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Immune Checkpoint Blockage - A Promising Strategy to Overcome Glioma Stem Cell Therapy-Resistance

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Abstract

Glioma stem cells (GSC) are postulated to provide a reservoir of cells with self-renewal capabilities, sustain tumor growth by generating differentiated non-stem tumor cells and may be responsible for recurrences after chemo-, radio- and surgical therapy. Elimination of the GSC population is regarded as a key to successful treatment of glioma, but high resistance of GSC to conventional glioma therapy remains a therapeutic challenge.

Preclinical studies show now that immune therapy for glioma using immune checkpoint inhibitors can achieve unprecedented efficacy that translates into significantly prolonged survival. Although these studies show a high anti-glioma efficacy of immune checkpoint inhibitors, especially when multiple pathways are inhibited, their impact on GSC is rarely addressed and the mechanisms behind it are far from fully elucidated. Nevertheless, recent evidence revealed that GSCs contribute more to tumor development than the non-GSC population by a more pronounced attenuation of immune surveillance. Therefore, abrogation of glioma-induced immunesuppression with immune checkpoint inhibitors seems to be a promising rationale to enhance anti-GSC efficacy. Clinical trials studying immune checkpoint blockage in high-grade gliomas have been recently designed with some of them already enrolling patients.

Keywords: Immune checkpoint; Glioma; Targeted therapy; Glioma stem cells

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Introduction

High-grade gliomas (HGG) remain one of the greatest challenges for cancer management, because current standard treatment has only achieved modest improvement of survival [1].

Despite improvements in surgical technique, radiation therapy and options for systemic cytotoxic therapy, the median survival for patients with newly diagnosed glioblastoma multiforme remains poor at 15 months with trimodality therapy [2].

Temozolomide (TMZ) remains the standard first-line treatment regimen for HGG despite the fact that more than 90% of recurrent gliomas do not respond to TMZ after repeated exposure[3].

Glioma stem cells (GSC), isolated from human glioma tissue by their high-level expression of stem cell markers, such as CD133, exhibit a pronounced malignant potential when injected into immunecompromised mice [4]. Subsequently, *in vitro* cultured GSC provide a reservoir of cells that maintains the tumor by generating differentiated non-stem tumor cells. Preclinical, as well as clinical studies show that GSC are responsible for recurrences after chemo-, radio- and surgical therapy [2, 5–7].

Neural stem cells and GSC share several common traits, such as sustained proliferation through asymmetric divisions and a highly efficient migratory capacity in the brain. There are also similarities between the neurogenic niche where adult neural stem cells reside, and the tumorigenic niche [8, 9]. Recently genome-wide transcriptional analysis identified two mutually exclusive GSC subtypes with distinct dysregulated signaling and metabolic pathways. Analysis of genetic profiles and phenotypic assays distinguished between a proneural and a mesenchymal GSC. Mesenchymal GSC display more aggressive phenotypes both *in vitro* and *in vivo* and are markedly more resistant to radiation than proneural GSC [10].

Evidence from preclinical studies employing patient-derived GBM cell lines suggests that GSC therapy-resistance is caused by the concomitant activation of multiple survival pathways and the presence of drug transporter genes that enable GSC to survive standard cytotoxic therapy [11–13]. It is also reported that GCS have the ability to remain in a quiescent state during chemotherapy resulting in very low number of chemotherapy-induced apoptosis and resuming proliferation after drug removal [14]. Resistance to Temozolomide (TMZ) in glioblastomas (GBM) is reported to occur due to a 32 to 56-fold increased expression of the DNA repair enzyme O6-methylguanine DNA methyltransferase (MGMT), which is increased in GSC compared to non-GSC from the same tumor [15]. These observations combined have led to the hypothesis that a large proportion of GSC may survive TMZ therapy, that ultimately gives rise to GBM recurrence. Immunofluorescence staining of human paired primary and recurrent GBM tissue showed that GSC are found in recurrent GBM at higher frequencies compared to their untreated progenitors (**Figure 1**).

While elimination of the GSC population is regarded as a key to successful treatment of cancer, the high resistance of GSC to conventional therapy remains a therapeutic challenge [15].

Recent preclinical studies suggest that glioma therapy using immune checkpoint inhibitors as monotherapy or in combination with various other therapies achieves unprecedented anti-glioma efficacy that translates into significantly prolonged survival [16]. Immunotherapy for gliomas represents one of the most promising strategies to overcome GSC resistance.

In the hereby-presented systematic review the authors summarize the studies assessing the anti-glioma efficacy of immune checkpoint inhibitors in preclinical studies and discuss the impact of this therapy on GSCs.

Search Methods

We selectively searched the PubMed database (http://www.ncbi.nlm.nih.gov/pubmed) for articles using the search terms “immune checkpoint and glioma”, “PD-L1 and glioma” and “CTLA-4 and glioma” employing the online search tool from the Endnote X7.2® citation program for Mac. All articles published until September 23, 2015 were included. The study authors carefully reviewed all full-text versions of the retrieved articles and summarized the studies characteristics and main findings in the presented systematic review. Articles without available full-text versions in English were excluded.

The authors state that no explicit review protocol was written and published and no funding or similar support has been received for conduction of this systematic review.

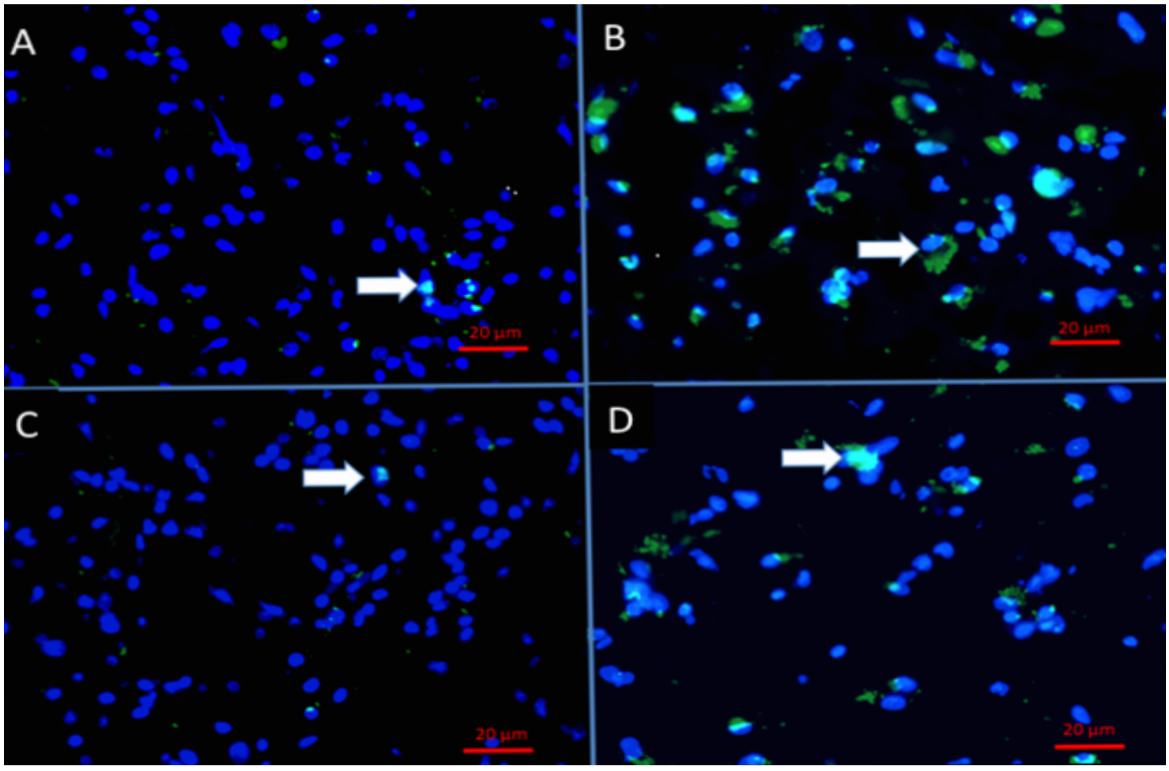

Figure 1 Glioma stem cells enrich in a recurrent glioblastoma following therapy-driven selection. Fluorescence microscopic image (40x magnification) of human glioblastoma multiforme paired primary and recurrent specimen harvested at the time of initial operation (A, C) and at the time of second surgery due to tumor relapse (B, D) after trimodality treatment with Stupp regimen. Nuclei were stained with 4´, 6-diamidino-2-phenylindole (DAPI; blue) and with stem cell markers anti-CD133 (green A, B) and anti-CD15 (green C, D). In the recurrent glioblastoma significantly higher frequencies ($p \leq 0.01$) of CD133+ and CD15+ glioma stem cells were encountered.

Review

Gliomas induce immunesuppression

Glioma cells express and secrete several immunosuppressive molecules that regulate immune cell functions [17, 18]. Therefore the central nervous system was considered suboptimal for sufficient antitumor immune responses, until recently. One of several glioma-induced immune compromising mechanisms includes the operation of immune checkpoints. The exact mechanisms of immunosuppression are only now being elucidated, but clearly involve a combination of factors including regulatory T cells (Tregs), tumor-associated PD-L1 expression, and CTLA-4 signaling [19]

Table 1 summarizes immune checkpoints and other regulators of intratumoral immune cells that have been pharmacologically controlled in preclinical glioma models to overcome glioma-induced immune suppression and to ultimately exert anti-glioma effects. Their physiological function is also described briefly in **Table 1**. While immune checkpoints help to prevent autoimmunity under physiological conditions, gliomas actively employ these mechanisms to evade immunological attacks. Immune checkpoints are frequently activated by glioma cells and exert their immune suppressive effects by direct suppression of the activity and number of glioma-attacking immune cells and indirectly by increasing the activity and number of immunosuppressive glioma-infiltrating Tregs and myeloid derived suppressor cells (MDSC). High-grade glioma patients suffer global compromise of their cellular immunity, characterized by dramatic reductions in CD4+ T cell numbers and function. It has been shown that this is attributed to an increased number of glioma-infiltrating Tregs [20]. Normally Tregs control tolerance of self-antigens by suppressing autoimmunity, while also enabling effective immune responses towards non-self-antigens. In GBM Tregs contribute to immunotherapeutic failure, ultimately leading to tumor progression [21]. The subpopulation of Tregs (CD4+CD25+Foxp3+) constitutes 5%-10% of CD4+ cells and plays a crucial role in suppressing anti-glioma immune response. The number of Tregs is significantly higher in patients with glioblastoma multiforme than in healthy controls and is inversely correlated with the frequency of glioma-infiltrating CD3+ activated T-cells. This may suggest that Tregs represent an important target for immunotherapy [22–24].

High-grade glioma (HGG) patients exhibit high expression levels of immune checkpoint-activating molecules within the tumor tissue and in serum. For example, CD200, a positive regulator of MDSC [25], and the immunesuppressive Fgl2 (Fibrinogen-like protein 2), are highly expressed in tumor tissue and serum. Fgl2 mRNA is significantly higher expressed in high-grade gliomas compared to low-grade gliomas; higher expression levels are therefore associated with shorter overall survival [26]. The clinically most important immune checkpoints PDL-1 (Programmed cell death protein 1 ligand), IDO (Indoleamine 2,3-dioxygenase) and CTLA-4 (Cytotoxic T-lymphocyte-associated protein 4) are also highly expressed in GBM and tightly correlated with survival [17, 24]. Several other immune regulatory molecules have been described to be of diagnostic and prognostic value and have been recently proposed as promising targets for immunotherapy of HGGs [27, 28].

Immunechekpoint inhibition

Immunotherapeutic strategies for the treatment of gliomas include the use of autologous stimulated lymphocytes, immunotherapy with cytokines and dendritic cells, immune checkpoint inhibitors, virotherapy, and tumor or peptide based vaccines and are currently under active investigation [29].

Especially immune checkpoint blockade with anti-CTLA-4 and anti-PD-1 have demonstrated encouraging results in clinical trials with other solid tumors and are yet to be performed for gliomas. Recent data suggest that this type of therapy may be particularly useful for tumors with high mutational burdens, which is the case in HGGs [30]. Therefore, it is thought that particularly immune checkpoint inhibitors will play a crucial role in immunotherapeutic approaches against HGGs [31].

Immune checkpoint molecules like PD-1, CTLA-4, and the T cell inhibitor TIM3 act as negative regulators of the immune system and are upregulated in GBM [32, 33]. Their immunosuppressive, glioma-propagating effects are substantially caused by their

Table 1 Regulators of intratumoral immune cells pharmacologically influenced in preclinical gliomas models to enhance anti-glioma immunity.

Regulator	Function
4-1BB (CD137)	Co-stimulatory molecule on activated T cells; drives proliferation of CD8+ T cells, increases pro-inflammatory cytokine production and plays an essential role in the formation of long-lived memory cytotoxic T cells (28).
CD200	Regulates myeloid derived suppressor cells; expressed predominantly by neurons in the central nervous system; down-modulating the activation state of perivascular macrophages and microglia through CD200R; regulates immune responses through multiple mechanisms including the activation of CD200R on myeloid-derived suppressor cells promoting tumorigenesis (24).
CD25	IL2 receptor alpha, expressed on activated Tregs and T cells (22).
CTLA-4	Immune checkpoint; inhibits T cells (29).
Fgl2	T cell inhibitor; generates and activates Tregs and myeloid-derived suppressor cells (30).
GM-CSF	Growth factor; stimulates dendritic cell maturation (31).
IDO	Suppresses T and NK cells; produced by Tregs and myeloid-derived suppressor cells (20).
Il-12	Cytokine; activates T cells and NK cells; polarizes naive helper T cells to adopt a TH 1 phenotype (32).
PD(L)-1	Immune checkpoint; inhibits activated CD8 T cells (33).
TIM-3	Immune checkpoint; inhibits CD8 T cells (34).

CTLA-4: Cytotoxic T-lymphocyte-Associated Protein 4; GM-CSF: Granulocyte-Macrophage Colony-Stimulating Factor; IDO: Indoleamine 2,3-Dioxygenase; Il-12: Interleukine 12; PD(L)-1: Programmed Cell Death Protein 1 (Ligand); TIM-3: T cell Immunoglobulin Mucin-3.

ability to activate and increase the frequency of intratumoral Tregs. The suppressive activity of Tregs has been implicated as an important factor limiting immune mediated destruction of tumor cells. Given the potently immunosuppressive function of Tregs, immune checkpoint inhibition is thought to inactivate Tregs, while simultaneously reactivating the cytotoxic lymphocyte response [34].

Table 2 summarizes the most important characteristics and main findings of preclinical studies that evaluated the anti-glioma efficacy of immune checkpoint inhibition in murine glioma models.

With few exceptions, immune checkpoint inhibitors administered as monotherapy have been reported to show little or no improvement of survival in preclinical trials. Whereas anti-CTLA-4 monotherapy lead to a survival advantage highly significant with 80% survival at day 100 in a study by Fecci et al. [20] and 50% survival at day 90 after tumor inoculation in a study by Grauer et al. [23], other authors report a significant effect only in early tumor stages [35] or no advantage in survival at all [36]. The variable response to anti-CTLA therapy is thought to be attributed to a time-dependent upregulation of CD25, CTLA-4, and other immune checkpoints on intratumoral Tregs during tumor growth [23].

PD-1 blockade has been uniformly reported to result in no survival benefit when administered as monotherapy [32, 33, 37]. Both, CTLA-4 and PD-1 blockade as well as anti-CD25 therapy exert their anti-glioma effects by reducing the number of highly suppressive Tregs within the growing tumor and provoking a CD4 and CD8 T cell dependent destruction of the glioma cells. Accordingly, anti-CD25 monotherapy results in similar survival advantages like anti-CTLA-4 monotherapy [23, 38].

The immune inhibitory molecules IDO and Fgl2 modulate the glioma-induced immunesuppression on a broader basis. Fgl2 generates and activates not only Tregs, but also MDSC and macrophages. Additionally to that, IDO physiologically suppresses T cells and NK cells. Hence, IDO blockage, as well as Fgl2 blockage lead to significant survival advantages over controls when administered as monotherapy [26]. This suggests that Fgl2 and IDO function as key immune-suppressive modulators and have potential as an immunotherapeutic target for treating GBM.

However, selective targeting of one component of a dysregulated pathway may be inadequate for a durable clinical response, given the intratumoral heterogeneity of GBM and hypermutated profiles displayed by tumor recurrences. Accordingly, in most studies the anti-glioma effects of combinations of two to three agents and radiotherapy have been assessed (**Table 2**). Whereas PD-1 blockage is ineffective as monotherapy the combination of PD-1 blockage and stereotactic radiotherapy results in a synergistic anti-glioma effect and long-term survivors [33, 37]. Given that tumor-infiltrating lymphocytes can express multiple checkpoints and expression of 2 or more checkpoints corresponds to a more exhausted T-cell phenotype, Kim et al. [33] added TIM-3 blockage to PD-1 blockage and stereotactic radiotherapy, which resulted in a significant improvement in survival compared with single and double treatment with an overall survival of 100% by day 146.

Also CTLA-4 blockage has been shown to be much more effective when combined with radiotherapy, with 40% survival of day 90 after tumor inoculation [39].

Noteworthy, Belcaid et al. [39] investigated the impact of timing of treatment start in relation to tumor cell inoculation on survival. Hence, three cohorts receiving radiotherapy on day 10 and starting with anti-CTLA-4 therapy on day 8, 10 and day 12, respectively, following tumor inoculation have been studied. Although the authors found no significant difference in survival, a trend is revealed of longer survival with earlier timing of anti-CTLA-4 therapy. Wainwright et al. [16], started triple immune checkpoint inhibition with CTLA-4 blockage on day 3 and day 7 post-inoculation and this resulted in 100% survival and 78% survival at day 90. Moreover, Agarwalla et al. [35] found significant anti-glioma efficacy of CTLA-4 blockage in the cohort that received treatment early after tumor inoculation (day 3), but not in the cohort starting treatment on day 12. Anti-CTLA-4 monotherapy starting as late as day 22 after tumor inoculation resulted in no significant survival advantage at all [36]. **Table 2** specifies the treatment starts after tumor inoculation for all treatment methods in reports studying immune checkpoint blockade in glioma models. The previously described preclinical data suggest that immune checkpoint inhibiton is efficacious only when tumor burden is very low. Translated into clinical situation this data suggest that immune checkpoint inhibition might only exert significant anti-glioma efficacy when administered after total/subtotal resection or in combination with multiple pathway blockage or other treatment modalities, e.g., radiotherapy.

Whereas reported long-term surival using multiple immune checkpoint blockages implicates considerable effects on GCS and therapy-resistance, the issue is rarely addressed experimentally in the existing studies. Huang et al. [40] studied the effects of NK therapy alone and PD-1 inhibited NK therapy on a stem cell enriched glioma cell line *in vitro* and GSC-enriched xenografts *in vivo*. Interestingly they found a significantly lower PD-L1 expression on GSC compared to the non-GSC differentiated cells. They hypothesized that GSC-resistance could be overcome by adding activated NK to the regimen, that have been reported to be effective against GSC. They demonstrated that inhibition of the PD-1/B7H1 pathway promotes the co-toxicity of NK cells against GSCs *in vitro* and in the intracranial GSCs model, mice that received PD-1-inhibited NK treatment showed longer survival and slower tumor growth.Recent evidence revealed that GSCs contribute more to tumor development than non-GSCs by a more pronounced attenuation of immune surveillance. GSCs express or secrete immune-suppressive factors that locally suppress the immune response. CD133+ glioblastoma cell populations secrete more TGFβ than CD133- glioblastoma cell populations [41]. This leads to a expansion of Treg population and attenuation of MHCII expression [42, 43]. Additionally GSCs are able to recruit tumor associated macrophages that support tumorprogression by enhancing immune-supressive microglia phenotypes [44]. Given that the GSC population especially exploits immune-suppression to promote tumor growth, abrogation of glioma-induced immunesuppression with immune checkpoint inhibitors seems to be a promising rationale to enhance anti-GSC efficacy.

Table 2 Characteristics and main findings of studies employing immune checkpoint inhibitors in preclinical glioma models.

Author/Year	Target(s)	Treatment/model	Therapy start (days after tumor inocuation)	Main findings
Kim JE et al., 2015	PD-1, TIM-3	Stereotactic radiosurgery+/- anti-PD-1+/- anti TIM/ Syngeneic glioma mouse model	Not specified in the article	Dual therapies demonstrated significant survival benefit over monotherapies. Triple therapy was synergistic and conferred a significant survival benefit with 100% survival by day 146.
Yan J at al., 2015	Fgl2	Anti-Fgl2/Syngeneic, orthotopic glioma mouse model	3	Reduced Tregs, M2 macrophages, PD-1 and myeloid-derived suppressor cells with anti-Fgl-2. Survival benefit of median 27 days with therapy compared to 17 days in control.
Huang BY et al., 2015	PD-1	NK cells +/- Anti-PD-1/ Syngeneic, orthotopic glioma mouse model	7	Dual therapy exhibits most pronounced cytotoxic effect towards GSC in vitro. Dual therapy shows significant survival benefit (median 44 days) over NK treatment alone (median 35 days).
Wainwright DA et al., 2014	IDO, CTLA-4, PD-L1	MT +/- anti-CTLA-4 +/- anti-PD-L1/Syngeneic, orthotopic glioma mouse model	7 (early) 14 (late)	Triple therapy shows significant increase in T-cell-mediated long-term survival with 100% survival (at 90 days) in case of early treatment start and 78% in case of late treatment start.
Moertel C et al., 2014	CD200(R)	Tumor lysate vaccination +/- anti-CD200(R)/ Syngeneic, orthotopic glioma mouse model	3	CD200R antagonist inhibited the expansion of murine myeloid derived suppressor cells in vitro and in vivo. CD200R antagonist peptide in glioma tumor lysate-derived vaccines slowed tumor growth and significantly enhanced survival.
Belcaid Z et al., 2014	4-1BB (CD137), CTLA-4	4-1BB activation or anti-CTLA-4 +/-focal radiation therapy/ Syngeneic, orthotopic glioma mouse model	10 for radiation 11 for 41BB activation 14 for anti-CTLA	Increased density of CD4+/CD8+ tumor-infiltrating lymphocytes and 50% long-term survival with triple-therapy (median 66.5 days), but not monotherapy.
Zeng J et al., 2013	PD-1	Stereotactic Radiosurgery +/- anti-PD-1; Syngeneic, orthotopic glioma mouse model	10 for radiation and anti-PD-1	Increased tumor infiltration by cytotoxic T cells, decreased Tregs and long-term survival in the combined treatment group (up to 40% survival at 90 days, median 52 days).
Vom Berg J et al., 2013	Il-12, CTLA-4	Intratumoral Il-12 +/- systemic anti-CTLA-4, Syngeneic, orthotopic glioma mouse model	21 for Il-12 22 for anti-CTLA	Drastic decrease of Tregs and increase of Teff with dual therapy, which led to tumor eradication even at advanced disease stages, but not with monotherapy.
Agarwalla P et al., 2012	GM-CSF, CTLA-4	GM-CSF expressing tumor cell vaccination +/- anti-CTLA-4, orthotopic syngeneic GBM mouse model	3 for GM-CSF tumor cell vaccination 3 (early) for anti-CTLA-4 12 (late) for anti-CTLA-4	Anti-CTLA-4 monotherapy improved survival only in early tumors stages; Vaccination improved survival over control. Sequential dual therapy synergistically prolongs survival and leads to 50% survival at day 60.
Grauer O et al., 2007	CD25, CTLA-4	Anti-CD25 +/- anti-CTLA-4/syngeneic murine orthotopic glioma model	-3 for anti-CD25 0 for anti-CTLA	Anti-CD25 monotherapy leads to decrease of Tregs and increase of Teff cell-depended glioma destruction: Anti-CTLA-4+ anti-CD25 leads to complete tumor eradication and 100% long-term survival (at day 90).
Fecci PE et al., 2007	CTLA-4	Anti-CTLA-4/Syngeneic murine orthotopic glioma model	7	Anti-CTLA-4 leads to normalization of CD4+ Teff cells and abrogates glioma-induced increases of Tregs and leads to 80% long-term survival.
El Andaloussi et al., 2006	CD25	Anti-CD25/Syngeneic murine orthotopic glioma model	7	Tregs isolated in murine brain tumors expressed FoxP3, CTLA-4, and CD62L. Mice treated with anti-CD25 lived significantly longer (median 40 days, 40% at day 70) than controls.

Fibrinogen-Like Protein 2; GM-CSF: Granulocyte-Macrophage-Colony-Stimulating Factor; MT: 1-Methyltryptophan; NK Cells: Natural Killer Cells; PD-1: Programmed Death-1; Teff: T Effector Cells; TIM: T-Cell Immunoglobulin Mucin-3; TMZ: Temozolomide; Tregs: T-Regulatory Cells.

Conclusion

Immunotherapy with immune checkpoint inhibitors is coming to the fore as a viable anti-cancer treatment modality, even in poorly immunogenic cancers such as GBM. Current data suggests that chemoradiation may not preclude the success of immunotherapeutics, as their effects may be synergistic.

Although these studies show a high anti-glioma efficacy of immune checkpoint inhibitors, especially when multiple pathways are inhibited, their impact on GSC is rarely addressed and the mechanisms behind it are far from fully elucidated. Nevertheless, recent evidence revealed that GSCs contribute more to tumor development than non- GSCs by a more pronounced attenuation of immune surveillance. Therefore abrogation of glioma-induced immunesuppression with immune checkpoint inhibitors seems to be a promising rationale to enhance anti-GSC efficacy.

Clinical trials studying immune checkpoint blockage in high-grade gliomas have been recently designed with some of them already enrolling patients.

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