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Immunotherapy advances for glioblastoma

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Survival for patients with glioblastoma, the most common high-grade primary CNS tumor, remains poor despite multiple therapeutic interventions including intensifying cytotoxic therapy, targeting dysregulated cell signaling pathways, and blocking angiogenesis. Exciting, durable clinical benefits have recently been demonstrated for a number of other challenging cancers using a variety of immunotherapeutic approaches. Much modern research confirms that the CNS is immunoactive rather than immunoprivileged. Preliminary results of clinical studies demonstrate that varied vaccine strategies have achieved encouraging evidence of clinical benefit for glioblastoma patients, although multiple variables will likely require systematic investigation before optimal outcomes are realized. Initial preclinical studies have also revealed promising results with other immunotherapies including cell-based approaches and immune checkpoint blockade. Clinical studies to evaluate a wide array of immune therapies for malignant glioma patients are being rapidly developed. Important considerations going forward include optimizing response assessment and identifiying correlative biomarkers for predict therapeutic benefit. Finally, the potential of complementary combinatorial immunotherapeutic regimens is highly exciting and warrants expedited investigation.

Keywords: glioblastoma, immune checkpoint, immunosuppression, immunotherapy, vaccine.

Outcome for patients diagnosed with glioblastoma (GBM), the most common malignant primary tumor of the central nervous system (CNS), remains dismal despite clinical investigation of varied therapeutic strategies including intensification of chemotherapy,[1] targeting dysregulated cell signaling pathways,[2] and most recently antiangiogenic therapy.[3,4] Innovative and novel therapeutic approaches are clearly needed.

The efficacy of immunotherapy for cancer has been recently validated by U.S.FDA approval of sipuleucel-T, a dendritic cell (DC) cancer vaccine, as well as ipilimumab, a humanized monoclonal antibody against the cytotoxic T-lymphocyte antigen-4 (CTLA-4) immune checkpoint.[5,6] In addition, other immune-based therapies, including blockade of programmed death 1 (PD-1) and its ligand PD-L1 as well as bioengineered chimeric antigen-receptor T cells, have achieved dramatic antitumor benefit for solid tumor and leukemia patients, respectively.[7–10] These exciting results have fueled current efforts to evaluate such approaches in neuro-oncology. Interest in exploiting immune responses has been heightened by 2 intriguing historical observations. The first of these, initially noted in the 18th century, was the association linking infection and cancer regression.[11] Formal investigations, as exemplified by Coley's toxins, also generated encouraging early results.[12] Similarly, anecdotal reports have linked improved outcome with postoperative infection in brain cancer patients,[13] and a recent retrospective analysis has documented a 2-fold survival benefit in GBM patients who developed perioperative infections.[14] The second observation derives from epidemiologic studies noting an inverse relationship between allergy and glioma risk.[15–17] Although mechanisms underlying this relationship remain to be elucidated, it is hypothesized that enhanced immunoreactivity associated with atopy may provide collateral antitumor benefit. These separate observations raise the possibility that harnessed and directed immunoreactivity could provide a therapeutic opportunity to improve outcome for GBM patients.

In this review, we will discuss the current status of active vaccine, cellular, and modulatory immunotherapy approaches for

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Table 1. Immunotherapeutic strategies for cancer therapy

Immunotherapeutic Approach	Class of Agent	Representative Therapeutic	Citations
Cellular	Adoptive T-cell transfer		70–73
	Chimeric antigen receptor-engineered T cell	CTL019	9,10,78–83
Vaccination	Tumor-associated antigen vaccine	ICT-107; IMA950	100,101
	Tumor-specific antigen vaccine	Rindopepimut (EGFRvIII)	103–109
	Whole tumor lysate vaccine	DCVax	98,99,126–136
	HSP96 vaccine	HSPPC-96	147,148
	Tumor-specific mutation vaccine		125
Immunomodulation	Anti-CTLA-4 MAb	Ipilimumab; tremelimumab	6,173,198,199–207,213–216,231,250
	Anti-PD-1 MAb	Nivolumab; labrolizumab; pidilizumab	8,208,250
	Anti-PD-L1 MAb	BMS-936559; MPDL3280A; MEDI4736	7
	Treg-depleting reagent	Dacluzimab	240,241

GBM patients (Table 1). We will highlight potential benefits, risks, and limitations of these approaches and conclude with future considerations.

Organization of the Immune System

The normal immune system is divided into innate (pattern-recognition based) and adaptive (acquired) components. The evolutionarily older innate arm provides plants and higher species with immediate responses against invading pathogens. The innate immune system comprises macrophages, neutrophils, natural killer (NK) cells, basophils, eosinophils, and complement and provides a first line of defense against predetermined, germ-line pathogen-associated molecular patterns (PAMPS) via interaction with Toll-like receptors (TLRs) and other pattern recognition receptors (PRRs).[18] Innate immune cells can also present antigens to the adaptive immune system. The adaptive immune system, comprising T and B lymphocytes, evolved phylogenetically with vertebrate species. Adaptive immune responses are highly specific and long lived, resulting in immunological memory. B cells provide humoral immunity, while T cells, which are classified primarily as cytotoxic (CD8+), helper (CD4+), and regulatory (CD4+ FoxP3+), generate cell-mediated responses.

The major histocompatibility complex (MHC) displays epitopes of self or nonself proteins degraded intracellularly on the cell surface for T-cell interaction. MHC class 1 molecules are expressed on all nucleated cells and present epitopes to specific cytotoxic CD8+ T-cell receptor (TCR) molecules. CD8+ T-cell–mediated killing ensues when a primary signal, composed of TCR interacting with its specific antigen presented by an MHC class I molecule, is generated along with a secondary signal triggered by binding of CD28 on the CD8+ cell to its ligands (B7-1, B7-2) expressed by the antigen-presenting cell. Primary and secondary signaling also leads to clonal expansion of antigen-specific CD8+ cells. MHC class II molecules are conditionally expressed by all cell types but normally occur on professional antigen-presenting cells, including DCs, macrophages and B cells. Interaction of a T-cell receptor (TCR) with its cognate antigen presented by an MHC class II protein leads to activation of specific and long-lived effector CD4+ T helper cells and immunoregulatory T cells. CD4+ T helper cells establish and optimize several effector programs including: (i) Th1 responses, which are mediated by interferon-gamma (IFN-γ) to activate bactericidal macrophages and production of opsonizing and complement-fixing antibodies by B cells; (ii) Th2 responses, which are mediated by interleukin-4 to activate humoral B-cell responses; and (iii) Th17 responses, which are mediated by interleukin-17 and -22 to provide protection from infections at mucosal surfaces. In contrast, activation of regulatory T cells (Tregs), following binding to antigen presented by MHC II proteins, suppresses immune responses to limit immune-mediated damage and prevent autoimmune reactions.

Immune Privilege of the Central Nervous System: Fact, Fallacy, or In Between

The CNS has traditionally been perceived as being immunologically privileged, implying that the immune system is inactive in the CNS and fails to interact effectively with the systemic immune system. This dogma, which originated from experimental data demonstrating prolonged survival of tissues grafted in the CNS that were rapidly rejected upon grafting elsewhere,[19] has been further advocated historically by: (i) limitations of leukocyte entry imposed by the blood brain barrier;[20] (ii) lack of CNS lymphatics; and (iii) absence of native T cells in the CNS.[21,22] In reality, privilege of the CNS is somewhat limited, and growing evidence argues that the CNS is immunocompetent and interacts dynamically with the systemic immune system.[23] The blood-brain barrier is disrupted by tissue injury and inflammation, including that associated with malignant gliomas,[24,25] thereby facilitating entry of immune cells into the CNS. In addition, CNS antigens and T cells access cervical lymphatic tissue via drainage through Virchow-Robin spaces.[26–28] Furthermore, activated peripheral T cells and antibodies can circumvent the CNS to interact with target antigens,[22,29,30] while antigen-specific T cells acquire effector function, proliferate, and are retained in the CNS tumor microenvironment.[31] In addition, the resident macrophage population of the CNS, known as microglia, can express MHC class II antigens and T-cell costimulatory cytokines when activated[32,33] and are capable of presenting tumor-associated antigens to T lymphocytes.[34–36] Finally, active immune responses are well documented in experimental autoimmune encephalitis[37] as well as common clinical entities including multiple sclerosis, neurodegenerative conditions, paraneoplastic syndromes, stroke, and autism.

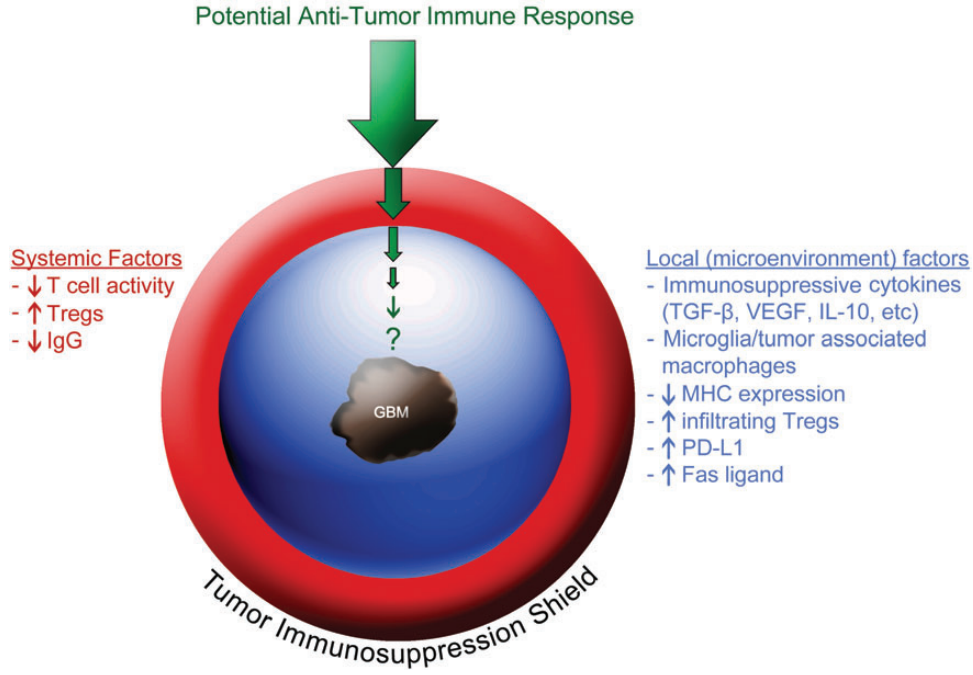

Fig. 1. Systemic and local (microenvironment) immunosuppressive adaptations elicited by glioblastoma tumors generating a protective shield against potential antitumor immune responses.

Suppression of Immune Activation: Systemic and Local Factors

Many cancers, including glioblastoma, exhibit multilayered protective responses that suppress endogenous antitumor immunoreactivity and may limit immunotherapy approaches.[38–40] Adaptive local and systemic immunosuppressive forces generated by tumors provide a protective shield to avoid immune rejection that instead fosters immune tolerance (Fig. 1). These forces are typically classified into systemic and local factors. Systemic factors, which act to diminish overall immune reactivity in GBM patients, include decreased T-cell responsiveness, immunoglobulin levels, and monocyte/DC function as well as increased circulating CD4+/CD25+/FoxP3+ Tregs.[40–44] In addition, corticosteroids, which are used to diminish cerebral edema, are immunosuppressive, while temozolomide chemotherapy ± radiotherapy can trigger prolonged lymphopenia.[45]

In addition, local immunosuppressive factors with complementary actions can act as a perimeter of immune defense within the GBM microenvironment and include: (i) MHC molecule down regulation;[36,46,47] (ii) production of immunoinhibitory cytokines such as transforming growth factor-β (TGF-β),[48] vascular endothelial growth factor,[49] prostaglandin E2,[50] interleukin-10,[51] and lectin-like transcript-1;[36,52–54] (iii) infiltration of immunosuppressive regulatory T cells (Tregs)[36,52,55–58]; (iv) polarization of glioma-infiltrating microglia/tumor associated macrophages which can account for up to 40% of glioma mass, toward the immunosuppressive M2 (alternatively activated macrophages) phenotype;[59,60] and (v) impairment of T cell function due to hostile physical factors such as hypoxia.[61] In addition, apoptosis of activated T cells can be promoted by Fas ligand expressed by malignant glioma cells.[62,63]

Many cancers, including GBM, also increase expression of immune checkpoint regulators such as programmed death-1 ligand (PD-L1).[64] Inhibitory immune checkpoint mediators are normally activated to attenuate appropriate immune responses in order to prevent autoimmunity and local tissue damage.

Cellular Immunotherapeutic Approaches for Glioblastoma

Adoptive T-cell transfer, a therapeutic strategy aimed at infusing T cells with high avidity against tumor antigens, originated from observation of a graft-versus-leukemia effect[65] and the use of donor lymphocyte infusions to achieve remission in patients undergoing allogeneic stem cell transplant.[66] Subsequent studies successfully utilized Epstein-Barr virus–specific T cells to treat posttransplant lymphoproliferative disease,[67] while Rosenberg et al pioneered the administration of expanded tumor-infiltrating lymphocytes to achieve durable responses in a subset of melanoma patients.[68] A follow-up study demonstrated that the coadministration of a lymphodepleting preparative regimen enhanced efficacy.[69]

Early adoptive transfer approaches for malignant glioma patients involved administration of T cells isolated from draining lymph nodes following subcutaneous injection of irradiated tumor cells with granulocyte-macrophage colony-stimulating factor (GM-CSF)[70,71] or ex vivo expanded autologous T cells cocultured with tumor cells.[72] More recently, the feasibility of expanding autologous, cytomegalovirus (CMV)-specific T cells from CMV-seropositive GBM patients has been reported,[73] and a phase I/II clinical trial is ongoing to evaluate the safety and antitumor activity of administering these cells to GBM patients (NCT01205334).

Next generation T-cell transfer therapy includes genetically modified T cells engineered for enhanced reactivity against tumor antigens. Chimeric antigen receptor (CAR) T cells are reprogrammed to express monoclonal antibody-binding domains that trigger T-cell activation and effector function upon tumor antigen binding.[74] CARs typically comprise an antigen-binding domain, an extracellular-spacer/hinge region, a transmembrane domain, and an intracellular-signaling domain. Second and third generation CAR T cells are under development that incorporate additional modular genetic modifications to enhance replicative potential, effector function, and in vivo persistence.[75,76] Theoretically, CARs can be designed to target any antigen. They also bypass MHC restriction based on their inherent direct antigen-binding capability. CAR T cells thus provide an attractive strategy to overcome MHC downregulation, an immunoevasive mechanism associated with many cancers including GBM.[47] In addition, by combining antigen-binding capability and T-cell activation function into one entity, CAR T cells may generate robust immune responses against tumor-associated antigens that are typically abrogated by central tolerance. Enhanced immunoreactivity may also trigger reactions against normal tissues, expressing target antigens as exemplified by a fatal cytokine reaction in a patient treated with ERBB-2 targeting CARs.[77] Nonetheless, dramatic proof-of-concept validation of CAR T-cell therapy has recently been achieved in refractory lymphoid leukemia patients.[9,10] CAR T cells also offer promise for CNS tumor patients.[78] Preclinical studies have shown benefit with CAR T cells engineered to target IL13Rα2l,[79,80] Her2,[81] and EphA2-positive[82] glioma tumors. Currently, clinical trials of CARs targeting EGFRvIII[83] (NCT01454596) and HER2 (NCT01109095) are ongoing for GBM patients.

Bispecific T-cell engagers (BiTEs) generate an immunological synapse between polyclonal cytotoxic T cells and tumor cells.[84] Composed of a single-chain variable antibody fragment (scFv) to the T-cell activation ligand CD3and linked to a tumor antigen-specific scFv, BiTEs are capable of mediating highly potent tumor cell lysis independent of traditional costimulatory molecules and MHC peptide recognition as well as clonal T-cell expansion and persistence.[85] Several BiTEs are in clinical development including blinatumomab,[84] a CD19/CD3 BiTE, that has induced durable cellular and molecular remissions in refractory acute lymphoblastic leukemia patients.[86] Systemic administration of a BiTE-targeting EGFRvIII has been recently shown in preclinical studies to effectively treat well-established EGFRvIII-positive intracranial GBM tumors.[87]

Vaccination Therapy for Glioblastoma

Vaccinations sensitize the immune system against target antigens and have successfully prevented or eradicated diseases such as tetanus, polio, smallpox, diphtheria, and pertussis by generating robust, yet specific, immune responses including immunological memory to trigger reactivation upon future antigen exposure. Nonetheless, only modest, inconsistent immune responses and antitumor benefits have been reported about cancer vaccines to date.

A major determinant of vaccine immunogenicity is antigen choice, and a wide array of antigens has been explored in cancer vaccine trials. Elucidation of factors optimizing vaccine immunogenicity will likely include careful evaluation of both quality and quantity of incorporated antigens. Tumor vaccine antigens are broadly classified as tumor associated and tumor specific (Table 2). Tumor-associated antigens (TAAs) are preferentially derived from native proteins or selectively expressed by tumor cells, but they may also be found on normal cells. Native proteins typically generate relatively weak immune responses due to central tolerance, which is the naturally occurring mechanism preventing immune reactivity against normal cells. In contrast, tumor-specific antigens (TSAs) are exclusive to tumor cells and thus typically elicit robust immune responses in a manner analogous to microbial antigens incorporated by infectious disease vaccines.

Table 2. Antigen categories for antitumor vaccination

	Tumor-associated Antigens	Tumor-specific Antigens
Examples	EphA2; Her-2; IL13Rα2; GP100; Mage-1; survivin; hTERT; Trp-1; Aim-2	EGFRvIII
Advantages	Multitude characterized Available for most patients Ability to combine multiple targets into cocktail vaccine Lowered risk of immune escape	Elicit potent, robust, and specific immune responses
Disadvantages	Reduced immune response due to central tolerance if expressed by normal tissues	Available for subset of patients Possible immune escape (growth of tumor cells that lack antigen expression)

Tumor-associated antigens offer several advantages. First, TAAs are prevalent and frequently expressed by gliomas.[88–99] Second, targeting TAAs can be done using off-the-shelf synthesized peptides for most patients, given the prevalent expression of these antigens by glioma tumors. In addition, cocktails of such peptides can be administered to elicit broad antitumor reactivity that reduces immune escape due to growth of nonantigen-expressing tumor cells. Finally, synthetic peptides can be designed that exhibit increased binding affinity for MHC molecules relative to the parent, tumor-derived molecules. Limitations of TAA vaccines include the generation of less robust immune responses compared with TSAs and human leukocyte antigen (HLA) restriction. Nonetheless, initial reports of synthetic TAA peptide vaccines in GBM patients have demonstrated encouraging findings. Nearly 60% of HLA-A2 recurrent GBM patients demonstrated positive ELISPOT or tetramer reactions following vaccination with α-type 1 polarized DCs loaded with synthetic glioma-associated antigen peptides specific for ephrinA2, interleukin-13 receptor-α2, YKL-40, and gp100 when combined with poly-ICLC as an immunoadjuvant.[100] There were no associated grade 3 or 4 adverse events (AEs) and no evidence of autoimmune reactivity in this study. Furthermore, radiographic responses were observed in 2 patients (9%), and progression-free survival (PFS) for at least 12 months was noted in 41% of patients. Further data evaluating a cocktail of tumor-associated antigens

has been recently reported.[101] In this study, median overall survival in HLA-A1/A2-positive newly diagnosed GBM patients who were vaccinated with autologous DCs pulsed with 6 different TAA peptides (HER2, TRP-2, gp100, MAGE-1, IL13Rα2, and AIM-2) was 38.4 months. Of note, tumors from all patients expressed at least 3 target TAAs by reverse transcription (RT)-PCR, while 75% expressed all 6 TAAs. A single-arm phase I/II study evaluating a variation of this vaccine approach is expected to initiate accrual of recurrent GBM patients in mid 2014 (NCT02078648). A randomized, placebo-controlled phase II study of a different TAA-based vaccine (referred to as ICT 107; Immunocellular Therapeutics) for newly diagnosed GBM patients has recently completed accrual (NCT01280552). An 11-TAA peptide vaccine (IMA950; Immatics Biotechnologies GmbH) is also in phase I/II clinical trials for newly diagnosed GBM patients (NCT01403285 and NCT01920191).

Vaccines that incorporate TSAs offer the advantage of generating highly potent immune responses, but these antigens are challenging to exploit because they are either patient specific or expressed by only a subset of patients. Currently, 2 TSA vaccine strategies are undergoing clinical evaluation for malignant glioma patients, while a third is in development. The mutated epidermal growth factor receptor variant III (EGFRvIII), formed by an 801 base pair, inframe deletion of exons 2 to 7, is a prototypic tumor-specific antigen that is expressed by ~30% of GBM tumors.[102] Rindopepimut (Celldex Therapeutics) is a synthetic 14 amino acid peptide mapping to the EGFRvIII-specific splice site, conjugated to the immune adjuvant keyhole limpet hemocyanin, and administered with GM-CSF.[103] In preclinical studies, EGFRvIII vaccination demonstrated survival benefit as well as EGFRvIII-specific humoral and cellular immune responses.[104] Subsequent clinical trials have confirmed that rindopepimut is well tolerated in EGFRvIII-positive GBM patients, and encouraging antitumor benefit has been observed (although treated patients had good performance status and minimal tumor burden).[105–109] In these trials, EGFRvIII-specific immune responses were detected and correlated with improved outcome. Importantly, coadministered temozolomide and its associated lymphopenia did not appear to abrogate the activity of rindopepimut. Of note, the majority of progressive tumors following rindopepimut vaccination no longer expressed EGFRvIII,[106,108] thereby providing proof-of-principle that tumor-specific antigen vaccination can generate an immune response capable of eradicating its intended GBM target population. A placebo-controlled, randomized phase III study of rindopepimut is ongoing for newly diagnosed EGFRvIII-positive GBM patients (Clinicaltrials.gov NCT01480479), while encouraging preliminary results have recently reported from a randomized phase II study evaluating the addition of rindopepimut to bevacizumab for recurrent GBM patients (Clinicaltrials.gov NCT01498328).[110]

Evaluation of a second TSA vaccination strategy involves targeting human cytomegalovirus (HCMV), a β-herpesvirus. DNA as well as HCMV proteins, but not infectious virions, have been demonstrated in most if not all GBM tumors, as well as the majority of grade II and III gliomas, but not in adjacent non-cancerous brain tissue.[111–114] Of note, HCMV has also been detected in several other malignancies.[115–118] Several immunotherapeutic strategies exploiting the presence of CMV in GBM tumors are underway and include vaccination with HCMV pp65-LAMP-loaded DCs with and without autologous T-cell transfer (Clinicaltrials.gov NCT00639639), a phase I vaccination study of HCMV peptide antigen for newly diagnosed GBM patients (Clinicaltrials.gov NCT01854099, and generation of polyclonal CMV-specific T cells for adoptive transfer (Clinicaltrials.gov NCT01205334).[73] Enthusiasm for targeting CMV as a therapeutic strategy for GBM has been heightened by demonstration of a potent pp-65 CD8+ T cell response following whole tumor lysate vaccination[113] and by recent data demonstrating prolonged survival in some newly diagnosed GBM patients treated with valganciclovir,[119] although these preliminary results require prospective validation.

A third approach utilizing tumor-specific antigens for vaccination is in development and exploits the presence of individual, tumor-specific mutations that generate unique mutant proteins.[120,121] With this approach, next-generation DNA sequencing of individual patient tumor and normal DNA is used to identify antigens derived from tumor-specific mutated proteins,[122,123] which are then classified based on predicted binding ability to patient-specific, class I HLA molecules.[124] Preclinical evaluation of this approach confirmed specific immunogenicity of mutant peptides as well as survival benefit following vaccination in a melanoma model.[125] A clinical trial evaluating this approach in advanced melanoma patients was recently initiated (NCT01970358), and similar strategies are in development in the United States and Europe for malignant glioma patients.

Vaccinations derived from tumor lysate preparations represent an approach that offers the advantage of incorporating both TAAs and TSAs on a patient-specific basis.[98,99,126–136] This approach is not restricted by HLA subclass and therefore could theoretically be available for every GBM patient with a resectable tumor. On the other hand, this approach requires surgery for vaccine generation plus several weeks for vaccine preparation. In addition, whole tumor lysates could theoretically trigger autoimmune reactions against normal host cells, thereby contaminating the vaccine product.

Preclinical studies demonstrating the feasibility and antitumor activity of tumor-lysate vaccination approaches[137,138] have led to a series of clinical trials[98,99,126–136] confirming the overall safety of this approach. Specifically, the most commonly observed toxicities included mild local reactions at vaccination site, low-grade fever, and some headache, while autoimmune reactions were not observed. Modest clinical benefit and variable degrees of tumor antigen immunogenicity have also been observed. A randomized phase III, placebo-controlled study evaluating DCVax-L (Northwest Biotherapeutics), a vaccine strategy consisting of autologous DCs pulsed with whole tumor lysate for newly diagnosed GBM patients, is ongoing (NCT00045968).

Two modifications of the tumor lysate vaccination strategy are currently under active investigation for GBM patients. The first adaptation incorporates vaccination with lysates or mRNA derived from glioma stem cells isolated from resected tumors. Targeting glioma stem cells is attractive because this tumor subpopulation fosters tumor growth,[139] resistance to chemotherapy and radiation therapy,[140,141] and immunosuppression.[142,143] In addition, glioma stem cells may express higher levels of some tumor-associated antigens than non-stem cells.[143,144] Preclinical studies have demonstrated the feasibility and antitumor activity of glioma stem cell vaccination strategies.[144,145] Based on these findings, several ongoing clinical trials are evaluating autologous DCs pulsed with glioma stem cell lysate or mRNA (NCT00890032, NCT00846456, NCT01171469, and NCT01567202).

Another innovative modification of the tumor lysate approach involves vaccination with tumor antigens bound to the 96 KD heat shock protein complex (HSP96). Heat shock proteins are upregulated by stress and are regarded as natural adjuvants based on their inherent ability to augment immune responses.[146] Preliminary evidence, which has demonstrated the immunogenicity of this approach and encouraged evidence of antitumor benefit in recurrent GBM patients[147,148] has led to a randomized phase II study evaluating HSP96 vaccination with bevacizumab for recurrent GBM patients (NCT01814813).

Determinants of Vaccine Immunogenicity: Additional Considerations

Several variables beyond tumor antigen choice may impact vaccine immunogenicity, and studies designed to optimize these variables in malignant glioma patients remain to be performed. A potentially important variable among vaccine components is the use of harvested autologous DCs. Dendritic cells are considered the most potent antigen-presenting cells of the immune system due to their high level of major histocompatibility complex and costimulatory molecule expression and their ability to induce tumor-specific effector T-cell responses.[149] Pheresed, autologous DCs can be readily activated ex vivo using cytokines such as GM-CSF and IL-4 and can then process tumor antigens generated by exposure to tumor cell lysates, mRNA, or purified antigens prior to administration back into the patient. Immune adjuvants induce DC maturation, which then demonstrate greater migration to draining lymph nodes and more potent upregulation of immunostimulatory molecules compared with immature DCs.[131,150] Several vaccine approaches incorporate ex vivo antigen sensitization of harvested autologous DCs, while others administer tumor antigen(s) with adjuvant directly. It remains unclear whether the additional labor, cost, and time required for pheresis and ex vivo processing of autologous DCs are required to optimize vaccine immunogenicity.

Another important vaccine variable is immune adjuvance, which augments vaccine-induced immune responses via several mechanisms.[151–154] In the absence of adjuvant-induced activation of DCs, immune tolerance may be promoted by Tregs.[155,156] Although several immune adjuvants have been evaluated in malignant glioma vaccine trials to date, the optimal immune adjuvant or combination remains unclear. TLR agonists such as imiquimod, polylysine, and carboxymethyl-cellulose (poly-ICLC) are mimics of pathogen danger signals that trigger DC activation and T-cell antitumor immunoreactivity.[157–160] cytosine:guanine nucleotide base-pairing, a synthetic oligodeoxynucleotide with unmethylated cytosine: guanine nucleotides motifs mimics microbial DNA and activates TLR9.[158] Keyhole limpet hemocyanin, a protein derived from the sea mollusk Megathura crenulata, is an active adjuvant that has been incorporated safely into active vaccination studies for GBM patients.[107,109,161] In addition, GM-CSF, a myeloid cytokine that can enhance DC maturation and function,[162] has been coadministered with vaccines and via gene-transduced tumor cells.[163–165] GM-CSF has improved outcome in some studies but has shown a detrimental effect in others.[164,166] One possible explanation for these mixed responses could be differential dose effects because low doses of GM-CSF can promote immune responses, while higher doses may enhance immunosuppression.[166,167] Another possibility is the dual role of GM-CSF in enhancing both effector and regulatory T-cell responses. Coadministration of a second signal, such as a TLR agonist, skews GM-CSF–elicited immunity toward tumor protection.[168]

Additional vaccine variables that have not been systemically investigated include site and frequency of vaccination. Data from a preclinical GBM model suggested that the vaccination site is important because the number and function of antigen-specific T cells increased with increasing distance of vaccination site from intracranial tumor.[169]

Patient-related variables that may also impact vaccine immunogenicity include age, degree of prior treatment, status of underlying tumor, size of tumor burden, and concurrent use of corticosteroids, although the latter 2 factors are frequently associated. Preliminary data suggest that younger patients respond more favorably.[127,128,170,171] In addition, minimal tumor burden has been associated with improved outcome, which may be due to immunosuppressive factors associated with tumor bulk.[127,128,171–172] For example, elevated systemic Treg cell levels at diagnosis decrease substantially following resection but then return toward baseline at tumor recurrence.[43,57,173] Future clinical trials should prospectively evaluate the impact of vaccine and patient-related factors on immunogenicity and overall outcome.

Finally, in addition to careful elucidation of factors contributing to vaccine immunogenicity, vaccine-induced tumor-specific immune responses must overcome substantive systemic and local immunosuppressive factors, which have been previously discussed in this review, if they are to generate meaningful antitumor benefit and improve patient outcome.

Immune Checkpoint Targeting Therapy for Glioblastoma

Underlying Biology

Cellular immunity, critically mediated by antigen-specific T cells, is temporally and spatially regulated through a complex series of sequential steps including: (i) clonal selection; (ii) activation and proliferation in secondary lymphoid tissues; (iii) mobilization to

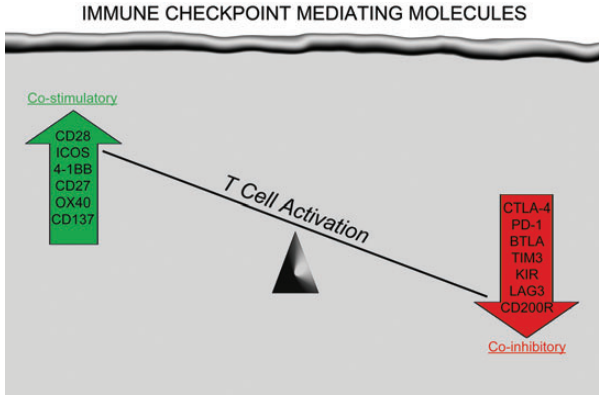

Fig. 2. Immunomodulatory molecules expressed on T cells that provide either a costimulatoryeffect to enhance T-cell activation or a coinhibitory effect to attenuate T-cell activation following interaction of antigen/MHC complex on antigen-presenting cells with the T-cell receptor.

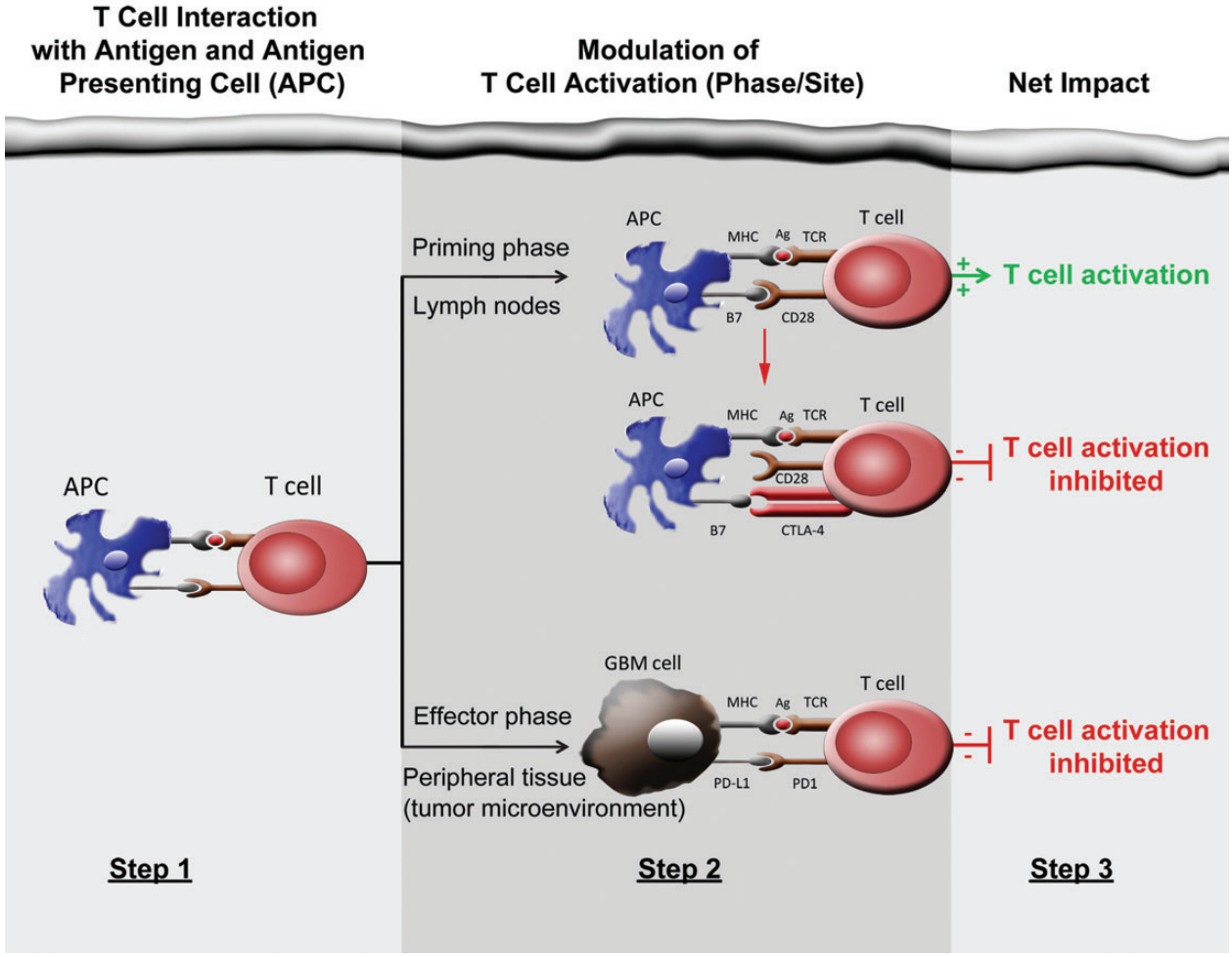

Fig. 3. Steps involved with T-cell activation as well as its negative modulation by CTLA-4 and PD-1/PD-L1. Note that CTLA-4 and PD-1/PD-L1 act by complementary mechanisms to inhibit T-cell activation. Monoclonal antibody blockade of CTLA-4 or PD-1/PD-L1 relieves immune checkpoint inhibition, thereby restoring T-cell activation.

sites of inflammation; (iv) initiation of effector functions at target sites; and (v) recruitment and activation of additional immune cells through cytokines and other signaling molecules. Both costimulatory/agonistic and antagonistic/inhibitory members of the B7/CD28 family modulate this process at key points referred to as immune checkpoints (Fig. 2). Immune checkpoint mediators function to optimize appropriate, normal T-cell immune responses while simultaneously maintaining self-tolerance that minimizes the risk of autoimmune reactions and potential collateral damage to normal tissues. Inhibitory checkpoint mediators function as brakes to attenuate normal T-cell immune responses (Fig. 3). Therapeutic blockade of inhibitory checkpoints, including cytotoxic T-lymphocyte-associated antigen-4 (CTLA-4) and programmed cell death protein 1(PD-1) or its ligands PD-L1 and PD-L2, have recently demonstrated exciting antitumor benefit against several cancers, including some such as metastatic melanoma,[6,8,174] which have historically responded poorly to conventional therapies.

CTLA-4 is solely expressed by T cells and inhibits early steps of the T-cell activation localized primarily within secondary lymphoid tissues. Surface expression of CTLA-4 normally upregulates on T cells upon antigen binding, with the degree of expression commensurate to the strength of T-cell receptor stimulation.[175] CTLA-4 outcompetes CD28, a costimulatory molecule required for augmenting T-cell activation, for its activating ligands CD80 (also referred to as B7.1) and CD86 (also referred to as B7.2).[176] Interruption of ligand binding blocks CD28 signaling and thus dampens antigen-specific T-cell activation. CTLA-4 can also suppress immune reactivity by decreasing helper T-cell activity and augmenting myeloid-derived suppressor cells as well as regulatory T cells, in which CTLA-4 is constitutively expressed.[177–179] The critical modulatory suppression of immune activation by CTLA-4 is underscored by lethal autoimmunity observed in *Ctla*-4-knockout mice.[180]

PD-1 also functions to suppress T-cell activation but does so in a distinct, yet complementary, manner relative to CTLA-4. Specifically, PD-1 primarily blocks T-cell activation and effector function at later stages and typically does so within peripheral organs and local sites of inflammation. However, PD-1 may also play an important regulatory role during T-cell priming by DCs. Inflammatory cytokines, particularly interferons, enhance PD-L1 expression. Analogous to CTLA-4, activated T cells also upregulate PD-1

expression.[181,182] In addition, chronic antigen exposure, which typically occurs with chronic viral infection or cancer, may trigger persistent PD-1 expression and lead to anergy or exhaustion of antigen-specific T cells.[183] Within the inflammatory or tumor microenvironment, PD-1 binding to PD-L1 suppresses T-cell effector activity by inhibiting signaling through interaction with the SHP2 phosphatase.[184] Of note, PD-1 promotes other aspects of immune suppression including proliferation of Tregs and attenuation of B cell and natural killer (NK) cell responses; thus, therapeutic targeting of PD-1 may enhance multiple aspects of immune reactivity associated with cytotoxic T cells, B cells, Tregs, and NK cells.[185,186]

Cancers notoriously subvert normal physiological processes to promote their survival. Among malignant gliomas, markedly upregulated angiogenesis and overexpression of the ubiquitous DNA repair enzyme methyl-guanine methyltransferase are 2 well-established examples of such adaptive behavior. Analogously, many malignancies overexpress the PD-L1 immune checkpoint ligand to counteract antitumor T-cell responses and proinflammatory cytokines.[187] Tumor cell PD-L1 expression can occur as an adaptive immune reaction in response to interferons, especially IFNγ, which are secreted by tumor cells or associated stroma.[188] In addition, increased PD-L1 expression has been linked to dysregulated oncogenic cell signaling pathways, including inactivating mutation of the phosphatase and tensin homolog (PTEN) tumor suppressor.[189] Among gliomas, PD-L1 expression correlates with grade[190] and has been demonstrated in established GBM cell lines,[191] primary tumors,[190–194] and CD133+ GBM stem cells.[190] In addition, a high percentage of tumor-infiltrating lymphocytes from malignant gliomas express PD-1.[194]

Preclinical Studies

CTLA-4 blockade led to 80% long-term survival among immunocompetent VM/Dk mice with established SMA-560 intracranial tumors.[195] CTLA-4 therapy was well tolerated with no evidence of autoimmune toxicity. Furthermore, anti-CTLA-4 therapy led to normalized CD4+ T cell counts and decreased $CD4^{+}CD25^{+}FoxP3^{+}$ Treg cells. In a separate study, intratumoral IL-12 therapy combined with systemic CTLA-4 blockade led to long-term survival in the majority of GL-261 tumor-bearing mice.[196] In addition, long-term surviving mice exhibited evidence of immunological memory when reinoculation of tumor into the contralateral cerebral hemisphere failed to generate tumor growth.

Limited preclinical data evaluating PD-1/PD-L1 blockade for GBM currently exist. Administration of an anti-PD-1 MAb improved median survival from 26 days to 52 days among C57BL/6 mice with GL-261 intracranial tumors when combined with a 10 Gy dose of radiation.[197] A cohort of long-term survivors was apparently cured; tumor growth was not observed upon flank rechallenge, which indicated that immunological memory had prevented recurrence. In addition, combination therapy led to increased tumor infiltration of cytotoxic T cells and decreased Treg infiltrate.

Clinical Experience

There are currently 2 fully human, CTLA-4 monoclonal antibodies under clinical evaluation for cancer patients including ipilimumab (Yervoy; Bristol-Myers Squibb) and tremilimumab (MedImmune LLC). Ipilimumab, an IgG1isotype, was approved for advanced, unresectable melanoma based on improved survival noted in a randomized phase III study.[6] The outcome for metastatic melanoma patients has historically been dismal due to failure to improve survival significantly by a variety of investigated therapeutic approaches.[198] In this study of 676 patients, median survival for those treated with either ipilimumab alone or ipilimumab plus gp100 peptide vaccine was 10 months, compared with only 6 months for those treated with vaccine alone (hazard ratio, 0.66; $P = .0026$). In addition, investigator-assessed radiographic response rate was 10.9% for patients treated with ipilimumab compared with only 1.5% for those treated with vaccine. A second randomized phase III study also noted a survival benefit in patients treated with ipilimumab plus dacarbazine compared with dacarbazine plus placebo.[174] Notably, the durability of antitumor benefit has been unprecedented, even though it was limited to a subset of patients. Recent long-term follow-up of 177 advanced melanoma patients, who were treated in early clinical trials of ipilimumab, revealed that the median duration of tumor response was 88 months.[199] Although it has not been approved by the FDA, antitumor benefits have also been observed in advanced melanoma patients treated with tremelimumab, an IgG2 CTLA-4 blocking MAb. Specifically, median overall survival was 12.8 months in tremelimumab recipients compared with 10.7 months for either temozolomide or dacarbazine chemotherapy recipients.[200] In addition, 10.7% of tremelimumab recipients achieved a radiographic response, which was durable for a median of 35.8 months. Of note, encouraging evidence of antitumor activity is emerging for both ipilimumab and tremelimumab among other solid tumors including lung,[201] prostate,[202,203] breast,[204] colorectal,[205] renal,[206] and pancreatic cancers[207] as well as mesothelioma.[208]

Dramatic evidence of antitumor benefit has also been observed with therapeutics blocking PD-1/PD-L1 signaling. In an initial phase I study of advanced solid-tumor patients treated with BMS-936559, a fully human IgG4 mAb that blocks PD-L1 binding to either PD-1 or CD80, a maximum tolerated dose was not reached, and 9% of patients experienced grade 3-4 treatment-related AEs that led to discontinuation of treatment for 6% of patients.[7] There were no treatment-related deaths. Evidence of meaningful antitumor benefit was observed at biweekly doses ≥1 mg/kg and was durable in an encouraging subset of patients; however, frequency of response varied by tumor type. Specifically, responses were noted in patients with melanoma (17%) as well as lung (10%), ovarian (6%), and renal cell cancers (12%) but were not observed in patients with colorectal or pancreatic cancers (although only small numbers of these latter tumor types have been published to date). In a simultaneously reported phase I study of nivolumab, a fully human IgG4 PD-1 blocking MAb, a maximum tolerated dose was also not defined despite dose escalation from 0.1 to 10 mg/kg biweekly.[8] Grade 3-4 drug-related AEs occurred in 14% of patients, while 5% of patients discontinued therapy due to treatment-related AEs. In addition, 3 deaths from pneumonitis were noted. Highly encouraging evidence of antitumor activity was again noted despite a significant degree of pretreatment in enrolled patients; however, benefit was also restricted by tumor type. Specifically, durable radiographic responses and improved PFS-6 rates were observed in melanoma, renal cell and lung cancer patients, but no radiographic responses were observed for prostate and colorectal cancer patients, although relatively small numbers of these tumors

have been evaluated. In addition, a higher rate of radiographic response was noted in patients with PD-L1–expressing archival tumor specimens. Significant single-agent activity was recently reported in advanced melanoma patients treated with lambrolizumab, a humanized, IgG4-kappa isotype, PD-1 blocking MAb, in a single-arm phase II study.[209] Three different dosing schedules were evaluated including 2 mg/kg every 3 weeks and 10 mg/kg every 2 or every 3 weeks. Specifically, 38% of all patients achieved a radiographic response by central review with a median PFS $>$ 7 months. Radiographic response rates were higher in patients treated at 10 mg/kg every 2 weeks (32%), compared with 10 mg/kg every 3 weeks (15%) and 2 mg/kg every 3 weeks (3%). Of note, responses were also observed in patients who had progressed on prior ipilimumab therapy. In this study, 13% of patients reported grade 3-4 treatment-related AEs, and one patient died. The incidence of treatment-related AEs was more common in patients treated with 10 mg/kg every 2 weeks.

With regard to toxicity, immune checkpoint blockade is associated with a diverse spectrum of well-characterized, immune-related adverse events (irAEs) including rash, colitis, hypophysitis, hepatitis, pancreatitis, iridocyclitis, lymphadenopathy/sarcoid-like syndrome, neuropathy, and nephritis.[210] Although most irAEs are mild to moderate, particularly if recognized early and treated appropriately, severe reaction have occurred including life-threatening toxic epidermal necrolysis and fatal colitis and pneumonitis. Similar irAEs have been noted in patients treated with CTLA-4 as well as PD-1/PD-L1 blockade, although the frequency and severity of irAEs appear to be more prominent following anti-CTLA-4 therapy that are presumably related to earlier and less specific inhibition of T cell activation.[211] AEs of any grade have been reported in 75% of patients on ipilimumab, and 15%–30% have experienced grade $\geq$ 3 irAEs.[6,174,210,212] Of note, a characteristic pattern of timing has emerged for irAEs. Specifically, dermatologic events typically occur first and are noted most commonly within 2–3 weeks of treatment initiation, while gastrointestinal and hepatic events are most common after 6–7 weeks of therapy. Endocrinologic AEs typically evolve after 9 or more weeks.[210,212] The frequency and severity of irAEs appear to be dose related.[212,213] Guidance for early recognition and appropriate intervention of irAEs has proven to be highly valuable for reducing their overall severity.[210,212]

Clinical evaluation of CTLA-4 blocking MAbs for neuro-oncology patients is thus far limited to patients with CNS metastases of systemic cancers. Ipilimumab has demonstrated encouraging benefit for patients with melanoma and brain metastases as a single-agent therapy[214] and in combination with radiotherapy administered using standard fractionated delivery or stereotactic radiosurgery.[215–217] Of note, concurrent corticosteroid use was associated with diminished activity in one study.[214] Clinical trials evaluating ipilimumab are in advanced development for both recurrent and newly diagnosed GBM patients (NCT02017717).

Several clinical trials evaluating PD-1/PD-L1immune checkpoint inhibitors are also in development for malignant glioma patients. Accrual has recently initiated to a phase II study which randomizes recurrent glioblastoma patients to receive nivolumab, nivolumab plus ipilimumab, or bevacizumab (NCT02017717). A phase I/II study is also expected to begin accrual soon for evaluating pidilizumab (CurTech), a humanized anti-PD-1 MAb, in patients with either recurrent malignant glioma or diffuse intrinsic pontine glioma (NCT01952769).

Combinatorial Approaches

To derive optimal patient benefit, immune-based therapies will need to generate potent, specific, and durable antitumor immune responses that are able to overcome systemic and local immunosuppressive factors. In order to achieve this goal, combinatorial approaches may be required. Intuitively, combining cytotoxic therapy with immunotherapy appears counterproductive because cytotoxic therapies can diminish overall immune function. In addition, recent data have associated radiation and alkylating chemotherapeutics, including temozolomide with selective lymphocyte toxicity, that have led to profound and persistent lymphopenia.[45,218–221] Paradoxically, growing data have also demonstrated that cytotoxic therapies may augment the magnitude, quality, and efficacy of tumor-specific T-cell responses via several mechanisms. First, dying tumor cells release tumor-specific antigens that can in turn initiate T-cell activation.[222,223] Second, lymphodepleting chemotherapeutics can induce homeostatic proliferation that preferentially enhances restoration of cytotoxic T cells faster than Tregs, leading to an increase in the CD8+:Treg ratio and improved outcome in both preclinical models and patients.[106,224,225] Third, some chemotherapeutic agents, including low-dose cyclophosphamide and gemcitabine, may deplete immunosuppressive Tregs and myeloid-derived suppressor cells.[226–228] Fourth, chemotherapeutics, including temozolomide, can induce intratumoral expression of immunostimulatory cytokines and chemokines that promote antitumor immune responses.[224] Finally, cytotoxic therapy may induce stress or danger signals that increase the susceptibility of tumor cells to immune attack.[229–231] Based on these considerations, several clinical trials combining cytotoxic therapy with glioma vaccination have been conducted, and encouraging preliminary efficacy has been reported.[101,105,107,132,133,135,136] Limited yet intriguing data have also demonstrated that cytotoxic therapy and immune checkpoint blockade may generate enhanced antitumor benefit; a recent report described an abscopal effect (defined as regression of metastatic tumor distant from sites of local radiation) in a patient with advanced melanoma treated with ipilimumab and focal radiotherapy.[232] Additional studies have suggested that immune checkpoint blockade, combined with chemotherapy, enhances antitumor benefit.[174,201] Immunologic benefit may vary between chemotherapeutic agents and may also depend on administration schedule.[201,233] Only one study evaluating combinatorial cytotoxic therapy and checkpoint blockade has been reported for GBM. In this recent preclinical study, significantly prolonged survival in an immunocompetent murine GBM model was achieved following combinatorial anti-PD-1 MAb plus stereotactic radiation.[197]

There is also a rationale for combining immunotherapy approaches with agents that block VEGF signaling. VEGF contributes to tumor immunosuppression by several mechanisms including: (i) inhibition of DC maturation and antigen presentation; (ii) induction of CD8+ T-cell apoptosis; (iii) promotion of Treg activity; and (iv) restriction of T-cell migration across tumor vascular endothelium into tumors.[234–237] Indeed, inhibition of VEGF signaling has been shown to augment antitumor immune responses.[49,238–240] Based on these considerations, a randomized phase II study evaluating rindopepimut with bevacizumab in recurrent GBM patiets is ongoing (Clinicaltrials.gov: NCT01498328).

A third combinatorial approach includes integrating immunotherapies with potentially synergistic mechanisms of antitumor

activity. The FDA approval of sipuleucel-T for metastatic prostate cancer and ipilimumab for advanced melanoma are noteworthy proof-of-principle achievements highlighting the value of immunotherapies for oncology. Nevertheless, the overall clinical benefit of each of these modalities is modest in that sipuleucel-T improves survival by only 4.1 months, and durable antitumor responses are achieved in only a minority of patients treated with immune checkpoint blockade. Such results underscore the likely need to combine complementary immune-based therapies in order to achieve broad and durable antitumor benefit. Fortunately, the spectrum of immune-based treatment strategies offers many exciting opportunities for combinatorial approaches in neuro-oncology. For example, based on preclinical antitumor benefit associated with either Treg depletion[241,242] or inhibition of Treg activation via IL-2 blockade,[241,243] a phase I/II clinical trial has initiated evaluating dacluzimab (Zenapax; Hoffman-La Roche Incl, Nutley, NJ), an IL-2 receptor MAb administered with EGFRvIII vaccination (Clinicaltrials.gov NCT00626015). (Clinicaltrials.gov NCT00626015). CMV antigen vaccination, combined with adoptive transfer of expanded CMV-specific T cells, is also currently under evaluation (Clinicaltrials.gov NCT 00693095).

Integration of tumor vaccination with immune checkpoint blockade has also been shown to enhance long-term survival in preclinical studies including GBM,[244–246] although optimal benefit may be schedule dependent.[247] Recent clinical studies in other cancer indications confirm the safety of combining immune checkpoint blockade with cancer vaccines.[202,248,249]

Finally, an exciting approach currently under wide clinical evaluation includes combination of immune checkpoint inhibitors. The rationale for such an approach is based on differences in timing and localization of T-cell activation regulated by CTLA-4 and PD-1/PD-L1 signaling.[211] Initial preclinical studies have revealed synergistic antitumor benefit following combined CTLA-4 and PD-1 blockade.[244,250] A recent clinical trial confirmed greater antitumor activity when CTLA-4 blockade was combined with anti-PD-1 therapy.[251] A clinical trial evaluating combined CTLA-4 and PD-1 blockade is underway for recurrent GBM patients (NCT02017717).

Additional Considerations

The ability to accurately assess response remains a significant challenge in neuro-oncology. The Response Assessment in Neuro-oncology (RANO) criteria were recently adopted because they address both pseudoprogression and pseudoresponse.[252] Complex imaging patterns of response have also been observed following immunotherapy and have included pseudoprogression due to inflammation associated with antitumor immune responses that led to enlargement of pre-existing lesions or the appearance of new lesions. In addition, tumors may initially grow, including the appearance of new lesions prior to the generation of antitumor immune responses.[253] To address these phenomena, immune-related response criteria have been integrated into immunotherapy clinical trials.[254,255] Critical considerations of these criteria include: (i) the ability of patients who are adequately tolerating therapy to remain on treatment beyond initial progression; and (ii) measurement of overall tumor burden rather than the appearance of new lesions to define progressive disease. A multidisciplinary working group is currently integrating immune response criteria into RANO for use in neuro-oncology patients undergoing immunotherapy clinical trials.

The holy grail of all cancer therapies, including immunotherapy, is the identification of correlative biomarkers to identify patients most likely to benefit.[256] Myriad assays of immune response have demonstrated variable association with survival benefit in GBM patients treated in immunotherapy clinical trials.[98–101,105–109,126–136,148,257] One proposed innovative use of immune parameters includes hierarchical clustering to predict response to immunotherapy treatments.[132,136] Recognition that significant variability in methodology and characterization of assay response poses a significant limitation to effective use of these data has led to a multinational effort to harmonize immunologic monitoring efforts across immunotherapy trials.[258]

Additional correlative data may be derived from tumor analyses. For example, a preliminary report has suggested that GBM tumors exhibiting a mesenchymal gene expression profile benefit more from tumor lysate vaccination than other subtypes due to induction of higher levels of CD3+ and CD8+ tumor-infiltrating lymphocytes.[133] Higher levels of response to anti-PD-1 therapy have been associated with tumor PD-L1 expression, although this result has not been validated by other studies.[8] Finally, because PTEN deficiency has been linked with increased PD-L1 expression, some investigators have suggested that GBM tumors exhibiting PTEN loss may be more likely to derive benefit from PD-1/PD-L1 blockade.[259] It will be imperative that hypotheses evaluating potential biomarkers be investigated as the clinical trials advance for immune-based therapies in malignant glioma patients.

Conclusion

Effective antitumor activity by the immune system offers the ability to both eradicate existing tumors and prevent future recurrence by generating immune memory responses. The CNS interacts in a effective manner with the peripheral immune system to generate meaningful immune responses. A wide array of exciting therapeutic approaches have been developed to generate effective antitumor immune responses and have demonstrated highly encouraging benefit for patients following active vaccination, immune checkpoint blockade, and adoptive cellular therapies. Nonetheless, several challenges exist including better understanding of immunosuppressive systemic and local adaptations frequently invoked by malignancies including GBM to foster immunotolerance. In addition, in order to maximize therapeutic benefit, systematic investigation of potential variables that may impact the optimal activity of immunotherapies and the evaluation of potentially informative biomarkers will be required. Finally, it is likely that combinatorial regimens with complementary mechanisms of action will be required to achieve broad and durable antitumor benefit.

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