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Critical Review

Irradiation to Improve the Response to Immunotherapeutic Agents in Glioblastomas

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Abstract

Purpose: Glioblastoma (GBM) remains an incurable disease despite extensive treatment with surgical resection, irradiation, and temozolomide. In line with many other forms of aggressive cancers, GBM is currently under consideration as a target for immunotherapy. However, GBM tends to be nonimmunogenic and exhibits a microenvironment with few or no effector T cells, a relatively low nonsynonymous somatic mutational load, and a low predicted neoantigen burden. GBM also exploits a multitude of immunosuppressive strategies.

Methods and Materials: A number of immunotherapeutic approaches have been tested with disappointing results. A rationale exists to combine immunotherapy and radiation therapy, which can induce an immunogenic form of cell death with T-cell activation and tumor infiltration.

Results: Various immunotherapy agents, including immune checkpoint modulators, transforming growth factor beta receptor inhibitors, and indoleamine-2,3-dioxygenase inhibitors, have been evaluated with irradiation in preclinical GBM models, with promising results, and are being further tested in clinical trials.

Conclusions: This review aims to present the basic rationale behind this emerging complementary therapeutic approach in GBM, appraise the current preclinical and clinical data, and discuss the future challenges in improving the antitumor immune response.

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Introduction

Glioblastoma (GBM) is the most common malignant primary brain tumor in adults and is an aggressive and invasive malignant neoplasm without a defined tumor boundary. Around the central core, there is a spread of infiltrating tumor cells that typically invade multiple lobes, even into both hemispheres of the brain.[1,2] The current standard of care for patients age <70 years with newly diagnosed GBM is maximal safe surgical resection, followed by radiation therapy (RT) and concurrent chemotherapeutic temozolomide (TMZ). The addition of TMZ improved the median survival time only slightly (by approximately 2.5 months),[3] and GBM remains one of the worst cancers in terms of survival rates. The median overall survival time is approximately 15 months, and the average 2-year survival rate is approximately 15%, which drops to a dismal 5% at 5 years.[4–6]

Ultimately, the tumor recurs, and most of the recurrences are within the radiation field. The median progression-free survival rate is approximately 30 weeks after the initiation of treatment.[7] Our current therapeutic strategy for this disease is clearly inadequate, as are the predictive markers at our disposal. In fact, the current treatment protocol was established in 2005 and has remained unchanged for more than a decade.[4] The only established marker is the methylation status of the O_6-methylguanine-DNA-methyltransferase promoter, which is a predictor of radiation[8] and TMZ resistance.[9] Hence, there is an urgent need for novel therapeutic concepts to improve local control and survival in this patient population.

Undeniably, one of the most promising therapeutic innovations in recent years, immunotherapy, has matured into a new treatment pillar against cancer with immune checkpoint blockade and the most successful modality to date. Immunotherapy is approved to treat several advanced cancer types and induces long-lasting clinical responses in a proportion of patients. However, certain cancers, such as GBMs, exhibit an innate resistance to most of these immune-modulating approaches.

At the forefront of new technologies that may help predict responsiveness to immunotherapies is next-generation sequencing for mutation load and neoantigen assessment, T-cell receptor sequencing, and multiplex immunofluorescence. The comprehensive picture that emerges incorporates genetic, epigenetic, gene expression, and proteomic information about the tumor with the immune signature of the tumor microenvironment (TME) and compiles the immune profile in the periphery.

High nonsynonymous somatic mutational load, high neoantigen burden, molecular signatures, DNA repair defects, and preexisting tumor-infiltrating cluster of differenciation 8+ (CD8+) T lymphocytes have all been correlated to the efficacy of immune checkpoint inhibitors in different tumor types.[10–12] These properties are presumably indicative of immunogenic tumors, at least those that are chemically induced.

In contrast, GBM and other poorly immunogenic tumors lack such properties and are unlikely to respond to single-agent immunotherapy. The question that remains is how to improve tumor immune recognition in the first place, and one suggested strategy is a combination of immune intervention and local irradiation. The rationale is based on the findings that irradiated tumors trigger an antitumor immune response better; therefore, immunotherapeutic agents are much more likely to enhance T-cell stimulation and drive antitumor reactivity in the context of RT.

In this article, we summarize the genetic, molecular, and immunologic contexture that dictates the lack of response to immunotherapy in GBM. Next, we detail the immunomodulatory effects of RT that could help improve the results of immunotherapies. Lastly, we report on the preclinical and clinical experimental results of radiation and immunotherapy combinations.

Genetic Features of Glioblastomas

In their analysis of mutational burden in different cancers, Alexandrov et al showed that GBM has one of the lowest burdens among 30 cancer types, with <1 somatic mutation per megabase. Yet, GBM clearly presents a characteristic mutation signature of mainly cytosine > thymine substitutions, which is probably associated with age.[13] Other teams have conducted an assessment of global mutational burden or nonsynonymous mutations among GBM samples and have confirmed the relatively low rate.[14,15]

The large data set of genetic, gene expression, and other features of human GBM samples generated by The Cancer Genome Atlas (TCGA) has led to a clinically relevant molecular reclassification of the disease. Based on gene expression profiling for instance, Verhaak et al defined 4 subtypes of GBM characterized by abnormalities in platelet-derived growth factor receptor-alpha (PDGFRA), isocitrate dehydrogenase 1 (IDH1), estimated glomerular filtration rate (eGFR), or neurofibromin 1 (NF1): Proneural (amplification of platelet-derived growth factor receptor-alpha and frequent IDH1 mutation), neural, classic (amplification of eGFR), and mesenchymal (loss of NF1 locus).[16]

Noushmehr et al identified another subset of GBMs that are hypermethylated and defined as glioma-CpG island methylator phenotype, which carries a mutation in the IDH1 gene and falls within the proneural subtype. Patients with the glioma-CpG island methylator phenotype are usually younger in age and show markedly better outcomes than the overall GBM population.[17] More recently, Ceccarelli et al performed a multiplatform

genomic analysis of 1122 diffuse gliomas from TCGA and identified DNA methylation-based subgroups. A subtype of IDH mutant glioma manifested relatively reduced DNA methylation, activation of cell cycle genes, and an unfavorable clinical outcome.[18]

eGFR amplification is a molecular characteristic of the classic subgroup and is observed in approximately 40% of GBMs. Approximately half of these patients harbor a subsequent eGFRvIII mutation. Some other genes are recurrently mutated in GBMs: Telomerase reverse transcriptase promoter (83%), TP53 (34.4%), phosphatase and tensin homolog (32%), PIK3CA (12%), and PIK3R1 (11.7%).[19] Basically, 3 major signaling cascades are genetically altered in GBMs: receptor tyrosine kinase/RAS/phosphoinositide 3-kinase pathway (altered in 88%), TP53 pathway (87%), and cell cycle retinoblastoma pathway (78%).[20]

A small number of newly diagnosed GBMs result from a germline biallelic mismatch repair deficiency and occur in the childhood. They demonstrate a hypermutator phenotype and harbor a high neoantigen load, which should result in increased tumor immunogenicity and responsiveness to immune checkpoint blockade (at least in theory). Similarly, genome sequencing in some gliomas that are recurrent after alkylating agent treatment revealed higher somatic mutation loads in relation to inactivating somatic mutations of the mismatch repair MSH6 gene.[21,22]

Antigenicity of Glioblastomas

The presentation of antigens is a prerequisite for any immune response, and tumors are no different. CD8+ T cells recognize antigenic peptides within the Major Histocompatibility Complex (MHC) class I on the tumor cell surface or dendritic cells during cross-presentation. This subset seems critical for tumor regression, but CD4+ T cells that recognize antigens in the context of MHC class II molecules assist in the tumor-specific antitumor immune response, as do other less specific immune effectors. In general, targetable antigens can be classified into 3 categories: tumor-associated antigens (TAAs), which are normal peptides that are aberrantly overexpressed in tumor cells; tumor-specific neoantigens (TSAs) expressed from nonsynonymous mutations; and viral tumor antigens.

The following TAAs have been tested as immunotherapeutic targets in GBM: interleukin (IL) 13Rα2 (testis antigen expressed nearly universally in malignant glioma cells)[23,24]; EphA2 (tyrosine kinase receptor overexpressed in GBMs)[23,25]; tyrosine kinase receptor human epidermal growth factor receptor 2 (overexpressed in up to 80% of GBMs)[26,27]; survivin (antiapoptotic protein expressed in a large number of distinct tumor types)[28,29]; melanoma-associated antigen-A1 (testis antigen)[26]; or gp100 (melanocyte protein).[26] One of the main concerns with targeting TAAs is that they make relatively weak antigens, and adverse effects owing to antigen expression on healthy tissues are possible.

In contrast, TSAs derived from nonsynonymous mutations are exclusively expressed on malignant cells and therefore keep nontargeted effects to a minimum. Two critical TSAs have already been identified and targeted in GBM. eGFRvIII, for instance, is the product of an in-frame deletion within the extracellular domain of eGFR, which is present in approximately 30% of GBM, but usually heterogeneously expressed within a patient's tumor.[19] The other well-known TSA is the result of R132H mutation in the IDH1 gene and is found in >70% of secondary GBMs but only sporadically in primary GBMs.[20] R132H mutation is thought to be a driver mutation, expressed by all tumor subclones, and is likely to be a good candidate for a targeted immunotherapy.

Lastly, human cytomegalovirus antigens, such as pp65, have been identified in the majority of primary GBMs but are undetectable in the normal surrounding tissue and provide another interesting target for the development of immunotherapies.[30]

Immunosurveillance in Glioblastomas

General wisdom dictates that the central nervous system (CNS) is immunoprivileged because of the protective role that the blood–brain barrier (BBB) plays, but recent data have challenged this paradigm and indicated that the peripheral immune system can indeed penetrate the CNS. The following are 3 potential ports of entry for T cells into the CNS: (1) postcapillary venules in the CNS parenchyma that are surrounded by the perivascular (Virchow-Robin) space; (2) leptomeningeal venules in the subarachnoid space; and (3) capillaries in the plexus choroid. For all 3 routes, T cells enter by a 2-step process and must first cross the vascular endothelium and then penetrate the glia limitans, which is comprised of astrocytic end-foot processes and covers the CNS.[31]

In analogy, CNS-derived antigens are drained into the deep cervical lymph nodes via 3 distinct pathways. The first is via the cerebrospinal fluid (CSF) that passes from the subarachnoid space through channels in the ethmoid bone to enter lymphatic vessels in the nasal mucosa and drain to cervical lymph nodes. Alternatively, CSF can drain to deep cervical lymph nodes via dural lymphatics. The third route is via the parenchymal interstitial fluid, which enters basement membranes in the walls of capillaries and arteries of the brain to once again reach the regional lymph nodes.[32]

Furthermore, GBMs are characterized by a compromised BBB owing to breaks in tight junctions and

decreases in BBB-associated pericytes. This loss of integrity of the BBB presumably facilitates the interactions between immune cells and GBM cell–associated antigens. Of note, the disruptive effect that brain tumors have on the BBB are further amplified by local irradiation, as demonstrated as early as 1990 by ^{99M}Tc-GH imaging,[33] which can be enhanced by radiation-induced vascular changes that encourage immune cell transexudation. Clearly, the dynamic to-and-fro of immune cells and the CNS is not something the concept of immune privilege can accommodate anymore.

Tumor-Infiltrating Lymphocytes in Glioblastomas

CD8+ cytotoxic effector T cells have the ability to directly kill target cells and are key for immune-mediated tumor regression. Upon tumor antigen recognition, they release cytotoxic molecules such as granzyme and perforin and secrete effector cytokines such as interferon (IFN) γ and tumor necrosis factor (TNF) α. Not surprising, tumor infiltration by CD8+ T cells tends to be a favorable prognostic factor in many malignancies[34–36] and a potential predictive biomarker for immune checkpoint inhibitors responsiveness.

Estimation of tumor-infiltrating lymphocytes (TILs) can be achieved via immunophenotyping techniques (immunohistochemistry or flow cytometry) or via T-cell–specific gene signatures analysis. In that respect, GBMs exhibit some of the lowest basal and preexisting TIL-associated genetic signatures across different solid tumor types.[15] For instance, using RNA-sequencing data from different TCGA tumor types, Rooney et al measured transcript levels of granzyme A and perforin and found that they were lower in GBMs than in other cancers.[37] Earlier, in 2013, Rutledge et al analyzed 171 GBMs from the TCGA and revealed that approximately half had no detectable lymphocytes at all, and one-third had lymphocytes in <50% of tumor tissue. Only 11% of GBMs had significant lymphocyte infiltration (ie, ≥50% of tumor tissue).[38]

For the most part, increased CD8+ TILs still positively correlated with survival in GBM,[39–41] but not in all cases.[42] Perhaps one of the most compelling aspects of this comes from the fact that immune infiltration appears to be different for different molecular subtypes of GBM. For instance, a strong positive correlation between TILs and survival was found in the case of mesenchymal transcriptional class–bearing mutations in the NF1 and retinoblastoma 1 genes, but much less so in the classic transcriptional class and in GBMs with eGFR amplification and homozygous phosphatase and tensin homolog deletion. This suggests that tumors that belong to the mesenchymal subtype are more immunogenic than those that belong to the classic one, but their response to checkpoint inhibitors is unknown. Future studies will have to examine effector and regulatory lymphocyte subsets in these tumors in more depth, including the functional activity of CD8+ lymphocytes.[38]

Glioblastoma-Associated Immunosuppression

High-grade gliomas profoundly modulate the immune system at both the systemic and intracerebral level. For one, GBMs are infiltrated by immunosuppressive immune cells. Approximately half of human GBM samples have detectable regulatory T cells (Tregs) coming in,[43,44] which is probably the result of the production of chemokine C-C motif chemokine ligand 2 (CCL2) by GBM-infiltrating macrophages and the increased expression of CC chemokine receptor 4 (CCR4) on other Tregs.[45,46] Intratumoral Tregs from GBM specimens strongly suppress effector T-cell proliferation and in turn reduce their ability to release proinflammatory cytokines IFNγ and IL-2.[47] However, the impact of GBM-infiltrating Tregs on survival remains controversial with both poor prognosis[48] or no impact on outcomes.[43,44]

Thirty to fifty percent of all cells in human GBMs are tumor-associated macrophages (TAMs), either intrinsic resident (microglia) or bone marrow-derived.[49] Glioma cells release several factors that attract TAMs to the tumor site, in particular SDF-1,[50,51] CCL2,[52] CSF-1,[53,54] and periostin.[55] In the TME, TAMs can acquire a tumor-promoting phenotype, designated as the alternative M2 phenotype that produces anti-inflammatory and immunosuppressive molecules (IL-10, TGFβ, arginase 1).[56–58] Other studies describe a continuum between the M1 proinflammatory phenotype and M2 immunosuppressive phenotype, including nonpolarized M0 macrophages and even monocytic myeloid-derived suppressor cells (MDSCs).[59,60]

STAT3 activation of TAMs appears to play a role in M2 phenotype polarization. Indeed, STAT3 inhibition can reduce the expression of immunosuppressive cytokines while stimulating the production of proinflammatory TNFα[61] and upregulating the expression of costimulatory molecules CD80 and CD86.[62] TAMs are also critical for glioma cell invasion and tumor growth, notably through the release of TGFβ and the activation of matrix metalloprotease-2.[63,64] The high proportion of TAMs in GBM and their protumoral properties make them an attractive therapeutic target. Different strategies are under investigation to prevent the recruitment of bone marrow–derived monocytes (eg, inhibition of SDF-1/CXCR4 signaling,[51] inhibition of CSF-1 signaling,[53] or converting protumorigenic TAMs into the tumor-attacking M1 phenotype).

MDSCs are increased in GBM tumor tissue and the peripheral blood of GBM patients, with a predominance of the CD15-positive granulocytic MDSC subpopulation

in the tumor tissue.[65] They express a high level of arginase, which inhibits immune responses mediated by T cells.[66] Moreover, glioma-infiltrating MDSCs upregulate programmed cell death-1 (PD-1) on infiltrating CD4+ T-effector memory cells and drive their functional exhaustion.[67]

Second, GBM cells express membrane-bound factors that directly inhibit the immune response. For instance, the majority of human GBMs are positive for immune checkpoint protein programmed death-ligand 1 (PD-L1),[68] although sometimes the level reported is as low as 3%.[69] PD-L1 confers immunosuppressive effects by promoting T-cell apoptosis, blocking T-cell activation, and inducing Tregs.[69] Its impact on survival in GBM is controversial, correlating with poor prognosis[69] or being unrelated to outcome.[68] Inhibiting the PD-1 axis has been shown to control tumor growth, generate long-term survivors, and induce a tumor-specific memory immune response, at least in syngeneic orthotopic murine GBM models,[70] but their relevance to the clinic can be questioned. There are several ongoing clinical trials that target the PD-1/PD-L1 pathway in newly diagnosed or recurrent GBMs (NCT02017717, NCT02617589, NCT02667587, NCT02311920, and NCT02336165).

T-cell immunoglobulin mucin 3 (TIM-3) is another immune-inhibitory molecule expressed on CD4+ and CD8+ effector T cells. TIM-3 is activated by its ligand, galectin-9, which leads to T-cell exhaustion and dysfunction.[71] TIM-3 expression has been observed in human GBM.[72] Interestingly, Liu et al showed that TIM-3 expression on CD4+ and CD8+ TILs and galectin-9 expression on tumor cells were higher in grade 4 than in grade 2 to 3 gliomas and that the level of both galectin-9 and TIM-3 correlated side-by-side.[73]

CD47 is expressed on GBM cells, and functions as a ligand for signal regulatory protein-α (SIRPα) on macrophages and dendritic cells. CD47 transmits a "don't eat me" signal. Willingham et al have shown that patients with GBM and increased CD47 expression had worse survival rates. They also demonstrated that anti-CD47 antibodies inhibited tumor growth and prevented metastasis in a murine model.[74]

Glioma cells also express other molecules that may inhibit immune effector cells, such as Fas-ligand that binds to its receptor on TILs and induces their apoptosis,[75] or lectin-like transcript-1 that represses natural killer (NK) cell activity.[76]

Third, GBMs produce various immunosuppressive factors. TGFβ is a versatile and powerful immunosuppressive cytokine that is highly expressed in GBM tumors and confers a poor prognosis.[77] TGFβ suppresses CD8+ T-cell activation and drives naive CD4+ T-cell differentiation into Tregs. TGFβ is produced by Tregs and TAMs in high quantities. Several anti-TGFβ therapeutic agents with different modes of action have been developed in GBM: TGFβ receptor I kinase inhibitor,[78–80] humanized anti-TGFβ monoclonal antibody fresolimumab,[81] and trabedersen (antisense oligonucleotide of TGFβ that downregulates the production of TGFβ at the translational level).[82] However, TGFβ biology is very complex, and its inhibition has so far led to disappointing results, possibly because no account has been taken of its receptor and other pathway mutations. Nevertheless, the inhibition of TGFβ may be a beneficial complementary approach to other immunotherapies under defined circumstances.[83]

Indoleamine-2,3-dioxygenase (IDO) is strongly expressed in GBM.[84] This enzyme catabolizes tryptophan to kynurenine, and is involved in the establishment of immune tolerance. In cancer, IDO contributes to an immunosuppressive microenvironment through the recruitment of Tregs[85] and suppression of effector CD8+ T cells. Not surprisingly, its expression is inversely correlated with survival in patients with GBM.[86] A few preclinical studies of IDO inhibitors against GBM have shown promise.[87,88] A phase 1 and 2 clinical study is ongoing to assess an IDO-inhibitor (indoximob) in patients with recurrent, TMZ-resistant GBMs (NCT02052648).

CCL2 is a chemokine that is secreted by GBM cells and GBM-infiltrating macrophages. CCL2 induces the recruitment of Tregs and MDSCs through CCR4 and CCR2 receptors, respectively, and contributes to immunosuppression in the TME. Low intratumoral CCL2 gene expression is associated with better survival in patients with GBM. In fact, the administration of a small-molecule CCR4 antagonist or CCL2-blocking monoclonal antibodies improved survival in orthotopic syngeneic mouse models and in an orthotopic human xenograft model.[45,89]

Type 1 IFN also affects the immunogenicity of GBM tumors. Silginer et al recently reported chronic constitutive autocrine IFN/STAT1 signaling in glioma cells and demonstrated that IFN/STAT1 impairs glioma immunogenicity and likely drives adaptive immune resistance. IFN drives PD-L1 and MHC class I and II expression alongside effects that are abolished by the disruption of its signaling. Glioma cells actually became more susceptible to NK cell-mediated lysis upon silencing of the IFN pathway, perhaps partly owing to compromised MHC expression.[90] Importantly, overexpression of an IFN/STAT1 pathway gene signature predicted poor outcome in the proneural GBM subtype.[91]

Although constitutive IFN signaling in glioma cells may impair their immunogenicity and confer a bad prognosis, IFN-I may also promote an antitumor immune response by bridging innate and adaptive immune responses. Using a preclinical model, Ohkuri et al showed that the production of IFN in the TME through stimulator of IFN gene (STING) activation allowed for the maturation of glioma-infiltrating CD11c + DCs and the subsequent activation of CD8+ T cells while decreasing the infiltration of Tregs. Equally, the intratumoral administration of STING agonists in glioma-bearing mice

enhanced tumor infiltration by effector cells and prolonged survival.[92] IFN is also a radiosensitizer but has a poor toxicity profile when administered extrinsically with RT.

Additional molecular factors are involved in glioma immune modulation. Glioma cells secrete other immunosuppressive cytokines, including IL-6,[93] IL-10,[94] and PGE-2.[95]

Lastly, systemic immune imbalances may additionally drive an immunosuppressive state. The blood neutrophil-to-lymphocyte ratio is one fundamental readout for systemic immune suppression, and when reaching levels >4 at the time of pretreatment, the results can be an independent prognostic indicator for poor overall survival in patients with GBM.[96] Similarly, circulating levels of MDSCs are often increased[65,66,97]; MDSCs express arginase, which diminishes the L-arginine level that is required for proper T-cell function.[97]

Patients with GBM usually present with lymphopenia that affects CD4+ T-cell subsets in particular and is often further negatively affected during RT and TMZ treatment. Patients continue to have low CD4 counts for at least the first full year of follow-up.[98] Tregs frequently represent an increased fraction of the CD4+ compartment in the blood, which results in an imbalance of Tregs relative to effector T cells.[99,100] This CD4+ lymphopenia affects clinical outcomes if CD4+ counts drop below 200/mm^3 2 months after the initiation of treatment, and CD4+ lymphopenia is associated with early death from tumor progression.[98]

Moreover, peripheral blood CD4+ T cells exhibit functional abnormalities, such as decreased proliferative responses, defective IL-2 production, and Th2-skewed cytokine profile.[101] Fecci et al showed that Treg removal from the peripheral blood of patients with GBM ex vivo eradicated T-cell proliferative defects and reversed Th2 cytokine shifts. Likewise, the systemic delivery of Treg-depleting anti-CD25 antibody allowed for the tumor rejection of established murine malignant astrocytoma SMA-560 in the absence of additional therapeutic intervention.[99]

Failure of Current Immunotherapeutic Strategies in Glioblastomas

Many immunotherapeutic agents are under active investigation in GBM, and yet no single agent has proven its efficacy to date. A case in point are the number of clinical studies that assess immune checkpoint blockade, mostly anti-PD1 and anti-PDL1, in newly diagnosed or recurrent GBMs. Available results are limited to date but do not seem to demonstrate any potential. CheckMate 143, a randomized phase 3 study comparing nivolumab (anti-PD1) to bevacizumab (anti-vascular endothelial growth factor) in patients with recurrent GBM, failed to show a survival benefit.

Vaccine-based approaches encompass a broad range of strategies, including peptide vaccines based on a single tumor-specific mutant protein (eGFRvIII; R132H mutant of IDH1) or on a panel of TAAs (autologous patient-derived dendritic cells [DC]) pulsed with autologous tumor lysate, peptide TAAs, tumor cell DNA or RNA, and viral-based vaccines, such as the CMV pp65-loaded DC vaccine. In the recent ACT IV randomized phase 3 trial, rindopepimut, an eGFRvIII peptide vaccine administered with standard treatment, failed to show a survival benefit among patients with newly diagnosed, eGFRvIII-positive GBM,[102] perhaps owing to antigenic modulation or loss.

The first results from a large international randomized phase 3 clinical trial of an autologous tumor lysate-pulsed DC vaccine (DCVax-L) added to TMZ maintenance therapy in patients with newly diagnosed GBM have been published.[103] Of note, all patients were allowed to receive the vaccine upon recurrence; as a result of this crossover trial design, nearly 90% of the patient population received DCVax-L. The vaccine was well tolerated, with a median overall survival of 23.1 months from surgery for the whole intent-to-treat population and an encouraging 34.7 months for patients with methylated O_6-methylguanine-DNA-methyltransferase. However, OS is not comparable between the 2 individual arms because of the study design.

Moreover, progression-free survival (primary endpoint) remains to be reported. In addition, phase 1 trials (notably GAPVAC-101 [NCT02149225] and Neo-Vax [NCT03422094]) have been investigating personalized neoepitope vaccine approaches, which is a compelling but highly complex therapeutic approach that requires the clonal tumor neoantigens for each patient to be identified.

Adoptive cell therapy with cytotoxic T cells are also being developed in the context of GBM in addition to genetically modified T cells that express a chimeric antigen receptor (CAR), bind to tumor antigens, and elicit T-cell responses in an MHC-unrestricted fashion. Different CAR T cells that target eGFRvIII,[104] IL-13Rα2,[105,106] human epidermal growth factor receptor 2,[107] and EphA2[108] are currently undergoing clinical testing, both with systemic administrations and intracranial infusions, but toxicity may be exacerbated in the brain, mainly owing to increased edema. They also may be toxic against normal tissue if they target non–tumor specific antigens.

Potential of Radiation to Improve the Success of Glioblastoma Immunotherapy

In addition to its direct cytotoxic effects, radiation can also generate T-cell tumor infiltrates in certain

circumstances. Most data are preclinical and indicate that RT might increase the immunogenicity of malignant cells. A plethora of mechanisms for this effect have been proposed: cell death and the release of TAA, upregulation of MHC I expression,[109] epigenetic modifications that lead to the re-expression of TAAs, and even genetic instability to increase the mutational load and the generation of neoantigens. Irradiation can also trigger an immunogenic cell death by releasing damage-associated molecular patterns. Immunogenic cell death is characterized by calreticulin translocation to the surface of the dying cells that acts as an "eat-me" signal for DCs[110]; the release of high-mobility group box 1 that binds to toll-like receptor-4 on DCs, promoting their maturation[111]; and the release of adenosine triphosphate that activates the inflammasome in DCs.[112]

In addition, the detection of DNA fragments in the cytosol after irradiation feeds into the cyclic GMP–AMP synthase STING pathway and stimulates the production of IFN type I, which is essential for the recruitment and activation of antigen cross-presentation.[113] The extent to which these multiple possible mechanisms are effective in human GBM is unknown, but current knowledge has certainly fed the notion that RT might prime and activate tumor antigen–specific immunity with effector T cells that traffic to the tumor.[114] There is a report that the T-cell receptor repertoire of TILs in a murine 4T1 mammary carcinoma model is broadened after irradiation.[115] RT can also increase the susceptibility of tumor cells to lysis by cytotoxic T and NK cells, in particular by upregulating Fas[116] and NKG2D-L.[117] Upregulation of several NKG2D ligands in irradiated mouse and human glioma cells rendered these more susceptible to NK cell-mediated cytolysis.[118] In other preclinical studies, in vitro and in vivo irradiation of glioma tumor cells increased their antigenicity by upregulating MHC I expression, which is associated with an concomitant increase in TILs[119] or ICAM-1 and CXCL16 levels.[120]

On the basis of these data, RT has been suggested repeatedly to generate a personalized endogenous antitumor vaccine in situ, and thus improve the clinical results of many immunotherapeutic agents, in particular immune checkpoint therapies,[121] but this is largely based on preclinical murine studies with highly immunogenic tumors and often using immune checkpoint inhibitors. However, some clinical case reports and a few retrospective studies do indicate synergistic effects of the RT and immunotherapy combination (again mostly using checkpoint inhibitors).[122]

Therapeutic Combination Strategies

In GBM, various strategies combining RT with agents that drive antitumor immune responses are being evaluated preclinically and clinically.

Preclinical Experience in Radiation Plus Immunotherapy Combinations

Removing the brakes

Stereotactic 10 Gy irradiation with PD-1 blockade produced durable complete tumor response and long-term survival in mice with intracranial gliomas when neither PD-1 blockade alone nor RT alone were able to. The authors demonstrated that a tumor influx of CD8+ T cells was the determining immunologic mechanism that mediated the combined treatment effect.[120] The same group investigated anti-CTLA4 monoclonal antibodies in the same experimental model and again found prolonged survival in the RT and immunotherapy combination arm without major impact of treatment timing.[123]

Pushing the accelerator

D137 (4-1BB) is a costimulatory receptor that is expressed by activated CD4+ and CD8+ T cells. Upon ligand binding, CD137 enhances the expansion, survival, and effector functions of antitumor T cells. Driving this pathway in the context of low-dose whole-brain RT (4 Gy × 2) can lead to significantly better survival than either modality alone, at least in mice, often pushing protective memory responses against a tumor rechallenge. CD8+ and CD4+ TIL density and the tumor-specific IFNγ production by splenocytes were much higher in mice that were treated with the combination therapy.[124] Glucocorticoid-induced, TNF receptor–related protein is another transmembrane costimulatory receptor, constitutively expressed on Tregs and inducible on CD4+ and CD8+ Teff cells. Binding of its ligand can provide dual benefits, namely inhibiting Treg activity while stimulating the Teff arm. Therefore, not surprisingly, a glucocorticoid-induced, TNF receptor-related protein agonist antibody given with 10 Gy irradiation induced significant tumor regression and prolonged survival in a murine intracranial glioma model. Of note, CD4+ Teff activation and skewing of macrophage polarization toward the M1 phenotype were some of the most obvious immune alterations.[125]

Betting on more than one horse

There is increasing evidence that glioma RT is best combined with immunotherapy that targets multiple pathways at once. A case in point is the success of the "triple bullet," which comprises RT with anti-CTLA4 and anti-CD137. This treatment not only resulted in prolonged survival, but also durable tumor-free survival in 50% of the mice.[123] Similarly, Kim et al demonstrated that only an aggressive triple glioma attack with 10 Gy RT with anti-PD1 and anti-TIM3 (another coinhibitory receptor) led to 100% long-term survival in the orthotopic murine GL261 glioma model. Both CD4+ and CD8+ T cell populations were shown to be critical for this response.

Long-term survivors demonstrated increased brain immune cell infiltration and activity and an immune memory.[72]

Intercepting complex immune-suppressive and protumor networks

Two preclinical studies have provided evidence that LY2109761, a specific inhibitor for TGFβ receptor I kinase, enhances the antitumor efficacy of fractionated radiation (5 × 2 Gy) in human GBM xenografts that grow intracranially[79] or subcutaneously.[78] On the other hand, the pharmacologic inhibition of IDO has been shown to synergize with chemoradiation and significantly prolong survival in the syngeneic orthotopic murine GL-261 glioma model compared with chemoradiation alone. Surprisingly, this effect was lost in mice deficient in complement component C3, which led the authors to conclude that IDO is masking a potent complement-dependent antitumor pathway that can be elicited by chemoradiation as long as IDO is blocked.[126]

Targeting vasculogenesis and myeloid recruitment may provide an additional therapeutic advantage when added to brain tumor RT. An HIF-1 inhibitor, small molecule inhibitor of the SDF-1/CXCR4 axis (plerixafor), and CXCR4 neutralizing antibody were all effective against an orthotopic GBM xenograft model when combined with RT. An influx of bone marrow-derived monocytes into the irradiated tumors, vasculogenesis, and tumor recurrence were all blocked by this treatment.[51]

Radiation and peripheral vaccination

Using the orthotopic murine GL261 glioma model, Newcomb et al found superior responses to low-dose whole-brain RT (4 Gy × 2) when administered with a peripheral vaccination with 25 Gy-irradiated, granulocyte-macrophage, colony-stimulating factor, transduced tumor cells compared with either treatment alone. Endpoints such as survival, immunologic memory, tumor MHC-I expression, and CD4+ and CD8+ lymphocytes infiltration were all measurably improved.[119]

Radiation and chimeric antigen receptor T cells

Weiss et al tested adoptive immunotherapy with chimeric antigen receptor (CAR) T cells engineered to express a chimeric NKG2D receptor in orthotopic, syngeneic, murine GBM models. They first demonstrated that NKG2D CAR-T cell treatment resulted in a significant proportion of surviving mice with long-term tumor control owing to the persistence of these cells at the tumor site. In light of previous data that indicate upregulation of NKG2D ligands on the glioma cell surface upon irradiation,[118] Weiss et al tested the combination of low-dose RT (4 Gy × 1) with NKG2D CAR-T cell transfer, which resulted in a synergistic antitumor activity, a prolonged survival, and reduced tumor volume in comparison with NKG2D CAR-T cells alone. Mechanistically, the authors observed improved trafficking of intravenously injected CAR-T cells to the tumor site and increased IFNγ expression by tumor-infiltrating CAR-T cells upon irradiation.[127]

Perhaps the biggest limitation of these preclinical studies is the lack of suitable murine models for GBM other than the GL-261 glioma, which is probably immunogenic and hence not representative for most GBMs in the clinic. Of note, many doses of RT are very low compared with what would be considered in the clinic, showing a more favorable tumor response.

Clinical Experience in RT and IT Combination

Several clinical studies are currently investigating RT in the context of immunotherapeutic agents (Table 1), and so far results are available from only a single study, namely the phase 2 clinical trial assessing RT with concurrent and adjuvant polyinosinic:polycytidylic acid (poly-ICLC) for adult patients with newly diagnosed GBM. Unfortunately, the trial was prematurely terminated after 31 patients were enrolled because of a change in the standard of care for GBM and the incorporation of TMZ into the treatment regimen. Compared with an appropriately matched historical group, the poly-ICLC plus RT treatment (without TMZ) appeared better, with a median survival time of 65 weeks, which suggests that poly-ICLC might have clinical activity against GBM. Moreover, the therapeutic combo was well-tolerated.[128]

Generally speaking, designing any clinical trial that combines RT and immunotherapy in GBM is challenging. The main goal is to improve local tumor control by leveraging the immunostimulatory properties of RT and enhancing the antitumor function of the immune system. Patient selection may be based on GBM subtype, and inflammatory or immune biomarkers should be tailored to the immunotherapeutic target.

The radiation treatment scheme and planning must be well defined to provide an optimal radiotherapeutic response while integrating the possibility of generating an immune response, which is not a trivial undertaking. For one, the radiation target volume must be limited because standard irradiation of a large GBM volume can induce severe lymphopenia. Yovino et al created a typical GBM radiation plan (8 cm tumor, 60 Gy/30 fractions) and estimated that the mean dose to the circulating lymphocytes was approximately 2 Gy, which is approximately lethal dose 50% of the radiosensitive blood cells.[129]

Second, concurrent TMZ chemotherapy and possibly the use of glucocorticoid agents to counter brain edema and neurologic symptoms can potentiate lymphodepletion. A study has shown that >40% of patients with GBM developed severe and persistent treatment-related lymphopenia (CD4+ lymphocytes <200/mm^3), which was significantly associated with poor overall survival.[98]

Table 1 Clinical trials that combine radiation and immunotherapy in glioblastoma

Immunotherapeutic target	Agent	New or recurrent glioblstoma	Tumor characteristic	Radiation scheme	Other associated treatment	Clinical trial identifier	Status
PD-1	Nivolumab	New	MGMT-unmethylated	Normofractionated	None	NCT02617589	Recruiting
PD-1	Nivolumab	New	MGMT-methylated	Normofractionated	Temozolomide	NCT02667587	Recruiting
PD-1	Nivolumab	New	None	Normofractionated	Temozolomide + GMCI (oncolytic adenovirus)	NCT03576612	Active, not recruiting
PD-1	Pembrolizumab	New	MGMT-unmethylated	Normofractionated	Temozolomide +/- HSPPC-96 (heat shock protein)	NCT03018288	Recruiting
PD-1	Pembrolizumab	New	None	Normofractionated	Temozolomide	NCT03197506	Recruiting
PD-1	Pembrolizumab	New	None	Normofractionated	Temozolomide	NCT02530502	Active, not recruiting
PD-1	Pembrolizumab	Recurrent	None	Hypofractionated	Bevacizumab	NCT02313272	Active, not recruiting
PD-1	Pembrolizumab	New	None	Normofractionated	Temozolomide + Vorinostat (HDAC inhibitor)	NCT03426891	Recruiting
PD-1 + CTLA-4	Nivolumab + Ipilimumab	New	MGMT-unmethylated	Hypofractionated	None	NCT03367715	Recruiting
PD-L1	Durvalumab	New	MGMT-unmethylated	Normofractionated	None	NCT02336165	Active, not recruiting
PD-L1	Durvalumab	Recurrent	None	Hypofractionated	None	NCT02866747	Recruiting
PD-L1	Atezolizumab	New	None	Normofractionated	Temozolomide	NCT03174197	Recruiting
PD-L1	Avelumab	New	IDH mutant grade II or III glioma transformed to gioblastoma after chemotherapy	Hypofractionated	None	NCT02968940	Recruiting
PD-L1	Avelumab	New	None	Normofractionated	Temozolomide	NCT03047473	Recruiting
GM-CSF	GM-CSF	New	None	Hypofractionated	Temozolomide	NCT02663440	Recruiting
GM-CSF + TLR3	GM-CSF + poly I:C	Recurrent	None	Not specified	None	NCT03392545	Not yet recruiting
TGF-β	LY2157299	New	None	Normofractionated	Temozolomide	NCT01220271	Completed
IDO	Indoximod	Recurrent	None	Hypofractionated	Temozolomide	NCT02052648	Active, not recruiting
CXCR4	Plerixafor	New	None	Normofractionated	Temozolomide	NCT01977677	Active, not recruiting
CSF1-R	PLX3397	New	None	Normofractionated	Temozolomide	NCT01790503	Active, not recruiting
TLR3	Poly ICLC (Hiltonol)	New	None	Normofractionated	None	NCT00052715	Terminated, has results

Abbreviations: CTLA4 = cytotoxic T-lymphocyte-associated protein 4; CSF1-R = colony-stimulating factor 1-receptor; CXCR4 = C-X-C chemokine receptor type 4; GM-CSF = granulocyte-macrophage colony-stimulating factor; GMCI = gene-mediated cytotoxic immunotherapy; HDAC = histone deacetylase; HSPPC = heat shock protein peptide-complex; IDH = isocitrate deshydrogenase; IDO = indoleamine-pyrrole 2,3-dioxygenase; Poly ICLC = polyinosinic:polycytidylic acid; TGF-β = transforming growth factor β; TLR3 = toll-like receptor 3; MGMT = O6-methylguanine-DNA methyltransferase; PD-1 = programmed cell death protein 1; PD1 = programmed death-ligand 1.

Treatment-induced lymphopenia can obviously also be a major barrier to the antitumor immune response; therefore, an RT strategy with a relatively small planning target volume is of interest, as in a hypofractionated scheme.

Regarding the best fractionation regimen to stimulate the antitumor immune response, preclinical data are controversial and favor anything between a hypofractionated,[130] single, very high dose[131] or conventional fractionated strategy.[132] However, recent data suggest that radiation administered in repeated high doses but below 12 Gy to 18 Gy optimally stimulates IFN-I production and adaptive immune responses.[133] The ideal timing of

Table 2 Potential immunotherapeutic agents to combine with irradiation in glioblastoma

Elimination of immune suppression	Checkpoint inhibitors	anti-CTLA4
		anti-PD1
		anti-PDL1
		anti-LAG3
		anti-TIM3
		TIGIT inhibitor
		anti-VISTA
		anti-NKG2A
	Anti-KIR	
	TGF-β receptor inhibitors	
	IDO inhibitors	
	MDSC recruitment inhibition	anti-HIF1α
		CXCR4 antagonist
		SDF-1 inhibitor
		CXCR2 antagonist
		CCR2 antagonist
		anti-CCL2
		anti-VISTA
	MDSC depletion	LXR agonist
	M2-M1 macrophage repolarizing agents	CSF1-R inhibitors
		anti-CSF1
		PI3K-γ inhibitor
		anti-VISTA
	STAT3 inhibitors	
	NFκB inhibitors	
	Inhibition of adenosine production	anti-CD39
		anti-CD73
		A2AR antagonists
Innate immunity:	TLR, RIG-I, MDA5 agonists	
Enhancement of APC function & maturation	STING agonists	
	Type 1 interferons	
	GM-CSF	
	Anti-CD40 agonist	
	"Don't eat-me signal" inhibitors	anti-CD47
		SIRPα antagonists
Enhancement of effector cells activity	Costimulatory agonistic antibodies	anti-CD137
		anti-OX40
		anti-CD27/anti-CD70
		anti-GITR
		anti-ICOS
	Cytokines	Interleukine-2
		Interleukine-12
Immunization	Active	Vaccine
	Passive	CAR-T cells

Abbreviations: A2AR = adenosine A2A receptor; APC = antigen-presenting cells; CAR-T cells = chimeric antigen receptor T cell; CCL2 = Chemokine (C-C motif) ligand 2; CD = cluster of differentiation; CTLA4 = cyototoxic T-lymphocyte-associated protein 4; CSF1-R: Colony-stimulating factor 1-receptor; CXCR2: C-X-C chemokine receptor type 2; CCR2: Chemokine (C-C motif) receptor type 2; CXCR4 = C-X-C chemokine receptor type 4; GITR = glucocorticoid-induced TNFR-related protein; GM-CSF = granulocyte-macrophage colony-stimulating factor; HIF1α = hypoxia-inducible factor 1 alpha; ICOS = inducible T-cell co-stimulator; IDO = indoleamine-pyrrole 2,3-dioxygenase; KIR = killer-cell immunoglobulin-like receptor; LAG3 = lymphocyte-activation gene 3; LXR = Liver X receptor; MDA5 = melanoma differentiation-associated protein 5; MDSC = myeloid-derived suppressor cells; NFκB = nuclear Factor kappa B; NKG2A = inhibitory receptor expressed on natural killer cells and CD8+ T-lymphocytes; OX40 = tumor necrosis factor receptor superfamily, member 4; PD-1 = programmed cell death protein 1; PD-L1 = programmed death-ligand 1; PI3K- γ = phosphoinositide 3-kinase-gamma; RIG-I = retinoic acid-inducible gene I; SIRPα = signal regulatory protein alpha; STAT3 = Signal transducer and activator of transcription 3; STING = stimulator of interferon genes; TGF-β = transforming growth factor β; TIGIT = T cell immunoreceptor with Ig and ITIM domains; TIM3 = T-cell immunoglobulin and mucin-domain containing-3; TLR = toll-like receptor; VISTA = V-domain Ig suppressor of T cell activation.

immunotherapy and radiation must also be defined and may vary with the mechanism of action of the immunotherapy applied. Therapies that promote cancer antigen presentation and T-cell activation should probably be administered starting before irradiation because available evidence suggest that later administration is ineffective. This may sound counterintuitive, but the aim is to develop systemic immunity with recirculating memory lymphocytes that can migrate to the tumor. Hypofractionated RT somewhat spares the lymphophenic effects and should be employed. Single fractions that are useful for the treatment of metastases are likely less effective for GBM.

Another clinically important topic is the appropriate imaging assessment of local tumor response and the distinction between pseudoprogression and real tumor progression. A transient increase in tumor volume is often observed after brain irradiation owing to an acute inflammatory and edematous reaction. On the other hand, a subset of patients who received immunotherapy also appeared to develop pseudoprogression, probably because of the infiltration of immune cells into the tumor site. To incorporate these considerations into imaging assessments, the immunotherapy Response Assessment for Neuro-Oncology criteria were established.[134]

Finally, monitoring the safety and tolerability of new combination strategies according to the Common Terminology Criteria for Adverse Events, version 4.03, is crucial. Immune-related adverse events are well known, and their management is standardized. These are likely increased in the proposed combination therapies, and there is also a possible enhanced risk of radionecrosis, which requires close monitoring by neurologic examination and imaging.

Conclusions and Future Challenges

To date, clinical trial data suggest that a single immunotherapeutic approach is unlikely to be sufficient to overcome the immune resistance of most solid tumors, and compared with other solid tumors, this topic remains largely underinvestigated in the context of brain cancer and especially GBMs. Cancer RT has significant immunomodulatory potential in its own right; therefore, combination radiation and immunotherapy has a strong rationale. Indeed, the results of preclinical studies are very encouraging in this regard, and data from ongoing clinical trials are eagerly awaited. Clinically, antitumor immunity will probably have to be tackled from multiple angles to really make a difference for irradiated GBM (Table 2).

Future clinical trials that combine immunotherapy with RT will need to incorporate an assessment of baseline immunity at the tumor site and in the blood to identify predictive biomarkers and tumor subtype. These correlative studies of immune monitoring should also schedule collections of blood and tissue specimens at various time points before, during, and after treatment to determine how effective treatment is at inducing specific immune responses and identify biomarkers correlated with clinical outcome. Lastly, a comprehensive immunobiologic analysis at recurrence or progression is also relevant to identify potential adaptive mechanisms.

Through the development of large databases, such as TCGA, our knowledge of recurrent genetic alterations in GBM has improved. Subsets of GBMs have been defined based on genetic profiles and have prognostic significance. More studies addressing the influence of tumor genotype on immunophenotype are now required to develop personalized treatment combinations. Immunotherapy clinical trials should also incorporate molecular subclass and determine treatment responses at least within the proneural, neural, classic, and mesenchymal subtypes.

In the upcoming years, we anticipate that new omics technologies (ie, genome/exome, transcriptome, proteome, epigenome, miRNome, metabolome, and microbiome), high-throughput data extraction and processing, multifactorial bioinformatics analyses, and collaborative multi-institutional efforts will allow for the identification of new biomarkers of radiation and immune response, the definition of new immune-related therapeutic targets, and the development of innovative personalized therapeutic strategies.

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