Importance of immune monitoring approaches and the use of immune checkpoints for the treatment of diffuse intrinsic pontine glioma: From bench to clinic and vice versa (Review)

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Abstract. On the basis of immunological results, it is not in doubt that the immune system is able to recognize and eliminate transformed cells. A plethora of studies have investigated the immune system of patients with cancer and how it is prone to immunosuppression, due in part to the decrease in lymphocyte proliferation and cytotoxic activity. The series of experiments published following the demonstration by Dr Allison's group of the potential effect of anti-cytotoxic T lymphocyte-associated antigen-4 (CTLA-4) paved the way for a new perception in cancer immunotherapy: Immune checkpoints. Several T cell-co-stimulatory molecules including cluster of differentiation (CD)28, inducible T cell co-stimulatory, 4-1BB, OX40, glucocorticoid-induced tumor necrosis factor receptor-related gene and CD27, and inhibitory molecules including T cell immunoglobulin and mucin domain-containing-3, programmed cell death-1 (PD-1), programmed cell death ligand-1 (PD-L1), V-domain immunoglobulin suppressor of T cells activation, T cell immunoglobulin and immunoreceptor tyrosine-based inhibitory motif domain, and B and T lymphocyte attenuator have been described in regulating T cell functions, and have been demonstrated to be essential targets in immunotherapy. In preclinical studies, glioblastoma multiforme, a high-grade glioma, the monotherapy targeting PD-1/PD-L1 and CTLA-4 resulted in increased survival times. An improved understanding of the pharmacodynamics and immune monitoring on glioma cancers, particularly in diffuse intrinsic pontine glioma (DIPG), an orphan type of cancer, is expected to have a major contribution to the development of novel therapeutic approaches. On the basis of the recent preclinical and clinical studies of glioma, but not of DIPG, the present review makes a claim for the importance of investigating the tumor microenvironment, the immune response and the use of immune checkpoints (agonists or antagonists) in preclinical/clinical DIPG samples by immune monitoring approaches and high-dimensional analysis. Evaluating the potential predictive and correlative biomarkers in preclinical and clinical studies may assist in answering certain crucial questions that may be useful to improve the clinical response in patients with DIPG.

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1. Introduction

Cancer exhibits a range of symptoms of differing severity, from mild ailments including headaches to complete organ failure; therefore, it is no surprise that cancer has been described as 'The Emperor of All Maladies' (1). Cancer has become so pervasive in the USA that, for many, a diagnosis of the disease is almost synonymous with a death sentence, and the statistics illustrate why. The American Cancer Society reported that, in 2016, there were ~1.7 million novel diagnoses of the disease and ~600,000 associated mortalities (2). For years, cancer therapy has been relatively unchanged, with surgery, radiotherapy and chemotherapy the three primary methods used to treat patients with cancer. Surgery offers a great chance for a cure for many types of cancer, principally those that have not metastasized. Radiotherapy is involved in many treatments of cancer; however, severe side effects can occur months to years following treatment. Additionally, certain tumor cells are resistant enough to tolerate, and recover from, the damage to their DNA caused by radiation therapy (3,4). Although

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chemotherapy remains an effective treatment for many types of cancer, it typically leads to side effects including fatigue, pain, diarrhea, nausea and vomiting, and blood and nervous system disorders (5). Resistance to several chemotherapeutic agents and molecularly targeted therapies, including vemurafenib, imatinib, nilotinib, erlotinib and trastuzumab, is the primary issue regarding current cancer research. These drugs are designed to discern molecularly transformed cells that may express, for instance, high levels of BRAF mutant, breakpoint cluster region-Abelson, epidermal growth factor receptor (EGFR) and human epidermal growth factor receptor 2 (HER2), from non-transformed cells. Through natural selection, transformed cells submitted to molecularly targeted therapies have also developed to escape from these therapies. Alterations in the drug target, activation of pro-survival pathways and ineffective induction of cell death are examples (6,7).

Hanahan and Weinberg (8,9) proposed a hypothesis in their observations defining critical aspects of cancer pathophysiology: Forms of symptomatic neoplastic disease and their association with acquired biological capabilities enable cancer cells to proliferate. They proposed to call this set of biological capabilities 'hallmarks of cancer'. Currently, in their conceptualization, there are eight hallmark capabilities that are common to a number, if not the majority, of forms of human cancer: Sustaining proliferative signaling, evading growth suppressors, resisting cell death, enabling replicative immortality, inducing angiogenesis, activating invasion and metastasis, deregulation of cellular energetics and metabolism, and avoiding immune destruction (8,9). Somehow, all these aspects impair the effectiveness of the therapy against cancer cells. Certain strategies, including cytokines (10), signal transduction inhibitors (11), oncolytic viruses (12) and angiogenesis inhibitors (13), have been attempted, generally with low rates of positive response. Thus, there is an urgent requirement to develop novel therapies for treating cancer.

A rapid increase in comprehending the mechanistic pathway of these principles, particularly avoiding immune destruction, has led to clinical success in the treatment of cancer. Robert Schreiber and Lloyd J. Old (the 'father of tumor immunology') demonstrated that T lymphocytes and interferon-γ (IFN-γ) assisted in inhibiting the development of spontaneous cancer in mice lacking the expression of recombination-activating 2, a gene which encodes a protein involved in the V(D)J recombination during T and B cell development (14). They also contributed in describing immunoediting and how cancer cells became less immunogenic than the starting population (15-19). Immunoediting is discussed in the present review. Currently, the function of the immune system in the recognition and elimination of cancer cells is beyond any doubt. The potential use of immunotherapy is to restore the immune system of patients in the attempt to stimulate it to reject and destroy tumors (20,24). Strategies including dendritic cell-based immunotherapy, T cell adoptive transfer, autologous immune enhancement therapy and genetically engineered T cells are being, with positive results, developed to improve the quality of life and increase the survival rates of patients with cancer (21,23-25). Recently, T cells have been genetically engineered to create specialized receptors on their surfaces known as chimeric antigen receptors (CARs), a personalized treatment that involves genetically modifying a patient's T cells to make them a target (24,26,27). Furthermore, there are several approaches for cancer immunotherapy already approved by the US Food and Drug Administration (FDA) or remain under investigation: Adjuvant therapy (recombinant *Listeria*, stimulator of IFN genes and Toll-like receptor agonist); adoptive T-cell therapy [autologous T cells, CAR-T cells and T cell receptor (TCR) transgenic T cells]; cytokine therapy [interleukin-2 (IL)-2, IFN-γ, IL-15, IL-18 and tumor necrosis factor-α (TNF-α)]; macrophage activation [cluster of differentiation (CD)40 agonists and CD47 antagonists]; natural killer (NK) cell therapy (*ex vivo* expanded NK cells); oncolytic virus therapy (engineered herpes simplex virus, measles virus and poliovirus); and vaccines (human papillomavirus vaccines and sipuleucel-T vaccine for prostate cancer) are class types and examples of cancer immunotherapy (25). However, in recent years, a novel and surprisingly effective method of immunotherapy has arisen: The immune checkpoint blockade. This novel form of therapy does not target cancer cells and also does not involve cytokines or vaccines to turn on the immune response; rather, it works by blocking inhibitory pathways (26). The best characterized of these immune checkpoints are cytotoxic T-lymphocyte-associated protein antigen-4 (CTLA-4) and programmed cell death-1 (PD-1). Immune checkpoint inhibitors blocking CTLA-4 and PD-1 molecules were approved by the FDA in 2011 and 2014, respectively. The present review makes a claim for the importance of investigating the tumor microenvironment (TME), the immune response and the use of immune checkpoint (agonists or antagonists) in preclinical/clinical diffuse intrinsic pontine glioma (DIPG) samples by immune monitoring approaches. The potential predictive biomarkers of tumor-associated cells and the TME in preclinical and clinical studies may assist in answering certain crucial questions that may be useful to improve the clinical response in patients developing DIPG, an orphan type of cancer representing the principal cause of mortality from pediatric brain tumors.

2. Immune checkpoint blockade as a potential approach to treat patients with cancer

Cancer immunotherapy was declared as the 'Breakthrough of the Year' in 2013 (28). The ecstasy is primarily grounded on a number of clinical successes of antibodies that modulate immune checkpoints mainly by targeting CTLA-4 and PD-1 (29). The idea of checkpoint blockade and consequently the renaissance of cancer immunotherapy, emerged when Dr James Allison's group interrogated why T cells were not being fully activated to attack cancer cells (30). The answer to the initial question led to the identification of a molecule called CTLA-4. This molecule exhibited a marked structural homology with CD28, but its function in stimulating or in dampening T cell activation was not completely understood. However, data provided by Tivol *et al* (31) and Waterhouse *et al* (32), using knockout mice, definitively revealed the inhibitory function of CTLA-4. The sequence of experiments in these studies paved the way to a new perception in cancer immunotherapy: Immune checkpoint blockade. In a preclinical study, the combination of anti-CTLA-4 and anti-PD-1 was more than twice as efficient as either therapy alone in generating an effector immune

response against murine melanoma and colon adenocarcinoma (33,34). The approval of immune checkpoint blockade targeting the CTLA-4 and PD-1 pathway motivated the interest in exploiting antibodies which also induce T cell activation. Immune responses are tightly regulated by a system of checkpoints that control positively or negatively the magnitude of the immune response in a wide range. Besides CTLA-4 and PD-1, the presence of several inhibitory immune checkpoints that block T cell responses including T cell immunoglobulin mucin domain-3 (TIM-3), lymphocyte-activation gene-3 (LAG-3), T cell immunoglobulin and immunoreceptor tyrosine-based inhibitory motif domain (TIGIT), V-domain immunoglobulin suppressor of T cell activation (VISTA), B and T lymphocyte attenuator (BTLA), B7-H3 and B7-H4 have emerged as novel targets for immune checkpoint blockade strategies. Conversely, stimulating T cells directed to molecules including CD27, CD28, OX-40 (CD134), glucocorticoid-induced TNF receptor-related protein (GITR) and inducible T cell co-stimulator (ICOS) has been used for mobilizing the immune system to attack cancer cells (35-42). Immunotherapeutic approaches to treat patients with cancer have been evaluated in the last few decades and, currently, immune checkpoints are the new paradigm for treatment of cancer. The FDA approved the antibody against CTLA-4 (ipilimumab) in 2011 for the treatment of metastatic melanoma (43). Clinical trials for the treatment of non-small cell lung carcinoma, small cell lung cancer, bladder and metastatic hormone refractory prostate cancer are being implemented (44-48). Antibodies against PD-1 (pembrolizumab and nivolumab) were approved in 2014 by the FDA for the treatment of patients with melanoma that did not respond to prior treatment. Antibodies against CTLA-4, PD-1 and programmed cell death ligand-1 (PD-L1) have exhibited an objective response against several types of cancer in clinical trials with rates of ~25% (49-52). This effect represents a particular challenge for immunotherapy, since certain types of cancer presented low mutation rates and high immune regulatory molecules, including VISTA, TIM-3, LAG-3 and TIGIT (53,54).

Tumor growth and development is associated with immunomodulation of T cell responses through the enhancement of co-inhibitory molecules. As expected, different types of immune cell exert different effects on tumor progression. In the vast majority of solid cancer types, tumor infiltration by cytotoxic lymphocytes, Th1 profile and mature dendritic cells are associated with a good clinical outcome (55). Nonetheless, studies have indicated that the increase in $CD8^+$ T cell infiltration is not always associated with a good prognosis in cancer, as could be observed in Hodgkin's lymphoma, diffuse large B-cell lymphoma, renal cell carcinoma, lung metastases from clear cell renal cell carcinoma and non-small cell lung cancer (56-60). These effects may be explained by the expression of immune checkpoints on infiltrating T cells or its ligands on tumor cells that are fundamental to immune escape in cancer. For instance, the anti-CTLA-4 (ipilimumab) therapy, which improved median overall survival in patients with metastatic melanoma (61), resulted in significant survival benefit in only 20% of patients. There is evidently a requirement to improve the therapeutic benefit of this treatment to more patients and also apply this approach, even if with only minimal success, to types of cancer with low survival rates, including DIPG. The immune monitoring studies of immune responses of patients to agents targeting immune checkpoints would be the best way to investigate the reasons for only a small proportion of patients with cancer responding to the treatment. The essential cause of resistance to immune checkpoint blockade may be explained through the failure of the effector T cells in becoming activated, mainly due to the low mutational neoantigen rates, the TME and the increase in co-inhibitory molecules which dampen T cell activity (62-64).

3. Glioma and DIPG

Brain cancer is rare for people of any age, but it may develop in children as well as in adults. When the supportive cells are transformed, and induced to proliferate by several mutation patterns, glial cancer known as glioma arises. The World Health Organization classifies glioma into low grade (grade I, pilocytic; grade II, fibrillary) or high grade [grade III, anaplastic; grade IV, glioblastoma multiforme (GBM)] (65). The capacity of gliomas to induce local and systemic immunosuppression restricts the immune response against tumor growth, development and progression, and it may impair the efficacy of immunotherapy (66). In this case, immune checkpoint agonists targeting GITR, ICOS, 4-1BB, OX40, CD27 and CD28 on the T cells may be a good strategy to improve clinical responses rates and also overcome the resistance to immunotherapeutic approaches in glioma. Accumulating evidence in clinical responses in a diverse group of advanced-stage cancer suggest that the combination with standard approaches and immunotherapy on the basis of immune checkpoints may also be beneficial. Brainstem glioma may be described by a diverse biological performance, and the prognosis and treatment depend on clinical symptoms, and their duration, location and mutational profile (67). The majority of pediatric brainstem gliomas begin within the pons, whereas the remaining 20% occur in the medulla, midbrain or cervicomedullary junction (68-72). Pontine cancer is a diffuse intrinsic brainstem glioma which behaves in an infiltrative manner and has a consistently poor prognosis (73).

DIPG is the pediatric malignancy with the poorest prognosis. It is defined as a high-grade glioma occurring in the ventral pons and accounts for between 10 and 15% of pediatric tumors of the brain, affecting an estimated 200-400 children of between 4 and 9 years of age in the USA annually (74). Conventional focal radiotherapy is the standard treatment for patients with DIPG; however, transient effects and minimal survival has been observed (75). Mutations in H3F3A or HIST3BHI which encode the histone H3.3 variant and H3.1, respectively, appear to be present in all DIPG cells (76-78). As a result of these mutations, a substitution of methionine for Lys^{27} (K27M) occurs, causing an altered binding of mutant H3 to Polycomb repressive complex 2, an essential developmental regulator of gene expression and it appears to be the main event of DIPG oncogenesis (79,80). Despite these mutations, the genomic landscape of DIPG cancer cells appear to have variations in activin A receptor type I, tumor protein 53, platelet-derived growth factor receptor A, phosphoinositide 3-kinase catalytic subunit α and c-Myc (81). Unfortunately, DIPG is not well understood, partly because of its low incidence, low biopsy and autopsy rates (82).

In order to solve this issue and to contribute to DIPG research, the international DIPG Registry (dipgregistry.org), a central resource of clinical information combines co-operative efforts throughout physicians and researchers from North America, Europe and Australia to consolidate and standardize the collection of clinical data and tumor samples from patients with DIPG. The aim of this effort is to support innovative research and ultimately find a cure for DIPG.

Currently, DIPG is the primary cause of brain tumor-associated mortality among children, with a median survival time of <1 year and with a 5-year survival rate of <1% (81). Their location within the brain and diffuse nature render them unfit for resection, and biopsies have rarely been conducted (83). The distribution of chemotherapeutic agents to the tumor has been prevented by the existence of the blood-brain barrier (BBB) and, even using the convection-enhanced delivery technique (CED), the effective dose has not been achieved (84). Doses of drugs that result in significant systemic toxicity have to be administered to obtain minuscule decreases in tumor growth (85). There have been >250 clinical trials designed at targeting several biological capabilities of DIPG and, despite numerous efforts, DIPGs have no effective treatment and no significant improvement has been made during the last 30 years (86). As aforementioned, DIPG is a type of cancer with epigenetic features that comprise histone modifications including methylation and acetylation. Certain agents targeting epigenetic factors including histone deacetylase inhibitors (HDACi; including panobinostat, vorinostat, belinostat, romidepsin and valproate) histone methylase and demethylase inhibitors, DNA methylation inhibitor and bromodomain and extra-terminal motif protein inhibitors are of importance in treating DIPG (87). Comprehending the epigenetic landscape of DIPG opened up the possibility for epigenetic modifiers, which may lead to regulation of this lethal cancer. Recently, several studies have demonstrated that HDACi including panobinostat or MS-275 (entinostat) are able to restore the aberrant gene expression associated with the K27M mutation, the dominant variation in genes encoding histones H3.3 and H3.1. Furthermore, HDACi are also able to enhance the immune response by increasing tumor-associated antigens (88), major histocompatibility complex (MHC) class I, II, CD40 (89) and NK cell-activating ligands (90). The effects of HDACi on immune cells have been reviewed previously (91). However, the potential molecular mechanism by which these agents upregulate or downregulate tumor ligands, T or NK cell molecules is unknown.

4. Immune system, gliomas and neoantigens

For a long time, the brain was believed to be devoid of a lymphatic system. However, this was idea was challenged in 2016 when Schläger *et al* (92) revealed that this system is part of the meninges (arachnoid and dura) and, even in physiological conditions, T cells do not cross the BBB; they are able to traffic between the leptomeninges and cerebrospinal fluid (CSF) through blood vessels (93). Furthermore, owing to the assumption that brain cancer is merely immunogenic, it was not considered that there was a function for the immune system in glioma. Immunotherapeutic strategies have altered this way of thinking, therefore important clinical trials in malignant glioma have been performed. At first, it was possible to realize, in principle, that tumor-associated antigens should be discernable to the immune system in the deep cervical lymph nodes and those immune cells would have access to brain cancer via the CSF and choroid plexus paths (94). Finally, as long as these barriers restrict access of immunotherapeutic approaches to the brain, certain strategies including direct infusion of antibodies, dendritic cells, T cells and other drugs by CED afford a possible opportunity for immediate delivery, decreasing the efflux of cells or molecules (95).

The lack of available and valuable cytotoxic therapies associated with prolonged poor clinical outcomes and the unmanageable landscape profile of DIPG warrant novel approaches that target DIPG cancer cells (96). Immunotherapy is being progressively considered as a 'weapon' for use in combination therapy or as a complementary approach to conventional treatments (97-100), particularly those that target glioma-associated antigens (GAAs). The function of the immune system and its importance in conferring protection against glioma development has been extensively investigated in the last 5 years. The genetic landscape of certain glioma antigens that allow the immune system to discriminate between cancer cells and non-transformed cells remains unclear. In the search for a source of antigens that are able to elicit specific T cell responses against melanoma, the Wölfel, and Rosenberg and Robbins groups initiated interest in tumor neoantigen as therapeutic targets in 2005 (101,102). Using expression-cloning approaches and *ex vivo* expanded tumor-infiltrating lymphocytes (TILs), the authors described antitumor T cell responses against melanoma antigens that were formed by somatic mutations: Neoantigens. The characterization of neoantigens has made an important contribution to cancer immunology and immunotherapy (103,104) and its understanding in DIPG, for example, may allow a better understanding of the innate and adaptive immune response to markedly improve novel immunotherapies strategies, clinical response rates and, eventually, patient survival. As a result of non-synonymous mutations, neoantigens may be identified using several tools which allow the comparison of DNA isolated from cancer samples with that of normal tissues. cDNA libraries, whole exome sequencing, transcriptome sequencing and MHC-binding prediction are examples (104). A comprehensive list of neoantigens is available at the Immune Epitope Database and Analysis Resource (www.iedb.org). Identifying driver and passenger mutations in GBM using genomic approaches was one of the first studies to be depicted by The Cancer Genome Atlas (cancergenome.nih.gov); however, studies in DIPG are required. These novel tumor-specific antigens may be the key to developing successful cancer therapies (105). An approach known as the cancer exome-based method has been used to determine the T cell reactivity against neoantigens (106). The success of immune checkpoint blockade, particularly using anti-CTLA-4 and anti-PD-1 in patients with melanoma and lung cancer may be explained by their potential formation of a neoantigen repertoire (107). In fact, it has been demonstrated that melanoma and lung cancer cells have increased mutation rates compared with glioma (108).

Pollack *et al* (109) evaluated the first clinical vaccination using human leukocyte antigen (HLA)-A2-restricted peptides from Eph receptor A2 (EphA2, a receptor tyrosine kinase

which in healthy cells regulates the cell growth negatively), IL-13 receptor subunit α2 [IL-13Ra2, a membrane glycoprotein that mediates activation of the transforming growth factor-β1 (TGF-β1) promoter upon stimulation by IL-13 or IL-4 and TNF-α] and survivin (an apoptosis inhibitor protein) for childhood brain cancer. The GAA peptide vaccination was well-tolerated, and exhibited initial evidence of an immunological and clinical response (109). Pollack *et al* (110) investigated the protein expression of three glioma-associated antigens in pediatric brain stem glioma and non-brain stem glioma; their results suggested that EphA2, IL-13Ra2 and survivin are reasonable targets for developing vaccines methods for pediatric glioma. Chheda *et al* (111) provided solid evidence to developing T cell-based therapy targeting neoantigens in DIPG. The authors identified and investigated a novel HLA-A*02:01-restricted neoantigen (10-mer peptide) containing the H3.3K27M mutation in DIPG neurospheres, NSC mice bearing intracranial U87H3.3K27M luciferase and donor-derived peripheral blood mononuclear cells (PBMCs). As a result, it was demonstrated: i) that the novel neoantigen was restricted to binding specifically and with a high affinity for HLA-A*02:01, but not for HLA-A*02:02, HLA-A*02:03, HLA-A*02:06, HLA-A*02:07 and HLA-A*02:17; ii) that the neoantigen induced specific T cell responses in DIPG-derived PBMCs, but not in healthy donors; iii) neoantigen-specific CTL reactivity; and iv) TCR transduction encoding the neoantigen inhibited progression of DIPG in xenograft mice. Ochs *et al* (112) demonstrated that the vaccination of neoantigen (27-mer peptide) encompassing the H3.3K27M mutation induced a marked Th1 immune response in transgenic mice (112). It has been demonstrated in preclinical models that a potent antitumor immune response, primarily by cytotoxic lymphocytes, is achieved when the combination of immune checkpoint inhibitors including anti-CTLA-4 (ipilimumab) and anti-PD-1 (nivolumab) are administered to markedly mutated types of cancer, including melanoma and lung cancer (113,114). Such evidence together with assumptions about the immunogenic profile of gliomas, the TME, the expression of negative immune checkpoints that lead to inappropriate T cell activity and the novel neoantigen candidates afford a rationale for improving the immune response by combining immune checkpoint inhibitors and peptide vaccines. In conclusion, this approach may lead the way to personalized immunotherapy. Gubin *et al* (115) identified that certain tumors are vulnerable to cancer immunotherapy. Using whole exome sequencing/RNA-sequencing and epitope prediction, the authors could identify specific tumor-specific mutations that work as tumor neoantigens (115). Neoantigens are privileged targets for T cells activated by checkpoint monoclonal antibodies; furthermore, neoantigens may be used in therapeutically effective and personalized cancer vaccines (115). By taking advantage of neoantigen discernments, Ott *et al* (116) established the feasibility, safety and immunogenicity of a vaccine that targeted 20 predicted personal tumor neoantigens. The vaccination induced marked multi-functional T cell responses in patients with melanoma; additionally, the vaccine-induced T cells discriminated mutated from wild-type antigens (116). Of six patients monitored, four had no recurrence at 25 months after vaccination, whereas two with recurrent melanoma were subsequently treated with anti-PD-1 therapy which led to complete tumor regression with neoantigen-specific T cells. Mutant neoantigens remaining in tumors are favored targets of T cells reinvigorated by checkpoint blockade therapy. These data offered a strong rationale for further development of this approach, alone and in combination with checkpoint blockade or other immunotherapies (116). The association between neoantigens and DIPG immune response remains unknown.

Zhou *et al* (117) demonstrated that B7-H3 (CD276), a type I transmembrane glycoprotein, is overexpressed in DIPG samples and the rates of B7-H3 expression were associated with malignancy grade in brainstem gliomas. There is currently no consensus on its biological function in DIPG; however, in several types of cancer, including prostate (118), colon (119), pancreatic (120), renal (121), ovarian (122) and bladder (123) cancer, B7-H3 appears to be responsible for promoting tumor invasion and metastasis.

The ability of immune cells to respond to several tumor antigens and traffic also boosts their attractiveness for the treatment of metastatic cancer. Medulloblastoma, a high-grade (IV) brain cancer, appears to have an immunosuppressive and hostile TME, mainly due to an immunosuppressive profile conferred by M2 macrophages (124). Increased expression of CD1d in a Sonic hedgehog-overexpressing mouse model of medulloblastoma has been demonstrated to be a therapeutic target and may be an exciting alternative for other types of glioma as well (125). The use of CAR-T cells specific to HER2 displayed efficacy against medulloblastoma in a murine model (126).

The antitumor immune reactivity within GBM, but not DIPG, has been investigated and, although there are quantitative antitumor effector cells present within the glioblastoma, these immune cells are non-reactive (127). This fact may be associated with an increased expression of co-inhibitory molecules on T cells or due to the immunosuppressive microenvironment.

In preclinical studies of GBM, the monotherapy targeting PD-1/PD-L1 and CTLA-4 resulted in long-term survival (128,129). The impact of immune checkpoint on gliomas is currently unmapped, and a better understanding of the pharmacodynamics and immune monitoring effect of immunotherapy on glioma, particularly DIPG, is expected to contribute to the development of this therapeutic approach in children with DIPG. A study by Berghoff *et al* (130) demonstrated PD-L1 expression in 88% (103/117) of patients with GBM. The expression of PD-L1 or other inhibitory molecules required to induce marked tumor-induced immune suppression or the level of expression that is associated with a therapeutic response is unknown.

Currently, according to clinicaltrials.org, there are 30 cancer immunotherapy clinical trials ongoing that are targeting GBM (compared with seven clinical trials for DIPG) which may be divided into five major categories: i) Vaccines [cytomegalovirus (CMV) antigen pp65-lysosome-associated membrane protein, mRNA-pulsed dendritic cells, autologous Wilms' tumor 1 (WT1) mRNA-loaded dendritic cells (DCs), tumor lysate-loaded DCs, brain tumor stem cell mRNA-loaded DCs, epitope enhanced peptides corresponding to IL-32Ra2, polyinosinic-polycytidylic acid stabilized with poly-l-lysine and carboxymethylcellulose (poly-ICLC) peptide vaccine, attenuated *Listeria monocytogenes* encoding EGFR variant III

(EGFRvIII) and NY-ESO, personalized peptide vaccine plus poly-ICLC/granulocyte/macrophage colony-stimulating factor (GM-CSF) and isocitrate dehydrogenase 1 (IDH1) peptide vaccine]; ii) checkpoint inhibitors (anti-PD-1, anti-LAG-3, anti-IL-15 and anti-CTLA-4); iii) combinations (DC plus T cell adoptive transfer, DC plus anti-PD-1, TGF-β receptor I inhibitor plus anti-PD-1, anti-CD27 plus anti-PD-1, anti-PD-L1 plus radiation therapy and anti-PD-1 plus oncolytic adenovirus); iv) adoptive T cell (CAR-T EGFRvIII, CAR-T IL-13Ra2, CAR-T CD133, CMV CAR-T HER2, CMLV CTL T cells, PD-1:CD28 switch receptor); and v) NK donor cells. The knowledge about epitope spreading and further T cell activation in autoimmune diseases are well-established; however, in brain cancer, these mechanisms require improved elucidation (96). Chongsathidkiet *et al* (62) suggested in a glioblastoma model that tumor cells present in the central nervous system inhibit T cell migration and induce sequestration of T cells in the bone marrow. Furthermore, GBM cells are able to stimulate IL-10, arginase-1, indoleamine 2,3-dioxygenase and tryptophan 2,3-dioxygenase responsible for converting monocytes into myeloid-derived suppressor cells (MDSCs) and regulatory T cells (Tregs), and inducing an immunosuppressive microenvironment (63,96,131). Immune checkpoint approaches (antagonists or agonists) may be useful to revert this phenotype in glioma. Regarding DIPG, there are 50 clinical trials ongoing (Table I), but only seven are concerned with immunotherapy approaches: Use of autologous dendritic cells (NCT02840123), H3.3K27M peptide vaccine (NCT02960230), Toll-like receptor agonist (NCT01400672), PegIntron® (PEGylated IFN-α2b; NCT00036569), anti-PD-1 (NCT01952769 and NCT02359565) and WT1 protein-derived peptide (NCT02750891). Except for NCT00036569, no results for these clinical trials have yet been released. The subsequent Phase II clinical trial tested the cytotoxicity and the efficacy of PegIntron. The study was sponsored by National Cancer Institute and has had 32 patients enrolled (median age, 6.28 years). The primary goal of the study was to evaluate whether there is a difference in the 2-year survival rate of patients treated with radiation alone compared with those patients treated with radiation and followed by PegIntron (132). The 2-year survival rate was not significantly different compared with the other group; however, the time for progression was increased to 7.8 months compared with 5 months in a similar population (132). As a monotherapy, the treatment was not satisfactory, but its use in combination with immune checkpoint blockade or other types of immunotherapy may be effective. Preclinical and immune monitoring studies are required to support the hypothesis that combining immunotherapy and standard treatment or their use as a monotherapy may benefit patients developing DIPG, an orphan type of cancer. Also, analyses involving TME and their ligands, T cell immune checkpoint profiles (stimulatory/inhibitory) and neoantigens may indicate which patients may respond to a certain type of immunotherapy and may also be used for the rational design of novel immunotherapy approaches. A thorough understanding of the molecular signatures and immunoprofiling as a predictor of patient response to cancer therapy and the use of several tools comprising the next generation of sequencing technologies which allow the understanding of, for example, a range of genes from different immune cell types, the epigenetic changes, the B and T cell receptor repertoire. The NanoString approach, whole exome sequencing, protein array, flow cytometry, mass cytometry by time-of-flight (CyTOF), immunohistochemistry (IHC), multiplexed ion beam imaging and systematic evolution of ligands by exponential enrichment have been used to pursue potential biomarkers and contribute to the future of cancer immunotherapy. There is therefore a requirement for studies aimed at DIPG, neoantigens and the TME. Preclinical studies using an orthotopic model or clinical studies, for example, would answer how certain standard therapies including radiotherapy or targeted therapy including HDACi or even combinations with immunotherapy may affect the immune response and the clinical outcomes.

5. Cancer immunoediting in glioma

Cancer cells exhibit several mechanisms of avoiding or suppressing the immune response in an attempt to prevent their destruction and consequently providing for their development and progression (15). This process is known as cancer immunoediting and comprises a well-established and co-ordinated subsequent process known as elimination, equilibrium and escape (16,17). In the last phase, transformed variant cells selected in the equilibrium phase undergo clonal growth in an immunologically regulated environment characterized mainly by the decrease in co-stimulatory molecules (18), antigen loss due to the downregulation of MHC molecules (133), resistance to apoptosis (19,134), the augmentation of $CD4^+CD25^+$ forkhead box P3 $(FoxP3)^+$ Tregs (135), IL-10-secreting T cells (136), M2 macrophages (137), MDSCs (138) and the increase in the expression of T cell-inhibitory molecules (immune exhaustion markers) including PD-1, PD-L1, PD-L2, CTLA-4, LAG-3, TIM-3, VISTA and galectin-9 (141,142).

The presence of several inhibitory immune checkpoints that block T cell responses and stimulate T cell responses offers particular strategies for mobilizing the immune system to attack cancer cells (139). CTLA-4 and PD-1 are strategic receptors on activated T cells that mediate immunosuppression in cancer. In the first case, CTLA-4 on the T cells binds to two ligands on antigen-presenting cells (CD80 and CD86), the same ligands required for CD28 activation. Owing to the higher affinity for both ligands, CTLA-4 is a competitive inhibitor for T cell activation (140). In the second case, PD-L1 and PD-L2, present on cancer cells or stromal cells interact with PD-1 receptor causing a downregulation of T cell responses (141). The immune checkpoint blockade mediated by anti-CTLA-4 and anti-PD-1 monoclonal antibodies remains to be elucidated (142). Immunotherapy with immune checkpoint inhibitors in certain types of cancer has revealed significant success in the last decade (143-146). Despite the accomplishments of these therapies, not every patient responds to immune checkpoint blockade and even the responders often experience toxic effects. Furthermore, there is an increasing requirement to identify potential biomarkers, primarily in immune cells, which may predict whether the patient with cancer may or may not respond to a particular immunotherapy, including immune checkpoint blockade or immune checkpoint agonist. Several clinical trials regarding immune checkpoint blockade are ongoing in glioblastoma and other types of brain cancer, but are required to be performed in DIPG.

Table I. Ongoing clinical trials in patients with diffuse intrinsic pontine glioma according to clincialtrials.gov.

Identifier	Status	Study results	Phase	Study type
NCT03101813	Recruiting	None available	NA	Observational
NCT01688401	Suspended	None available	Phase I	Interventional
NCT02420613	Recruiting	None available	Phase I	Interventional
NCT02233049	Recruiting	None available	Phase II	Interventional
NCT02992015	Recruiting	None available	Early Phase I	Interventional
NCT01182350	Active, not recruiting	None available	Phase II	Interventional
NCT01165333	Completed	None available	Phase I	Interventional
NCT00996723	Completed	None available	Phase I	Interventional
NCT01777633	Completed	None available	Phase I/Phase II	Interventional
NCT01400672	Completed	None available	Phase I	Interventional
NCT01106794	Recruiting	None available	NA	Observational
NCT01393912	Completed	None available	Phase I	Interventional
NCT00890786	Active, not recruiting	None available	Early Phase I	Interventional
NCT03086616	Recruiting	None available	Phase I	Interventional
NCT02840123	Recruiting	None available	Phase I	Interventional
NCT01222754	Active, not recruiting	None available	Phase I	Interventional
NCT03126266	Not yet recruiting	None available	NA	Interventional
NCT00036569	Completed	Available	Phase II	Interventional
NCT02960230	Recruiting	None available	Phase I	Interventional
NCT02717455	Recruiting	None available	Phase I	Interventional
NCT03178032	Recruiting	None available	Phase I	Interventional
NCT00600054	Completed	None available	Phase II	Interventional
NCT01922076	Recruiting	None available	Phase I	Interventional
NCT01517776	Terminated	None available	Phase II	Interventional
NCT02274987	Recruiting	None available	NA	Interventional
NCT01952769	Active, not recruiting	None available	Phase I/Phase II	Interventional
NCT02758366	Recruiting	None available	Phase II	Interventional
NCT01644773	Recruiting	None available	Phase I	Interventional
NCT01058850	Terminated	None available	Phase I	Interventional
NCT01189266	Active, not recruiting	None available	Phase I/Phase II	Interventional
NCT02750891	Active, not recruiting	None available	Phase I/Phase II	Interventional
NCT02359565	Recruiting	None available	Phase I	Interventional
NCT03243461	Not yet recruiting	None available	Phase III	Interventional
NCT02607124	Recruiting	None available	Phase I/Phase II	Interventional
NCT01469247	Active, not recruiting	None available	Phase I/Phase II	Interventional
NCT02644460	Recruiting	None available	Phase I	Interventional
NCT00879437	Active, not recruiting	None available	Phase II	Interventional
NCT00028795	Completed	None available	Phase II	Interventional
NCT00278278	Unknown	None available	Phase III	Interventional
NCT01836549	Completed	None available	Phase II	Interventional
NCT02742883	Active, not recruiting	None available	Phase II	Interventional
NCT01445288	Recruiting	None available	NA	Observational
NCT01837862	Recruiting	None available	Phase I/Phase II	Interventional
NCT01884740	Recruiting	None available	Phase I/Phase II	Interventional
NCT02444546	Recruiting	None available	Phase I	Interventional
NCT00561691	Completed	None available	Phase III	Observational
NCT01878266	Recruiting	None available	Phase III	Interventional
NCT02644291	Recruiting	None available	Phase I	Interventional
NCT01502917	Recruiting	None available	Phase I	Interventional
NCT02343406	Recruiting	None available	Phase II	Interventional

Table II. Selected markers and approaches which may be used in cancer or peripheral blood mononuclear cells for immunomonitoring in diffuse intrinsic pontine glioma.

Mass cytometry	Flow cytometry	Immunohistochemistry	Imaging mass cytometry	NanoString
CD68, PD-L1, VISTA, CD70, CD73, FoxP3, BTLA, 4-1BBL, ICOSL, CD80, B7-H4, CTLA-4, CD3, TIM-3, CD27, CD86, PD-1, CD28, KI67, 4-1BB, TIGIT, CD4, CD8, OX40, CD326, ICOS, LAG-3, CD11c, CD11b, CD44, CD62L, galectin-9, galectin-1,galectin-3, NY-ESO1, HVEM, B7-H3, CD45, GITR, PD-L2, OX40L, HLA-DR and CD56	CD4, CD8, ICOS, FoxP3, PD-1, 4-1BB, OX40, CTLA-4, CD14, CD16, CD56, CD69, NKp44, NKp30, NKG2A, NKG2C, NKG2D, PD-L1, Eomes, T-bet, Blimp-1, Bcl-6, c-Myc, CCR7, CD45RA, CD45RO, ROR-γ and VISTA	H3K27M mutant, GFAP, human nestin, Olig2, Ki67 human vimentin, CD4, CD8, CD45RO, GrB, ICOS, CD68, PD-L1, PD-1, arginase-1, iNOS and VISTA	CD31, CD68, CD3, CD4, CD8, CD45, actin, β-catenin, nestin, PDGFRA, ACVR1, FGFR1, α-SMA, histone H3, histone K27M mutant, CD44, c-Myc, VISTA, vimentin, EGFRvIII, CD133, galectin 1, galectin 3, FoxP3, CD25, PD-1, PD-L1, p53 and SHP2	CSAG2, MAGEA3, MAGEC2, IL13RA2, PRAME, CSPG4 and SOX10, CD45RO, CD20, CD57, FoxP3 and granzyme B

Data taken from (103,191-215,217-226).

The concept of cancer immunoediting has been explored in many types of tumor, but only recently has attention turned to glioma. Efforts have been made to understand how the immune system is able to support the glioma cancer cells to develop and grow, and at the same time dampen an anti-tumor immune response. Numerous antigens from glioma have been described, including IL-13Ra2 (109,111,147,148), N-acetyl-β-glucosamine (149), sex-determining region Y box (SOX)2 (150,151), EGFRvIII (152), SOX6 (153), glioma-expressed antigen 1/2 (154), paired helical filament 3 (154), NK group 2D (NKG2D), NKp30, MHC class I polypeptide-related sequence A/B (MICA/B) (155,156), UL16-binding protein 1 (157), EphA2 (109,110), EphB6 (158), antigen isolated from immunoselected melanoma-2 (159), squamous cell carcinoma antigen recognized by T cells 1/3 (160,161), HER2 (162), tyrosinase-related protein 2 (163,164), glycoprotein 100 (165), melanoma antigen-1 (166) and NY-ESO1 (167). Potential mechanisms in glioma immunoediting include evasion of adaptive T cell responses by altering the MHC class I antigen processing/presentation and a decrease in levels of cell-surface MHC I (168), antigen loss (169), a decrease in β2-microglobulin (170), latent membrane protein 2, transporter associated with antigen processing 1 and B7 expression (171), upregulation of B7-H3, impairing NK cell recognition by releasing NKG2D ligands (172,173) and increasing the expression of HLA-E and HLA-G, and, in certain cases, MHC class I expression (172,174) and increasing the immunosuppressive profile conferred by TGF-β, IL-10, prostaglandin E_2 and Treg promotion (175-178). Understanding the glioma immunoediting process will assist in unraveling the mechanisms in DIPG which abrogate an effector immune response. Unfortunately, all these mechanisms in DIPG are currently unknown.

Cancer immunoediting decreases the immunogenicity of developing tumors and supports tumor development. Therefore, immune monitoring studies have the potential to reveal the immunological machinery of antitumor responses, evaluate disease progression, assess the therapeutic effect, describe and identify novel candidates for immunotherapy, and act as predictive and correlative markers of clinical outcome.

6. Immune monitoring approaches to DIPG

To obtain an improved understanding of the DIPG microenvironment and the use of immune checkpoint approaches which may benefit a robust immune response against DIPG cancer cells, it would be interesting to conduct immune monitoring studies in patients with cancer, or an orthotopic mouse model for developing DIPG (Table II). As aforementioned, the immune checkpoint blockade is minimal in those tumors that have few somatic mutations and consequently poor neoantigen repertoire. Several studies have demonstrated that even by using immune checkpoint blockade including anti-CTLA-4 and anti-PD-1, several negative immune checkpoint molecules on T cells (TIM-3, LAG-3, VISTA, TIGIT and BTLA) are overexpressed, acting to dampen the immune activation in an attempt to achieve the immune homeostasis (179-190). These results were only possible due to the design of several immune monitoring approaches in the genomics and proteomics field. In a number of types of human cancer, the expression of immune checkpoints has been associated with a good or poor outcome. However, given the lack of clarity, the present review endorses the importance of immune monitoring studies in DIPG. Studies regarding immune monitoring approaches to DIPG have the potential to elucidate immunological mechanisms of antitumor responses, evaluate the therapeutic effect,

monitor disease progression, serve as a prognostic marker of clinical and outcome and also identify potential candidates for cancer immunotherapy. Immunological responses may be determined by alterations in T cell infiltrate and the quality of T cells regarding co-stimulatory/co-inhibitory molecules, the ratio of $CD8^+/CD4^+$ Tregs, cytokine production (inflammatory or immunosuppressive profile), the presence and macrophage differentiation (type 1 or type 2 macrophages), the expression of certain trivial tumor ligands including PD-L1, PD-L2, B7-H3, B7-H4, MICA/B and myeloid C-type lectin receptors. Blood and tissue samples may be analyzed to evaluate specific types of neoantigen or gene that may activate a robust immune response in DIPG. Furthermore, neoantigen-specific T cells may be assessed for immunogenicity using enzyme-linked immunospot (ELISPOT) and tetramer analysis. Overall, the immune monitoring profile from preclinical/clinical blood and tissue samples is the best way to assess and characterize the quality of immune response from those patients who are undergoing several therapeutic approaches. Studies which aim to estimate the impact of several therapies on T cell responses to DIPG neoantigens in tumor samples and to investigate the association between T cell neoantigen responses and other immunological parameters to drug-associated toxicity and radiographical responses would be interesting to investigate certain biomarker candidates in patients developing DIPG.

The increased availability of tissue samples for preclinical investigation, the development of novel experimental model systems, the advent of next-generation sequencing, IHC, flow cytometry and CyTOF provide useful tools to monitor the future of immunotherapy. Immune monitoring studies as proposed in the present study would benefit a better understanding of whether several types of immunotherapy and combinations are able to restore the quality of antitumor responses in DIPG. Serum cytokines, angiogenic factors and chemokines should also be evaluated using ELISPOT, ELISA or other relevant multiplex-based protein assay methods. Comprehensive analysis in immune profiles may lead to the identification of immune-based biomarkers.

The preclinical approaches may provide the opportunity to investigate in depth the effects of cancer immunotherapy and its possible use of adoptive transfer strategies including $CD8^+$, CAR-T cells, NK cells, immune checkpoints and drug delivery on gliomas. So, the primary goal of performing immune monitoring studies in DIPG specimens is to determine an immunological profile in PBMCs and the potential changes in tumor samples (tumor ligands and TME) in an attempt to understand the TME and also the systemic immune response. The establishment of immunological biomarkers may promote the development of practical immunotherapeutic approaches. To determine the adaptive immune signatures in the TME at the baseline and during the course of the treatment using the immune checkpoint blockade combination (anti-CTLA-4 and anti-PD-1), Chen *et al* (66) performed, using melanoma tissue samples, an immune profiling study by analyzing a 12-marker IHC panel and gene expression pertaining to common cancer signaling pathways using the NanoString approach. By comparing responders and non-responders during the CTLA-4 and anti-PD-1 blockade, it was demonstrated that, at baseline, no change was observed in any of the biomarkers measured, including CD45RO, CD20, CD57, CD68, FoxP3, granzyme B, PD-1, LAG-3, CD14, CD33, CD163 and CD206. However, there was a significantly increased density of $CD8^+$ T cells in responders compared with in non-responders during the treatment. Additionally, increased expression of CD45RO, CD20, CD57, FoxP3 and granzyme B was detected in responders compared with in non-responders. Together, these results are relevant in the prediction of biomarkers of response and resistance to the immune checkpoint blockade, while offering a mechanistic understanding of immune checkpoint blockade as associated with enhanced cytotoxic activity, antigen processing and the IFN-γ signaling pathway. In another study using the NanoString approach, Beard *et al* (191) investigated the potential candidates for immunotherapy by comparing the gene signatures between melanoma and healthy tissue. In melanoma samples, chondrosarcoma-associated gene 2/3 protein, melanoma-associated antigen A3 (MAGEA3), melanoma-associated antigen C2 (MAGEC2), IL-13Ra2, preferentially expressed antigen in melanoma, chondroitin sulfate proteoglycan 4 and SOX10 were overexpressed when compared with the healthy tissue (169).

In glioma cancer, the NanoString approach has been used extensively to identify driver genes as potential therapeutic targets and also to predict signature disease specific-survival and recurrence-free survival times (192-206). However, there is an evident lack of studies involving patients with DIPG.

The significance of an integral immune surveillance in controlling cancer progression and development has been known for a number of years. Several studies have demonstrated a marked association between TILs in cancer tissue and favorable prognosis in numerous malignancies (85,207-209). $CD8^+$ T cells and the ratio of $CD8^+$ effector/$FoxP3^+$ Tregs appears to be associated with improved prognosis and long-term survival in a number of solid tumors. CyTOF has been used to define immune cell populations, to identify potential biomarkers and in drug development (210). By measuring 34 parameters simultaneously in single cells, Bendall *et al* (211) defined the hematopoietic hierarchy from healthy human bone marrow. Spitzer *et al* (212) analyzed the immune responses in several tissues following anti-PD-1 and PD-L1 immunotherapy by developing intuitive models (Scaffold map) to visualize single cell data with statistical implication (212). In their study, the authors were able to demonstrate the crucial involvement of the systemic immune response in cancer rejection. Wei *et al* (213) performed a comprehensive profile on the effects of checkpoint blockade (anti-CTLA-4 and anti-PD-1) on the tumor immune infiltrate in humans and mice. It was demonstrated that the tumor-infiltrating T cells were markedly similar between tumor models, but there were differences in the subsets of tumor-infiltrating T cell populations; whereas the anti-PD-1 therapy induced the expansion of exhausted $CD8^+$ T cells, anti-CTLA-4 induced the expansion of the Th1-like $CD4^+$ effector cell population. In a phase II clinical trial designed to determine the efficacy of metronomic temozolomide in recurrent glioblastoma patients, Omuro *et al* (214) determined, using mass spectrometry genotyping/iPLEX®, tissue from 38 patients (28 glioblastomas and 10 grade III), the presence of certain mutations previously described in glioma. Mutations were identified in EGFR (EGFR_P596L, EGFR_C620Y, EGFR_G598V, EGFR_I91L, EGFR_T263P and EGFR_V651M), IDH1 (IDH1_R132L_H and R_132C_G_S) and ERBB2 (ERBB2_L49H) (214).

Together, these results provided substantial insights that CyTOF and the tools available for high-dimensional analysis, including Scaffold, SPADE, viSNE and CITRUS, offer a feasible way of correlating and predicting immune biomarkers, which has not yet not been performed in DIPG.

7. Conclusions

The present review makes a claim for the importance of immune monitoring studies in DIPG, an incurable brain cancer representing the principal cause of mortality from pediatric brain tumors. Growing diffusely in the ventral pons, the tumor causes disabling neurological symptoms that gradually abolish the co-ordination of the face, pharynx and body. Unfortunately, surgical resection is not a feasible option, radiation therapy results only in temporary stabilization of symptoms, and several chemotherapy trials developed for adult glioma have not demonstrated satisfactory results to date (215). Novel and useful therapies are urgently required, and immunotherapy may be an excellent strategy to modulate the immune response to this devastating disease. Several immune checkpoint molecules have been proposed in the present review as having an involvement in a potential strategy for mobilizing the immune system for the treatment of DIPG. Also, by sharing the recent achievements in other types of cancer, the present review may serve to stimulate the scientific community in developing preclinical and clinical immune monitoring studies in DIPG. An improved understanding regarding TME, cancer immunoediting and neoantigens is required.

Randomized clinical trials have allowed the advance of a consistent evidence-based medicine. Currently, several tools may be used to characterize the biology of several types of cancer, and the present review highlights the requirement for designing more descriptive clinical trials which are expected to result in more marked treatment effects for a more significant portion of treated patients. In the present review, certain immunological landscape approaches including flow cytometry, CyTOF, IHC and imaging mass cytometry, as well as certain biomarker candidates, were proposed as potential approaches that may be useful to perform immune monitoring studies in tissue and PBMCs from patients developing DIPG. It is therefore clear that molecular tools which allow the identification of i) alterations at the genomic level (DNA/RNA) including single-nucleotide variations, insertion/deletion, loss of heterozygosity and translocation; ii) epigenomic modifications including DNA methylation (CpG islands), DNA methylation (non-CpG islands) and histone modification (acetylation or methylation); and iii) protein expression, post-translational modification, and protein complexes and interactions (whole exosome sequencing, whole genome sequencing and bisulfite sequencing), as well as targeted identification (multiple reaction monitoring) are essential to detect, diagnose, monitor, estimate and predict therapeutic interventions likely to benefit the patient (216). As the immune system is a multifaceted cellular system that is not entirely understood, particularly in the cancer microenvironment, the importance of monitoring the immune responses from those patients undergoing several treatment approaches has become crucial for understanding the cancer biology and potential biomarkers and also for designing new drugs. Several activating receptors on T cells including CD27, CD28, OX40, 4-1BB, CD40 ligand, DNAX-accessory molecule 1, transmembrane activator and calcium modulator and cyclophilin ligand-interactor, B cell maturation antigen, ICOS, GITR, B cell-activating factor belonging to the TNF family receptor, and inhibitory receptors including CTLA-4, PD-1, PD-L1, CD160, CD200 receptor, LAG-3, 2B4, TNF superfamily member 14, BTLA, VISTA, TIGIT, B7-H3, B7-H4 and killer cell lectin-like receptor subfamily G member 1 have been used to design agonists or antagonists, respectively, as immune checkpoint targets to improve immune responses against several types of cancer. The robust clinical responses, but not universally, in a diverse group of advanced cancer suggest that immune checkpoint immunotherapy may be also be beneficial in the treatment of DIPG. Assuming that in the glioblastoma, numerous infiltrating Tregs and high levels of immune-suppressive cytokines dictate the immune suppression, the T cell exhaustion profile and also the potential neoantigens in DIPG should be considered. In the present review, the idea of immune monitoring approaches has been discussed, and the use of antagonists or agonists as immune checkpoint approaches for the treatment of DIPG are also supported. The recognition that the immune response is dysfunctional in patients with cancer and also the current immune checkpoint strategies including agonists/antagonists have prompted certain questions regarding why only certain patients with cancer exhibit marked responses or how the efficacy, specificity and safety of cancer immunotherapy strategies may be increased, or what would be the best model system and biomarkers to address these questions. Monoclonal antibodies that block PD-1 and CTLA-4 proteins further exemplified the power of cytotoxic T lymphocytes in the control of tumor development by reversing cancer-induced immunosuppression and inducing durable therapeutic responses in certain patients with cancer. Immune checkpoint therapy targeting co-inhibitory or co-stimulatory molecules on T cells is a new paradigm for cancer treatment. Rather than targeting the tumor cell, this approach targets molecules on immune cells that regulate their activity to sustain immune responses to cancer and achieve elimination of tumors and immunity to recurrence. This strategy has proven effective in treating different types of cancer and is now the standard of care for metastatic melanoma and lung cancer. The high-dimensional analysis of tissue and blood samples from patients with cancer is being performed with several technologies and different software. Examples of these technologies are flow cytometry and CyTOF; the analyses are made using specific software including FlowJo, viSNE, SPADE, Scaffold and CITRUS.

In conclusion, substantial improvement has been made in the development of cancer immunotherapy. Immune monitoring studies have identified certain biomarkers which are able to reveal the complexity of the cancer microenvironment (217-226). The novel way of thinking and managing patients with cancer under immunotherapy perspectives by inducing the immune system to track down and destroy cancer cells it is not hyperbole, but rather a reality. Patients with only weeks or months to live are now surviving for years following treatment. Personalized cancer immunotherapies targeting tumor-specific mutant neoantigens are leading to combinatorial therapies, where several strategies act to kill cancer cells. Such effects have been accomplished in preclinical tumor

models and can now be extended to human patients with cancer including DIPG.

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