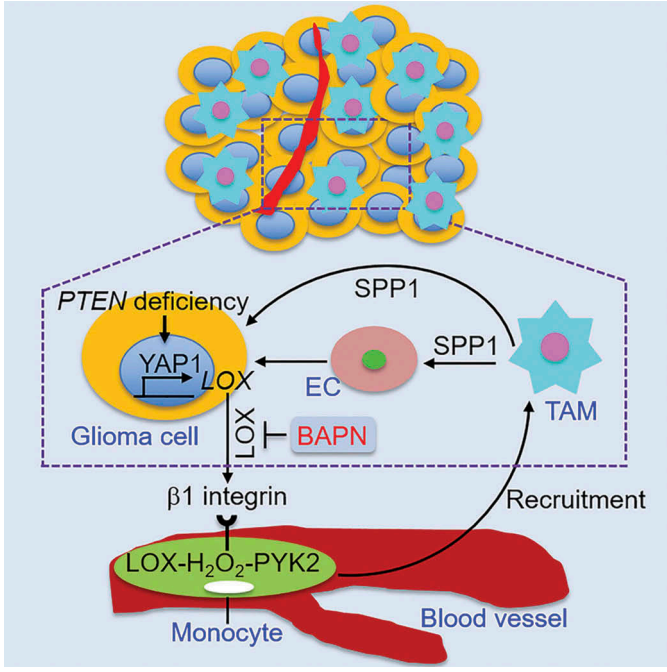

Figure 1. Overview of the p53 signaling pathway and therapeutic approaches. A schematic of the main p53 signaling pathway is shown. (a). Hypoxia, UV radiation, ionizing radiation, and chemotherapy initiate DNA damage, which recruits ATM and ATR to phosphorylate CHK1 or CHK2, respectively. In unstressed cells, p53 is suppressed by MDM2 and MDM4. Additional downstream components of this pathway, feedback loops, and parallel p53 isoforms are not shown but also play key roles in modulating p53 and gliomagenesis. Several methods to target mutant p53 include genetherapy and vaccination to reintroduce wild-type p53 into cells with mutant p53. Other methods include p53-MDM2 targeted agents (e.g., nutilins, RITA) to interrupt the p53-MDM2 interaction to allow p53 to induce cell senescence. (b) CHK1, CHK2, and DNA damage activate p53 that binds to p53 response elements in the genome to upregulate a variety of genes in GBM. Furthermore, mutant p53 up- and down-regulates a distinct set of genes in GBM. A method targeting mutant p53 includes agents that attach to mutant p53 (e.g., PRIMA-1, PhiKan083, SCH529074, MIRA-3, STIMA-1) and revert it to a wild-type form. By normalizing wild-type p53 or otherwise disrupt the effects of mutant p53, these treatments may impact tumors. ATM ataxia telangiectasia mutated, ATR ataxia telangiectasia related, MIRA-3: mutant p53 reactivation and induction of rapid apoptosis, PRIMA-1 p53 reactivation and induction of massive apoptosis-1, RITA reactivation of p53 and induction of tumor cell apoptosis, STIMA-1: SH group targeting and induction of massive apoptosis. Reproduced with permission (England et al. Current understanding of the role and targeting of tumor suppressor p53 in glioblastoma multiforme, *Tumor Biology*. August 2013, Volume 34, Issue 4, pp 2063–2074).

2.3. *p53*

p53 is a classic tumor suppressor that regulates many genes involved in the cell cycle and apoptosis cascades (Figure 1). Inactivation of p53 occurs by a variety of mechanisms, including amplification of p53 inhibitors such as murine double minute (MDM) 2 or MDM4, deletion of p53 stabilizers such as p14/ARF, or mutation in the *TP53* gene which occurs in 85% of GBMs [8,27]. p53 deficiency confers a growth advantage of glioma cells and facilitates malignant transformation of primary cortical astrocytes [28,29]. Strategies attempting to restore the p53 pathway using gene therapy or pharmacological approaches in a variety of cancers, including GBM, have shown little clinical efficacy [30]. However, MDM2 inhibition has emerged as a promising option to restore the p53 pathway, albeit only in 8-10% of GBMs with MDM2 amplification and wild-type *TP53* [31]. Novel nutlin-based agents showed efficacy in MDM2-amplified, *TP53* wild-type GBM

xenograft models, with adequate blood-brain barrier (BBB) penetration for clinical trials [32].

Mutant p53 is stabilized by chaperone activity of heat shock protein 90 which in turn is upregulated by histone deacetylase (HDAC) 6 by K294 deacetylation [33]. HDAC inhibitors such as suberoylanilide hydroxamic acid can therefore preferentially degrade mutant p53 and have shown anti-GBM activity in several studies [34–36].

2.4. GBM heterogeneity

Unfortunately, the efforts to target these oncogenic pathways have been hampered by tremendous molecular heterogeneity of GBM. Even individual cells within the same tumor exhibited mosaic expression of receptor tyrosine kinases and showed gene signature reflecting multiple glioblastoma subtypes [10]. Furthermore, cells from different locations of a multifocal GBM, as well as tumors from local and distant recurrences demonstrated divergent genetic profiles and varying drug responses [37]. Therefore, monotherapy targeting a single oncogenic pathway is unlikely to be beneficial in GBM, even with appropriate target engagement, because GBM will adapt to utilize other non-targeted mechanisms, conferring rapid drug resistance. One approach to address this problem is by tailoring treatment based on genetic makeup of the individual GBM [38]. However, this is prohibitively expensive and therapeutic agents targeting the unique genetic alterations may not be available. Another approach is to drive an immune response against a wide array of tumor antigens via dendritic cell vaccination, although the specific therapeutic targets are unclear [39–41].

Given these limitations due to GBM heterogeneity, we believe that the greatest potential for therapeutic breakthrough in GBM lies not in targeting the process of gliomagenesis *per se*, but its pro-angiogenic, immunosuppressive microenvironment. This strategy is unlikely to result in a cure, but may transform GBM into a manageable disease.

3. Selective targeting of tumor microenvironment

3.1. Angiogenesis

GBM is a highly vascularized tumor requiring extensive recruitment of blood vessels to combat hypoxia. Inhibition of vascular endothelial growth factor (VEGF) mediated angiogenesis is a therapeutic strategy at the center of several clinical trials for GBM [4,42,43]. However, a meta-analyses of 14 clinical trials found that these drugs do not improve overall survival (OS) in GBM, either as a single agent or in combination with chemotherapy. While anti-VEGF therapies such as bevacizumab, cediranib, and enzastaurin showed promising radiographic response rates and increased progression-free survival (PFS), they do not impact OS and are currently only offered as salvage therapy in refractory cases at our institution [44].

Besides VEGF, many other pathways and factors play important roles in the angiogenesis cascade. Integrins are membrane bound heterodimeric proteins involved in modulating the proangiogenic vs antiangiogenic microenvironment, activated by ligands of the extracellular matrix (ECM) [45]. Integrins αvβ3 and αvβ5 have been shown to be highly expressed on activated endothelial cells within the tumor compared to normal brain endothelial cells [46]. These ligated integrins have also been shown to regulate migration, invasion, and survival of endothelial cells. Disruption of the interaction between these integrins and their ligands in *in vitro* as well as *in vivo* promotes vascular regression, making them viable clinical targets [47,48]. Matrix metallopeptidases are also involved in these regulatory processes between the ECM, integrins, and endothelial cells by releasing pro-angiogenic factors into the ECM by proteolytic cleavage [45].

Other targets include the delta-like ligand 4/Notch pathway, shown to have a proangiogenic effect *in vivo*. A decoy delta-like ligand protein demonstrated reduced tumor angiogenic sprouting, vessel perfusion, pericyte coverage, and tumor growth, showing promise for clinical translation [49]. The Wnt family member 5A (WNT5A) pathway has been shown to mediate GBM stem cell (GSC) differentiation to endothelial cells, which recruit existing endothelial cells to support peritumoral satellite lesions [50]. These impactful preclinical studies show promise for the therapeutic targeting of these pathways.

3.2. Tumor-associated microglia and macrophages (TAMs)

The immune response to GBM is dominated by myeloid cells, consisting of CNS resident microglia and CNS infiltrating peripheral macrophages [51]. It is widely believed that these TAMs belie the immunosuppressive nature of the GBM microenvironment, reflected by the relative dearth of effector lymphocytes. Classically, TAMs were functionally classified into tumor suppressive M1 polarized phenotype in response to interferon γ (IFN γ) or toll-like receptor 4 ligands versus tumor supportive M2 polarized phenotype in response to IL4, IL-10, and/or IL-13 [52]. M2 polarized TAMs, defined by expression of cell surface markers such as CD206 and characterized by production of immunosuppressive IL-10, increases in number with glioma grade [52]. However, single cell sequencing of TAMs has revealed frequent co-expression of both M1 and M2 gene signatures in individual cells, suggesting that TAMs exhibit highly plastic phenotypes *in vivo* [53]. Classification of TAMs as M1 or M2 based on single marker such as CD206 may be inappropriate [54]. Nonetheless, this notion of M1/M2 TAM polarization has persisted as a useful conceptual framework, forming the basis for attempts to redirect TAMs to possess antitumor activity.

3.2.1. TAM reprogramming

Initial attempts to therapeutically reprogram TAMs in GBM targeted colony stimulating factor 1 receptor (CSF1R), critical for macrophage differentiation and survival. BBB-penetrant small molecule CSF1R inhibitors significantly increased survival in a proneural mouse model and slowed intracranial growth in patient-derived glioma xenografts by decreasing TAM accumulation, inhibiting M2 polarization, and increasing phagocytosis [55]. However, phase 2 trial demonstrated safety but no efficacy [56], which may be due to acquired macrophage expression of insulin-like growth factor 1 (IGF1) that provides

redundant signaling [57]. Future trials investigating CSF1R inhibitors in combination with other immunotherapy are being planned.

Other attempts to elicit M1 polarization have focused on intratumoral delivery of IL-12 using genetically modified viruses. Oncolytic herpes simplex virus (HSV) expressing IL-12 elicited M1 polarization, increased macrophage infiltration, and increased T effector to T regulatory ratios in xenograft and syngeneic models, which in combination with checkpoint inhibitors drastically improved survival [58]. Another study utilized replication incompetent adenovirus that can be induced to express IL-12 by administration of veledimex. In a syngeneic mouse glioma model, this approach led to preferential induction of IL-12 and IFN γ within the tumor, increased infiltration of effector lymphocytes, and tumor regression [59], and recent phase I trial using this approach showed promising results [60].

Another study showed that TAMs facilitate GBM by stimulating GSCs through pleiotrophin (PTN) secretion and activation of receptor protein tyrosine phosphatase receptor type (PTPRZ1). In addition, PTN and PTPRZ1 expression correlated with Cd11b+/CD163M2 TAMs and poor prognosis. Implantation of M2 macrophages without PTN inhibition promoted tumor growth, while implantation with simultaneous PTN inhibition abrogated the pro-tumorigenic activity of the M2 macrophages, highlighting the therapeutic potential of targeting this pathway in TAMs [61].

Accumulating data have demonstrated that STAT3 is a central mediator of immunosuppression in GBM [62–65]. STAT3 pathway is activated by various cytokines (e.g. IL-6 and IL-10) and growth factors (e.g. EGF), leading to the translocation of phosphorylated STAT3 into the nucleus and induction of target genes. In particular, STAT3 activation in TAMs limits their activation whereas STAT3 activation in GSCs promotes IL-10 secretion and inhibition of microglia/macrophage phagocytosis [66,67]. Given such a comprehensive role for STAT3 in the complex interplay between GBM and TAMs, STAT3 is an attractive therapeutic target. Indeed, STAT3 inhibitors were able to restore macrophage activation and their pro-inflammatory state in TAMs isolated from surgical specimens and peripheral blood mononuclear cells [63,65]. In addition, miR-124 inhibited STAT3 activation *in vivo* and showed promising efficacy in multiple preclinical glioma models [68].

3.2.2. Targeting TAM recruitment

TAM recruitment is mediated by chemokines such as CCL2 and fractalkine (CX3CL1). For instance, induction of CCL2 in a syngeneic rat GBM model promoted TAM accumulation and more aggressive tumor growth [69]. CCL2 has also been implicated in recruitment of monocytes that differentiate into immunosuppressive myeloid-derived suppressor cells (MDSCs) [70]. CX3CL1 signaling also promotes recruitment of TAMs, which is dependent upon CX3CL1 mediated CCL2 transcription and inhibited by the presence of the common V249I *CX3CR1* polymorphism [71,72]. Given the important role of CCL2 in TAM recruitment in GBM, targeting its receptor CCR2 is an attractive therapeutic strategy, with efficacy demonstrated in preclinical models [73].

Combined profiling and functional studies of PTEN deficient GBM models show that PTEN deficiency activates YAP1, which directly upregulates lysyl oxidase (LOX) expression. LOX acts as a chemoattractant for macrophages, which infiltrate and secrete osteopontin (SPP1), sustaining glioma cell survival and stimulating angiogenesis (Figure 2). LOX has been identified as a therapeutic target by a study showing that its inhibition suppresses tumor progression by abrogating macrophage infiltration [74]. SPP1 itself is a key driver of TAM recruitment in GBM, and its receptor integrin αvβ5 is highly expressed by M2 polarized TAMs [75]. GSCs additionally secrete periostin that recruits M2 TAMs via the integrin αvβ3 signaling, and disruption of this pathway inhibits glioma progression [76].

3.2.3. Enhancing TAM function

CD47 was identified as a 'don't eat me' signal on tumor cells that binds to SIRPa on macrophages leading to a signal cascade within the macrophages that inhibits phagocytosis [77]. Monoclonal antibodies blocking the CD47-SIRPa axis have shown efficacy treating GBM *in vitro* and *in vivo*, leading to phagocytosis of GBM cells by TAMs. The anti-CD47 treatment led to significantly higher levels of M1 macrophages in the tumor microenvironment, showing that anti-CD47 treatment either increases M1 polarization or enhances M1 macrophage recruitment from the periphery [78]. Despite the M1 bias, however, phagocytosis of tumor cells was enhanced in both M1 and M2 macrophages in response to CD47 blockade.

Recent studies have demonstrated that checkpoint inhibitors against programmed cell death protein 1 (PD-1) and programmed cell death ligand 1 (PD-L1), while traditionally thought to impact cytotoxic lymphocyte – tumor interaction, also affects TAM function as well. PD-1 is expressed by TAMs in a mouse model of colorectal cancer and in samples from colorectal cancer patients. PD-1 positive TAMs had a reduced capacity for phagocytosis of tumor cells, which was restored by PD-1/PD-L1 blockade, resulting in improved survival [79]. In an immunocompetent mouse GBM model, PD-L1 blockade enhanced macrophage phagocytosis of tumor cells and mediated radiation induced abscopal effect [80].

3.3. Checkpoint blockade

PD-1/PD-L1 checkpoint blockade has shown profound efficacy for malignancies such as non-small cell lung cancer, renal cancer, chronic Hodgkin's lymphoma, and gastric cancer [81–84]. PD-1 is expressed by activated T cells and its binding by PD-L1 tumor cells drives T cell apoptosis, anergy, and exhaustion which prevent cytotoxic T cell mediated tumor cell killing [85–87]. High levels of PD-L1 expression in tumors have been demonstrated to predict clinical efficacy of PD-1 inhibition [88–90]. Studies have shown that between 61% and 88% of GBM patients have tumors expressing PD-L1, indicating the potential use for PD-1 blockade in GBM treatment [91,92]. Despite promising success in preclinical studies, clinical trials have initially shown disappointing results for patients receiving PD-1 blockade therapy [93–98]. However, a recent randomized multi-institutional clinical trial showed that patients receiving neoadjuvant pembrolizumab had significantly increased overall survival compared to patients

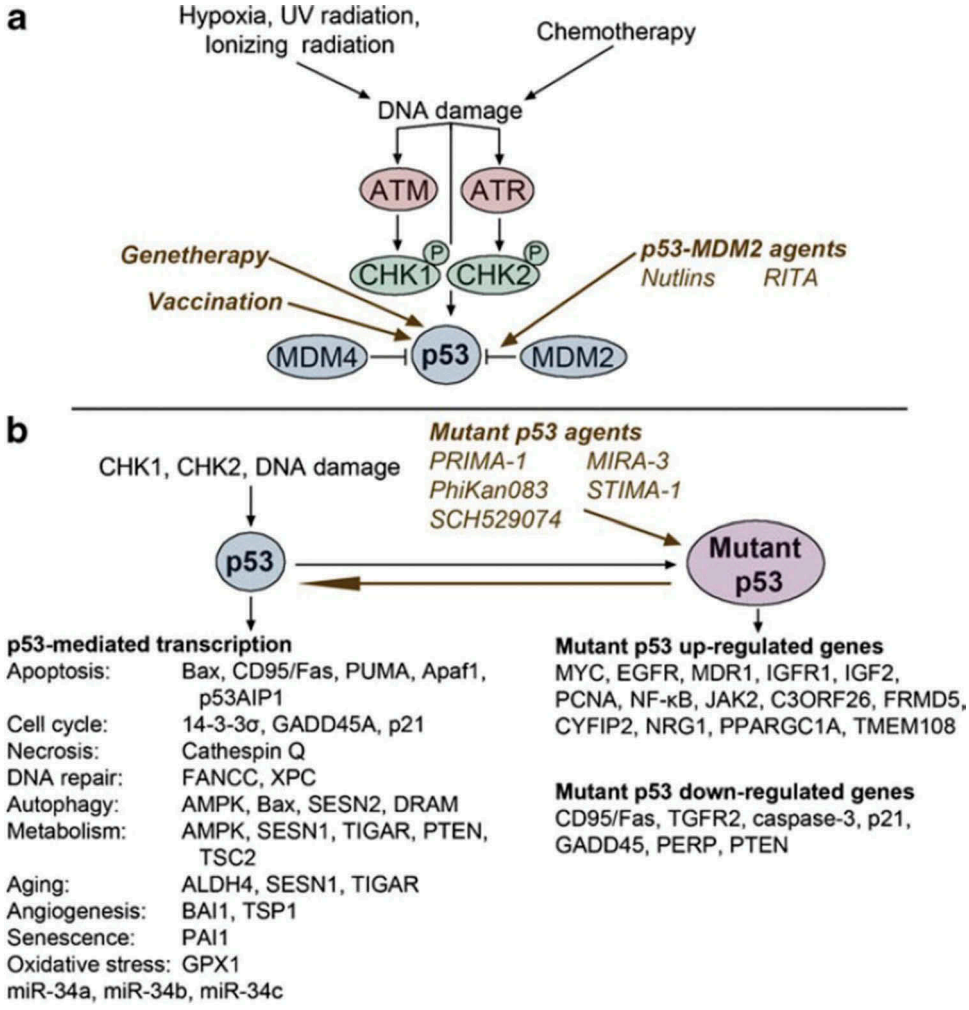

Figure 2. Infiltrating macrophages secrete SPP1, which sustains glioma cell survival and stimulates angiogenesis. In PTEN-null GBM models, LOX inhibition markedly suppresses macrophage infiltration and tumor progression. Correspondingly, YAP1-LOX and β1 integrin-SPP1 signaling correlates positively with higher macrophage density and lower overall survival in GBM patients. This symbiotic glioma-macrophage interplay provides therapeutic targets specifically for PTEN-deficient GBM. Reproduced with permission (Chen et al. symbiotic macrophage-glioma Cell interactions reveal synthetic lethality in PTEN-null glioma. *Cancer Cell*. Volume 35, Issue 6, 10 June 2019, Pages 868–884.e6.).

randomized to receive adjuvant pembrolizumab. Tumor infiltrating lymphocyte density was shown to be associated with survival, while presurgical tumor volume, postsurgical tumor volume, percentage resection, gross total resection, and dexamethasone dosage at time of registration were not, indicating that the survival benefit is mediated by augmenting the preexisting immune response [99].

Cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) is another checkpoint receptor extensively studied for cancer immunotherapy. CTLA-4 inhibition increases CD28, which allows for sustained T-cell activation [100]. Combination treatment of IL-12 and anti CTLA-4 antibodies in a syngeneic mouse GBM model led to tumor regression even at advanced disease stages in which monotherapy failed. The concurrent treatment led to a significant decrease in forkhead box P3 (FoxP3) positive regulatory T cells and an increase in effector T cells [101].

In the last few years, a number of primary tumors metastatic to the CNS have seen a dramatic improvement in prognosis with the application of various immunotherapies. Most recently, the use of combined nivolumab and ipilimumab in patients with melanoma brain metastases was shown to confer a significant survival benefit [102]. Despite the exciting developments of immunotherapies for other primary malignancies, applications in GBM remain difficult. The unique immunosuppressive profile of GBM is ultimately the greatest challenge to immunotherapy development. Contributing factors are the low number of infiltrating T cells and expression of 'exhaustion' markers in T cells [102–104]. Additionally, the combination standard of care treatment for GBM including chemotherapy, temozolomide, radiotherapy, and corticosteroids is known to further contribute to immunosuppression [104].

Recently, stimulator of IFN genes (STING) agonists has emerged as a promising approach for increasing T cell infiltration into the GBM microenvironment, rendering the tumor more susceptible to checkpoint blockade. In GBM, STING is activated by double-stranded DNA from necrotic tumor cells, driving type I IFN production and upregulation of chemokines that recruit T cells. Intratumoral delivery of STING agonists

elicited *Ccl5* and *Cxcl10* upregulation, increased the number of IFN γ producing CD8T cells, and extended survival in a mouse GBM model [105].

Other studies have shown that inhibition of FGL2 is another potential strategy to reverse immunosuppression in GBM and allow for more effective checkpoint blockade. Low levels of FGL2 expression along with high levels of granulocyte-macrophage colony-stimulating are associated with higher cytotoxic T cell infiltration and longer survival, while FGL2 overexpression increases CD4+ foxP3 regulatory T cells and M2 polarization of TAMs (Figure 3) [106,107]. In addition, FGL2 also inhibits dendritic cell maturation, antigen presentation capabilities, and ability to stimulate allogeneic T cell proliferation [107].

3.4. Oncolytic viruses – stimulating the anti-GBM immune response

Oncolytic viruses (OVs) are viruses that have been genetically modified to selectively replicate in and lyse tumor cells, emerging as a new treatment for GBM. Several OVs have entered clinical trials for patients with glioblastoma including herpes simplex virus, adenovirus, polio virus, measles virus, H-parvovirus, reovirus, and Newcastle disease virus [108].

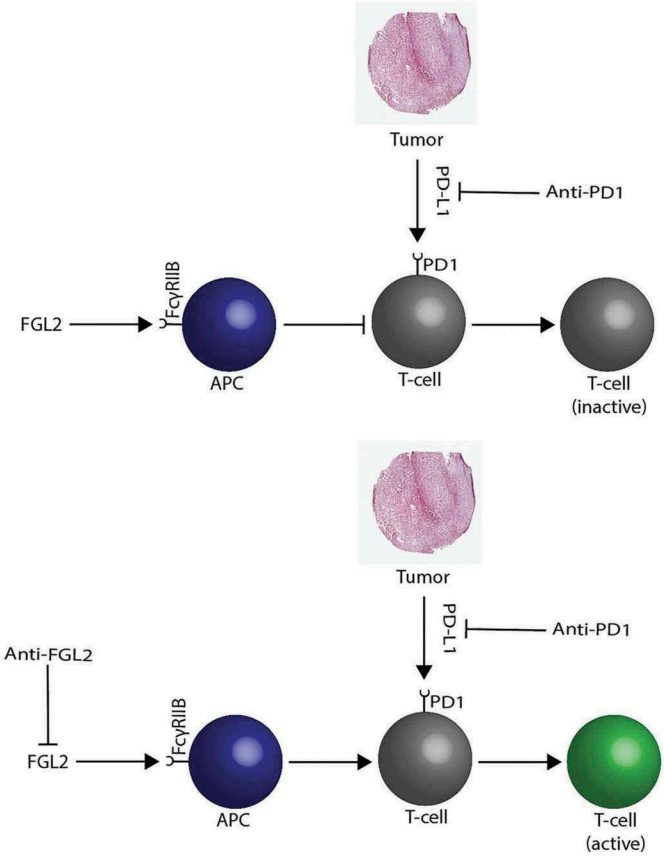

Figure 3. Schematic of FGL2 function. FGL2 activates the FcγRIIB on antigen-presenting cells (APC) which can override PD1 (programmed cell death) blockade, resulting in T-cell inactivation. FcγRIIB blockade will subsequently result in T-cell activation in conjunction with anti-PD1 antibody therapy. Reproduced with permission (Patel et al. Fibrinogen-like protein 2: a potential molecular target for glioblastoma treatment. *Expert Opinion on Therapeutic Targets*. 23:8, 647–649, DOI:10.1080/14728222.2019.1628220).

Traditionally, tumor eradication from oncolytic virotherapy was thought to be achieved through direct oncolysis. A recent paradigm shift in OV function now highlights the virally induced immune responses against the tumor that plays a critical role in their efficacy [109].

Combining checkpoint inhibitors with oncolytic viruses is an exciting approach that combines a promising immunotherapeutic strategy with the cytotoxic delivery system of oncolytic viruses. Despite promising results for checkpoint inhibitors in other brain malignancies [110], applications in GBM remain difficult. Possible contributing factors include poor penetration into the microenvironment through the BBB as well as low number of infiltrating T cells [102]. Saha et al. demonstrated the potential for this approach by engineering HSV to express IL-12 as well as the checkpoint inhibitors anti-PD-1 and anti-CTLA-4. This combination cured most mice in two separate glioma models. The authors showed that the treatment was associated with macrophage influx and M1-like polarization as well as increased T effector to T regulatory cell ratios, suggesting that microenvironment manipulation is necessary for the survival benefit [58]. Another study used HSV-1 armed with anti-PD1 antibodies to successfully treat two immunocompetent mouse models of GBM with a durable antitumor response rejecting second challenges of GBM implanted in the contralateral hemisphere [111].

Studies have also explored the use of viruses expressing immunotherapeutic agents other than checkpoint inhibitors. The recombinant polio:rhinovirus chimera virus (PVSRIPO) is currently in clinical trials for use against recurrent glioblastoma. A mechanistic study found that the virus activates the immune system by two separate mechanisms. Cytotoxic infection of malignant cells releases tumor-specific antigens for immune system priming. Additionally, sublethal infection of dendritic cells and macrophages yields type I interferon-dominant activation as well as development of tumor antigen-specific T cell responses [112]. G47Δ is another virus capable of modulating the immune system. G47Δ is a modified G207 virus with α47 gene deletion in order to disrupt the ICP47 protein, thus activating major histocompatibility complex class I antigen presentation [113]. Another study used HSV OV as a gene therapy vector carrying the human IL-12 cytokine to enhance T-cell-mediated immunologic effects [114,115]. Ultimately, the immunotherapeutic effects of OVs that stimulate an innate antiviral immune response and an adaptive anti-tumor T cell are now recognized to be essential to the success of this treatment strategy (Figure 4). Identifying viruses that produce robust immune responses in clinical trials and combining these viruses with neoadjuvant and adjuvant therapies will be central in the future of virus-based immunotherapy.

4. Conclusions

Traditional approaches that target oncogenic pathways that are intrinsic to the tumor have been met with limited success because of the molecular heterogeneity of GBM. Understanding and manipulating the GBM microenvironment is the key to addressing translational gaps. Abrogating the ability of GBM to sustain itself through angiogenesis and immunosuppression is a promising way to circumvent its defense mechanisms.

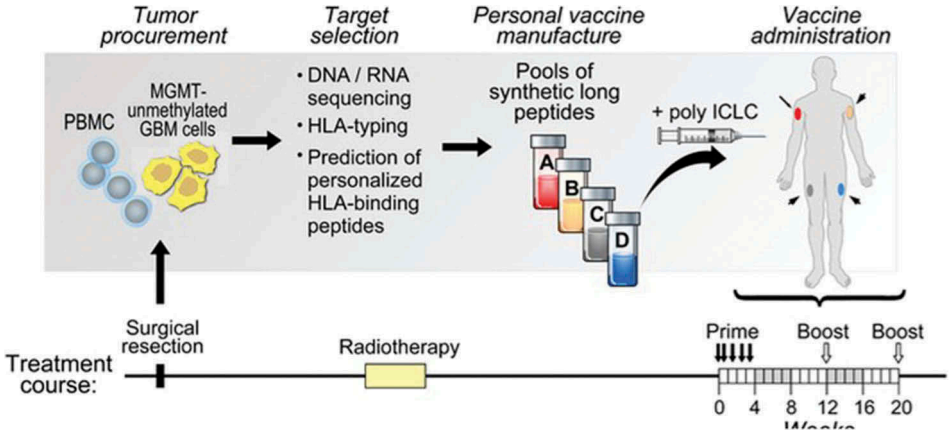

Figure 4. Schematic picture summarizing the events and factors related to successful oncolytic immunotherapy. From Martikainen and Essen, Virus-based immunotherapy of glioblastoma. *Cancers (Basel)*. 5 February 2019. doi: 10.3390/cancers11020186.

5. Expert opinion

Despite decades of research, overall survival for patients diagnosed with GBM remains dismal. Detailed molecular characterization of GBM revealed several key oncogenic mutations. However, the efforts to target these tumor intrinsic pathways have met with little success, best exemplified by clinical failure of the peptide vaccine against EGFRvIII. Despite achieving its intended effect and driving EGFRvIII antigen loss, the agent failed to extend survival in a phase III trial. This highlighted the greatest barrier to targeting oncogenic pathways in GBM; that is, GBM exhibits tremendous amount of heterogeneity from each tumor cell to tumor cell, from each distinct region of the tumor to another, and between *de novo* and recurrent tumors. Any attempt to downregulate one particular aspect of gliomagenesis may simply allow for another protumoral signaling to predominate, rapidly resulting in drug resistance. Therefore, while other monotherapeutic agents targeting other oncogenic pathways such as IDH1 mutant inhibitors are in pre-clinical development, it is difficult to imagine that they will be able to overcome this problem. One attempt to circumvent the thorny issue of GBM heterogeneity is 'personalized therapy.' For instance, dendritic cells pulsed with the patient's own tumor lysate or multivalent peptide vaccines have been shown to invoke an anti-tumor immune response against multiple tumor antigens. Personalized therapy is incredibly expensive and given the relatively rare incidence of glioblastoma, it may be cost prohibitive to actualize. It remains to be seen whether this strategy will be effective, but we believe that another promising approach in GBM therapeutics is targeting of the GBM tumor microenvironment.

The cells that comprise the GBM microenvironment – endothelial cells, microglia/macrophages, and T cells – are presumably genetically stable and less impacted by heterogeneity as the tumor cells. In addition, signaling pathways in these non-transformed are more accurately and easily recapitulated in pre-clinical models than tumor-intrinsic pathways because they do not have to account for the complex GBM heterogeneity, and wide range of genetic tools such as cell-type-specific knockout or reporter mice are available, facilitating clinical translation.

Various therapeutic targets in TAMs affecting their recruitment, polarization, function, and interaction with T cells have been identified, placing this immunosuppressive cell type at the forefront of the quest for effective GBM treatment. For example, STAT3 inhibitors and viral mediated inducible delivery of IL-12 are already in clinical trials. In order to advance the field even further, a few gaps in knowledge need to be addressed. For instance, the relative contribution of CNS resident microglia versus peripheral macrophages is still unclear. Deep immunophenotyping of these two similar cell types using discriminatory markers such as TMEM119 or CCR2 within different GBM niches would address this question. In addition, characterization of TAMs should transition from strict M1/M2 dichotomy to a truly functional definition based, for example, on their secretome or phagocytic capacity.

There has also been burgeoning interest in checkpoint inhibitors for GBM, given the remarkable clinical efficacy of these drugs in several systemic malignancies and their CNS metastases. However, their application in GBM have been hampered by the immunosuppressive microenvironment characterized limited numbers of infiltrating T cells that express high levels of exhaustion markers. Fortunately, STING agonists, FGL2 inhibitors, or oncolytic viruses may be able to reinvigorate this adaptive effector response, and may render GBM susceptible to checkpoint inhibition when used as combination therapy. Ultimately, we believe the greatest potential for alleviating the devastating prognosis of GBM lies in better understanding and therapeutic modulation of the immunosuppressive microenvironment.

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Declaration of interest

The authors have no relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript. This includes employment, consultancies, honoraria, stock ownership or options, expert testimony, grants or patents received or pending, or royalties.

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