IMMUNOTHERAPY FOR TUMOR IN THE BRAIN: INSIGHTS FROM – AND FOR – OTHER TUMOR SITES

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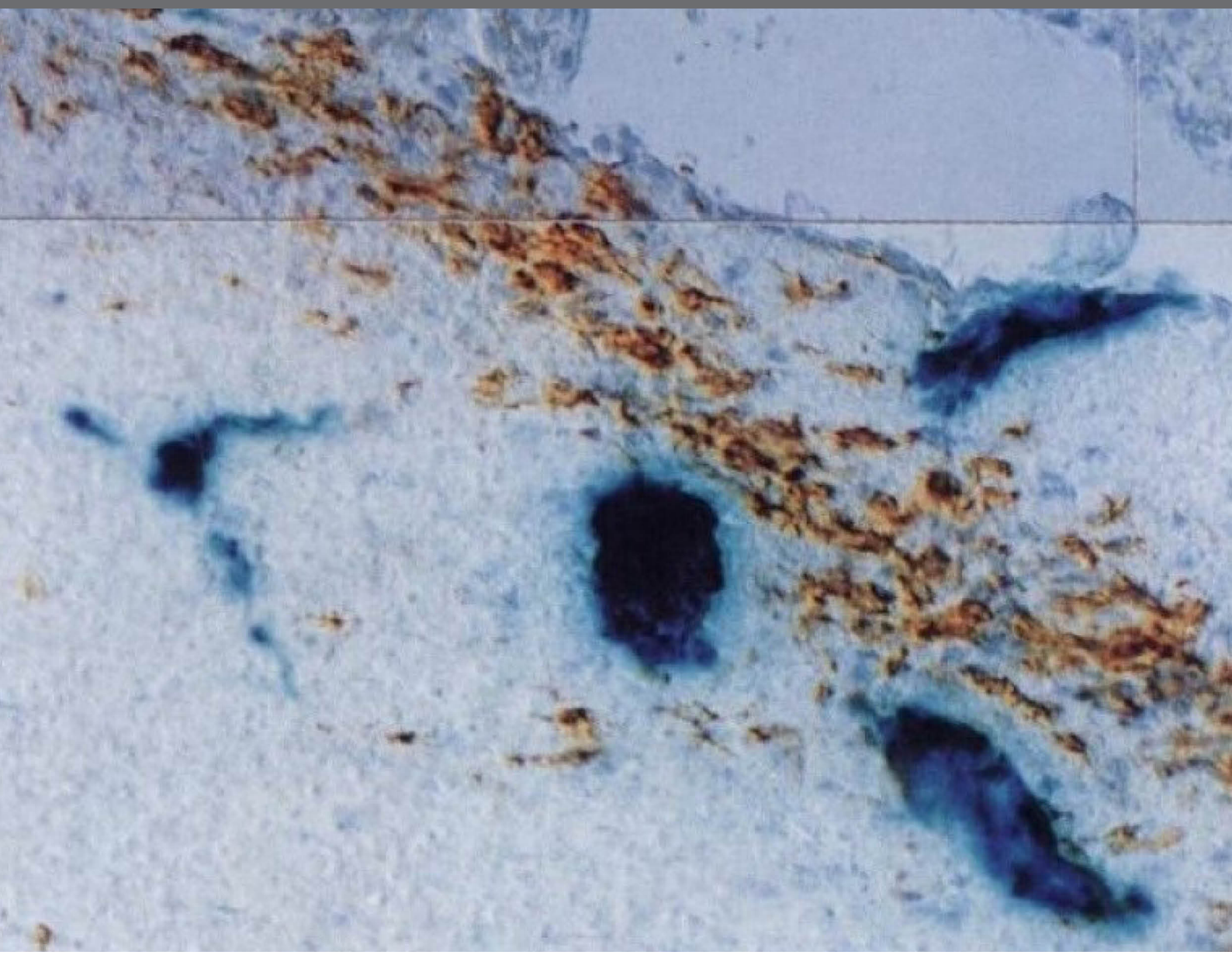

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IMMUNOTHERAPY FOR TUMOR IN THE BRAIN: INSIGHTS FROM – AND FOR – OTHER TUMOR SITES

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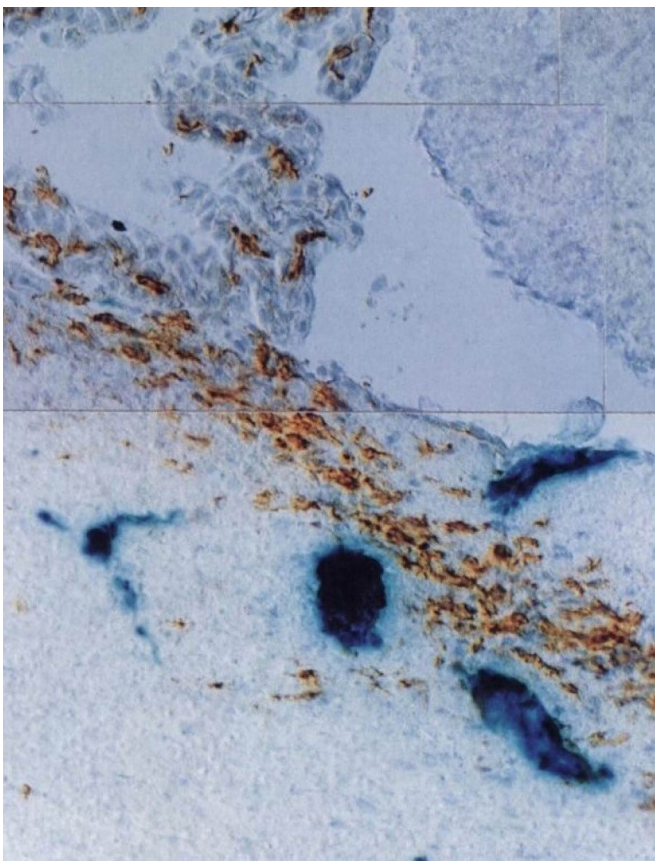

Growing tumor (bright blue) and leukocytes (dark brown) in a rat brain. Adapted from Lampson et al, (1992), fig. 3B, with permission.
Lampson LA, Wen P, Roman VA, Morris JH, Sarid JA. Disseminating tumor cells and their interactions with leukocytes visualized in the brain. *Cancer Res.* (1992) 52:1018–25.

Tumor immunotherapy has now shown its promise for many, its disappointments and failings for others. Going forward, brain tumor patients can both benefit and contribute.

Tumor immunotherapy is steadily progressing. As experience accumulates, it is important to consider its generality. The reviews herein emphasize the brain's place among other tumor sites. Two major topics are addressed.

THE SITE: WHAT CAN WE EXPECT FROM IMMUNOTHERAPY WHEN THE TARGET IS IN THE BRAIN?

Experience with immunotherapy for different targets in the brain, including tumor and also pathogens, is reviewed. Long-standing assumptions are confronted. The potential for beneficial responses is stressed.

BRAIN TUMOR IMMUNOTHERAPY: WHAT HAVE WE LEARNED SO FAR?
Clinical experience with brain tumor immunotherapy, from a variety of centers, is reviewed. Primary tumors, emphasizing glioblastoma, and brain metastases are each considered.

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EDITORIAL
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Editorial: Immunotherapy for Tumor in the Brain: Insights From—and For—Other Tumor Sites

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Keywords: brain tumor, brain immunology, brain tumor immunotherapy, glioblastoma, brain metastases, immune privilege, blood–brain barrier, tumor antigen

Editorial on the Research Topic

Immunotherapy for Tumor in the Brain: Insights From—and For—Other Tumor Sites

TUMOR IMMUNOTHERAPY: COMMON GROUND

Accelerating progress in tumor immunotherapy reflects a balance between what is particular to a given tumor and what cuts across tumor types and sites, including primary and metastatic brain tumors. Although there are tumor-specific differences, the epidermal growth factor receptor (EGFR) is an important target for both glioblastoma and non-small cell lung cancer, among others, making insight into basic EGFR biology broadly relevant (1). Indirect manipulation of the immune response acts even more broadly, with checkpoint inhibitors giving durable responses against the individual antigens expressed by multiple tumor types (2). Understanding of the nature of tumor antigen is still evolving. General insights into the practical requirements for specificity (3) and the importance of neo-antigens (2) complement identification of tumor-specific targets (4).

Insights into tumor biology show a similar mix of the general and the specific. For any therapy, the eventual outgrowth of therapy-resistant tumor is common. Although resistance mechanisms vary, a general insight applies: it is now appreciated how often the potential for resistance, whether as clones with pre-existing mutations or as alternative regulatory states, is already present within the original tumor (5–8). Also appreciated is the importance of interactions between individual tumors and their local micro-environment (9–11), including those that favor immunosuppression. In this case, many details are also common, with many of the same components, such as regulatory T cells (Tregs) or cytokines (IL-10, etc.), implicated in the brain as other sites (Dutoit et al.; Perng and Lim).

BRAIN TUMOR: LIMITS AND CONCERNS

Given these shared properties, it might be expected that tumor in the brain would show the same benefit from immunotherapy as tumor at other sites. In practice, it has been difficult to show definite benefit for brain tumor patients. This does not necessarily mean that brain tumors are more intractable. For primary brain tumors, there are practical limitations on clinical trials. The most common primary brain tumor in adults is glioblastoma (also referred to as glioblastoma multiforme or high-grade glioma). Although it's grim prognosis gives glioblastoma prominence in public awareness and as a research focus, among all tumors it is rare (12).

Brain metastases are far more common. They are characteristic of some of the most common primary tumors, including those of the lung and breast, and of the best-studied example of successful immunotherapy, metastatic melanoma. As these and other tumors come under better control at other sites, brain metastases are increasingly important as a site of recurrence. Survival after

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conventional therapies for brain metastases can be just a few months, and toxicity, especially after radiation therapy, is of great concern. Despite this background, patients with brain metastases have often been excluded from clinical trials (Cohen and Kluger). Reasons have ranged from pessimism, given the poor prognosis, to specific concerns about immunotherapy.

RETHINKING "PRIVILEGE" AND THE BLOOD–BRAIN BARRIER

A concern that has been relevant for all brain tumors, whether primary or metastatic, has been whether safe immunotherapy was even possible. A widespread, deeply entrenched assumption that the brain is, or should be, "immunologically privileged" was supported by awareness of detrimental responses, including autoimmune disease, such as multiple sclerosis, or a pathological response to neural virus. Fortunately, this concern is increasingly understood to be outdated (Huber and Irani).

From many contexts, especially work with neural viruses, it is clear that the immune response in the brain can be beneficial, is necessary, and, just as in other organs (3), is under regulatory control. Although, just as in other organs, a mis-regulated response can cause its own pathology, the immune response can safely control virus in the brain (13) (Huber and Irani; Huber et al.).

A related concern has been whether the blood–brain barrier (BBB) would prevent immune effectors from reaching brain tumor sites (14). In the normal brain parenchyma, passive entry of antibody protein is indeed blocked by the BBB. Nonetheless, antibody can affect the brain. The BBB is plastic; it changes as tumor grows. Indeed, leakage of immunoglobulin into the brain is a classic sign of pathology. The extent to which antibody can enter the brain, or accumulate, at sites of pathology, and the mechanisms by which even small amounts may be beneficial are of current interest for tumor, especially microscopic tumor (3, 14), and more broadly (15).

Although the same term is used, BBB has a different meaning for effector cells (cytotoxic T cells, etc.) than for antibodies. Metabolically active, migratory cells are well able to enter the tissues, including the brain, if appropriate signals are present (Huber et al.) (13, 16). Indeed, precursors to antibody forming *cells* can enter the brain, and make antibody from within it. Precedent is seen in the life-long production, within the brain, of antibody to neural viruses (Huber et al.) (13); this potential has not yet been intentionally exploited against brain tumor (14).

WHERE WE ARE

A more optimistic view of immunotherapy for the brain is consistent with accumulating experience with brain tumors, as described by the papers herein. The clearest benefit has been seen for brain metastases (Cohen and Kluger). Many approaches are also being taken for glioblastoma (Ampie et al.; Dutoit et al.; Van Gool; Yamanaka and Hayano), although the work is at an earlier stage. The immune response encompasses a multitude of effector cells, molecules, and mechanisms; although broadly shared, their balance and regulation vary (3) (Huber et al.; Perng and Lim; Dutoit et al.). As targets, the biology of glioblastoma is very different from that of most metastases (3, 14); the optimal immunotherapy strategy need not be the same.

Today, we have seen that the immune response is able to control human tumor, and that the response can be intentionally enhanced (2). The field is young. Not every patient benefits, many tumors recur, and often responses are not well-controlled. The papers herein illustrate growing appreciation that immunotherapy, its potential and its challenges, are just as relevant for the brain as for other sites. Achieving a balance between immunotherapy and autoimmunity is a general challenge, not only for tumors, and has been a special concern for the brain. As the brain's relevance becomes accepted, insights gained from the brain and other sites should reinforce each other.

AUTHOR CONTRIBUTIONS

The author confirms being the sole contributor of this work and approved it for publication.

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REVIEW
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Immunotherapy of Malignant Tumors in the Brain: How Different from Other Sites?

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Immunotherapy is now advancing at remarkable pace for tumors located in various tissues, including the brain. Strategies launched decades ago, such as tumor antigen-specific therapeutic vaccines and adoptive transfer of tumor-infiltrating lymphocytes are being complemented by molecular engineering approaches allowing the development of tumor-specific TCR transgenic and chimeric antigen receptor T cells. In addition, the spectacular results obtained in the last years with immune checkpoint inhibitors are transfiguring immunotherapy, these agents being used both as single molecules, but also in combination with other immunotherapeutic modalities. Implementation of these various strategies is ongoing for more and more malignancies, including tumors located in the brain, raising the question of the immunological particularities of this site. This may necessitate cautious selection of tumor antigens, minimizing the immunosuppressive environment and promoting efficient T cell trafficking to the tumor. Once these aspects are taken into account, we might efficiently design immunotherapy for patients suffering from tumors located in the brain, with beneficial clinical outcome.

Keywords: brain tumors, glioma, tumor immunotherapy, tumor microenvironment, brain homing

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The immune system, thanks to its power and specificity, has extraordinary potential to achieve long-lasting tumor remissions, with no side effects on normal tissues. Manipulating the immune system to achieve such a goal is the objective of cancer immunotherapy, which has been under intense investigation for more than 20 years, with some successes, but also room for improvement. In particular, T cell immunotherapy aims to generate, *in vivo* or *in vitro*, efficient tumor-specific T cells able to reach the tumor microenvironment and provide long-term antitumor function. This approach comes with many complexities, namely the choice of a tumor antigen, the source of tumor-specific T cells, the need to elicit strong immune responses, and to target the immunosuppressive tumor microenvironment. Immunotherapy has been developed for many malignancies, which now includes tumors in the brain. Decades of research have helped understanding the fundamentals of immune responses to tumors and showed that tumor-specific immune responses were able to occur, but were limited by the mechanism of tumor immunoediting (1). These studies also revealed that antitumor immune responses were able to occur in the brain, following similar rules to those applying to peripheral organs (2). However, the brain, as an immune specialized site, is endowed with additional hurdles to overcome before efficient immunotherapy can be achieved. Here, the means and requirements for successful immunotherapy will be identified and potential additional requisites for efficient immunotherapy of tumors located in the brain will be discussed. Ongoing immunotherapeutic clinical trials will finally be described to appreciate the current status of these approaches.

TUMOR IMMUNOTHERAPY: CURRENT APPROACHES

The aim of T cell-based tumor immunotherapy is to provide patients with tumor-specific T lymphocytes that will patrol the body to detect and kill tumor cells. This can be accomplished by either active or passive approaches.

Therapeutic Vaccination

Therapeutic vaccination relies on the patient's immune system to react to an injected tumor vaccine. Tumor vaccines aim to raise an immune response against tumor antigens using specific peptides, proteins, tumor cells (including lysates and eluates), mRNA, or DNA, in some cases pulsed onto dendritic cells (DCs) (3). One major advantage of peptide vaccines is that the antigen is well characterized, ensuring a precise targeting of the tumor with possibly little damage to normal tissue. In this regard, the best tumor antigen is a tumor-specific antigen (TSA), resulting from a tumor-specific mutation. Whereas such TSA are the ideal targets, they are not shared by the majority of patients and were until recently not frequently exploited for peptide vaccines. However, advances in personalized vaccine approaches will most probably revive their use, as patient-specific tumor mutations can now be relatively easily identified and used as vaccine antigens (4). In contrast to TSA, tumor-associated antigens (TAAs) are shared by a larger proportion of patients and have been widely used in cancer vaccines over the years. TAA derive from proteins overexpressed in cancer cells but retaining some expression in healthy tissues, which varies depending on the antigen. This is the major drawback to their use, as potential harm to normal cells cannot be excluded, which can be fatal depending on the cells or organ involved. Although TAA-based peptide vaccines have not shown major toxicity thus far (3), adoptive cell therapy has been faced with severe adverse events including deaths due to TAA expression by normal tissues (5), as will be discussed later. Another advantage of TAA is that they are shared among patients and can thus be exploited to design multipeptide vaccines with the aim to prevent tumor escape by antigen downregulation, a phenomenon observed occasionally with single-peptide vaccination (6, 7). It is hypothesized that the latter can be circumvented by the process of epitope spreading, whereby immune responses are directed toward additional tumor antigens liberated from lysis of the initially targeted cells (8, 9). Nonetheless, the use of well-defined antigens is limited by the need for identification and many groups have therefore chosen to vaccinate with whole tumor cells or tumor mRNA (10, 11). This approach has the advantage of providing patient-specific and multiple tumor antigens for vaccination but also presents the risk of inducing immune responses to non-tumor antigens present in the preparation. In addition, the requirement for sufficient tumor for vaccine preparation restricts their use to a subset of patients in malignances where small tumor samples are received, as is the case for tumors in the brain. To overcome this hurdle, some trials are using allogenic tumor cell lines for vaccine preparation (12). Finally, the use of undefined vaccine antigens makes immunomonitoring challenging, possibly hindering correlation between vaccine-induced immune responses and clinical outcome. Regardless of the antigen source, peptide and tumor vaccines have been injected with or without DC, the latter being used to bridge innate and adaptive immunity and more efficiently initiate vaccine-specific immune responses (13).

The Need for Adjuvants

Most antigens used in tumor vaccine are derived from self-proteins and therefore are not recognized by pattern recognition receptors of innate immunity (14). Therefore, in most ongoing clinical trials, tumor vaccines are injected with an adjuvant, which aims at stimulating innate immunity and augmenting vaccine immunogenicity. Many different adjuvants have been used since the beginning of cancer vaccine administration, but the current development of more and more ligands for innate pathogen recognition receptors such as TLR, RLR, or STING ligands, among others, is likely to improve vaccine efficacy (15). TLR and RLR are sensors that detect viral/bacterial DNA or RNA, or bacterial, fungal, or protozoan lipoproteins/peptidoglycans and induce type I interferons. Synthetic TLR3, TLR4, TLR7, and TLR9 ligands are being tested in cancer patients as single agent or in combination with cancer vaccines (15) and ligands for other TLRs are in development. STING ligands induce type I interferon after detection of intracellular DNA and have shown impressive antitumor effect in preclinical models (16–18), which should stimulate rapid translation into the clinic. In addition to the use of adjuvants, it was shown that inducing inflammation at the vaccine site by vaccination with recall antigens (tetanus and diphtheria toxoids, Td) prior to tumor antigen DC vaccine improved patient survival by increasing DC migration to the vaccine draining LN, a process which was dependant on CCL3 (19).

T Cell Therapy

T cell therapy does not rely on patient vaccination but on the adoptive transfer of high numbers of autologous tumor-specific T cells. The latter can be generated from tumor-infiltrating lymphocytes (TIL) or from antigen-specific T cells enriched from peripheral blood. Alternatively, peripheral T cells can be engineered to express a high-avidity tumor-specific TCR (TCR-transgenic T cells) or an antibody fragment [chimeric antigen receptor (CAR) T cells] (20). Adoptive transfer with TIL is based on the demonstration that T cells found at the tumor site are tumor-specific and endowed with tumor killing activity, reflected by the fact that, in many malignancies, infiltration by activated CD8 T cells correlates with patient outcome (21). However, few tumors are highly immunogenic and thus infiltrated by lymphocytes. In addition, the fact that tumor-derived T cells might be exhausted and might not persist long enough after injection for efficient tumor eradication has prompted the development of adoptive transfer with modified peripheral blood T cells (22). One option is TCR-engineered T cells that are made to express the α and β chains of a high affinity well-characterized HLA-restricted tumor-specific TCR; these can be relatively rapidly generated and infused to any patient sharing the cognate HLA and expressing the specific tumor antigen (23). An alternative approach is CAR T cells that are engineered to express a tumor-specific antibody as a single chain fragment to redirect T cell recognition to the tumor (24). They are not HLA-restricted as

their moiety for antigen recognition is an antibody and can therefore be given to any patient expressing the cognate antigen; an additional benefit is that this overcomes the mechanism of tumor evasion by MHC downregulation. One advantage of TCR-transgenic and CAR T cell transfer is that the large majority of the infused cells are tumor-specific, which provides the patient with considerable numbers of tumor-reactive cells. In addition, the antigen recognition domain of TCR-transgenic and CAR T cells can be mutated to increase affinity to the antigen, making infused cells of high avidity to the target antigen. Another key advantage of cell therapy with genetically modified T cells is the possibility to optimize the T cell product in terms of *in vivo* cell persistence, resistance to T regulatory T (Treg) cells, and effector functions (25). At the same time, increasing avidity and efficiency of infused cells renders the choice of antigen even more critical. As mentioned above, the level of adverse events observed in clinical trials using TCR-transgenic or CAR T cells is high and these can be fatal (5). On-target, off-tumor toxicities due to recognition by TAA-specific TCR-transgenic T cells of antigen expressed on healthy tissues are observed in the majority of patients treated (26). Severe adverse events due to cross-recognition of non-targeted antigens by high affinity mutated TCRs were also observed (27). To safeguard against this, many construct used to generate CARs now incorporate a suicide gene, with the aim to quickly deplete the transfused cells if life-threatening toxicity is seen. Cytokine storm, which is an early and potentially fatal adverse event resulting from the rapid activation of transferred T cells can usually be managed *via* treatment with anti-IL-6 antibodies (28). Another appealing solution was recently offered by the publication of a proof-of-concept study in mice illustrating eradication of established solid tumors by transfer of high-avidity TCR-transgenic T cells specific for one single neoepitope (29). Hence, the development of mutation-specific TCR-modified cells, even if targeting a single epitope, could allow the design of safe and powerful clinical trials by inducing epitope spreading, as seen with other tumor-specific cell therapies (30, 31).

The Challenge of the Tumor Microenvironment

One of the greatest hurdles for efficient tumor immunotherapy is the fact that tumor-specific T cells have to exert their effector function in a hypoxic environment, in which chronic inflammation and tumor cells stimulate immunosuppression (32). Among the many mechanisms evolved by the tumor to escape immune response are the secretion of immunosuppressive cytokines (TGF-β and IL-10, among others), the recruitment or induction of immunosuppressive cells [Tregs, myeloid-derived suppressor cells (MDSCs), tumor-associated macrophages (TAMs)], the depletion of essential nutrients [by indoleamine dioxygenase (IDO) and arginase] and the expression of inhibitory molecules (FasL, PD-L1). Treg constitute an important fraction of tumor-infiltrating $CD4^+$ T cells and inhibit tumor-reactive T cells either by direct cell contact or through TGF-β and IL-10 production (33). TAMs contribute to IL-10 and TGF-β production, to Treg recruitment by the secretion of CCL22, and promote tumor growth and invasion through production of endothelial growth factor, vascular endothelial growth factor (VEGF), and platelet-derived growth factor (PDGF), among others (34). MDSCs mostly act by inhibiting T and NK cell function through arginine depletion and production of nitric oxide and reactive oxygen species (35). Tumors also evade immune recognition by downregulating molecules required for T cell recognition, such as MHC, the antigen itself, or molecules implicated in antigen processing (32). Targeting these mechanisms is required to fully benefit from the efficacy of vaccine-induced or modified tumor-specific T cells.

Immune Checkpoint Inhibitors

The immune checkpoint molecules expressed during normal immune responses to prevent immune overactivation are also playing a substantial role in antitumor immunity. Many of these molecules are expressed in tumor-specific T cells, probably due to chronic antigen stimulation occurring at the tumor site, and their expression correlates with an exhausted phenotype and loss of effector function (36). On the other hand, ligands for many immune checkpoint molecules are upregulated in the tumor environment by tumor cells, stromal cells, DCs, or MDCS and participate in antitumor response inhibition (37, 38). The physiological relevance of immune checkpoint molecules is supported by the outstanding clinical efficacy of immune checkpoint blockade (ICB) antibodies (39). Anti-CTLA4 and PD1 antibodies are now approved for several malignances and are being tested for virtually all tumor types together with anti-PD-L1 antibodies, and antibodies targeting Tim3 and LAG3 are in clinical trials, mostly in combination with anti-PD1 antibodies.

Immune checkpoint inhibitors work by allowing pre-existing immune responses to TAA or TSA to occur. However, efficacy of anti-CTLA4 and anti-PD1 as single agents has been greatest in malignancies that harbor a high rate of mutation, such as melanoma and some lung carcinoma (40, 41), suggesting that TSA-directed immune responses are prevalent. Accordingly, studies in melanoma have shown that the majority of tumor-reactive T cells found in TILs were recognizing TSA and not TAA (42) and response to ICB has been shown to be associated with detection of neoepitope-specific T cell responses (40). A critical question for the use of ICB for malignancies harboring low rates of mutations is thus to be able to determine the minimal mutation load required to achieve efficient tumor destruction with these agents. In that regard, one study in melanoma showed that patients harboring tumors with >100 mutations were more prone to benefit from anti-CTLA4 treatment (40), suggesting a threshold for ICB molecule efficacy. Nonetheless, this is not absolute, as some patients still benefit from treatment with IBC despite low-mutation rate (43). In addition to allowing neoantigen-specific immune responses to occur, immune checkpoint inhibitors are also able to amplify vaccine-induced immune responses and trials of peptide vaccination against melanoma antigens in combination with a soluble LAG3 have been reported, which showed the safety of the approach (44, 45). Combination with other immunotherapies is ongoing and is likely to be an important contribution of ICB antibodies to cancer treatment in the future.

IMMUNE RESPONSES TO TUMORS ARISING IN THE BRAIN

With the exception of primary central nervous system (CNS) lymphoma (PCNSL) arising from B cell transformation, most primary brain tumors (astrocytoma, oligodendroglioma, oligoastrocytoma, and ependymoma) derive from glial cells. They account for approximately 2% of all cancers, but the associated mortality is very high, the 5-year survival rates for grade III astrocytoma and glioblastoma (GBM, grade IV), the most common, being 30 and 3%, respectively (46). The major characteristics of GBM are its highly invasive nature and extraordinarily low rate of metastasis outside the brain. Regarding PCNSL, it is a rare disease, representing about 2% of all primary brain tumors in immunocompetant hosts (47), but, similar to gliomas, is very aggressive and associated with poor prognosis. Likewise, it also exceptionally metastasizes outside the brain, for reasons that are not yet clear.

Although it was long thought that the brain was an immune sanctuary, it is now established that immune responses toward tumors located in the CNS are able to occur. This is substantiated by both animal models of intracranial tumors, which show that strong antitumor immune responses are able to control tumor cells (48, 49), and by observations in humans revealing that T cells are detected at the tumor site and positively influence survival (50–52). Antigen-specific spontaneous B and T cell immune responses have been detected in patients with glioma (53–56), although less frequently than in other malignancies such as melanoma. PCNSL are associated with a robust inflammatory response, including infiltrating activated macrophages and reactive T cells, the latter being associated with a favorable outcome (57, 58).

The mechanisms of immune system activation by tumors located in the brain have been explored in the last decades. Features of the brain, which are different from other sites, namely lack of conventional lymphatic draining, absence of resident DCs in the brain parenchyma, and existence of the blood–brain barrier, are no longer regarded as obstacles to initiation of immune responses but might present a high threshold to be reached before efficient spontaneous antitumor immunity is induced. In spite of these, it has been shown that antitumor immune responses were able to occur. Antigens are able to drain from the brain parenchyma to reach the cervical lymph nodes (59, 60) where they are presented by DCs to T cells (61), leading to the proliferation of tumor-specific cells that will be able to home to the brain *via* expression of, among other molecules still to be discovered, VLA4/α4β1 and CXCR3 (62, 63). These T cells are retained at the tumor site *via* expression of αEβ7 (62) and could potentially represent tissue-resident memory cells poised to be reactivated upon re-encounter of tumor-expressed antigens (64).

However, brain tumors, similar to tumors arising in other sites, are able to resist immune attack through various means including MHC downregulation (65), release of immunosuppressive cytokines such as TGF-β (66), VEGF (67), prostaglandin E2 (68), IL-10 (69), and of enzymes such as IDO (70) and arginase (71), attraction of Tregs (72), and MDSCs (71, 73). In particular, Tregs have been shown in mice models of spontaneous glioma to be present at the tumor site very early, even before symptoms are visible (35). IDO, which can inhibit conventional T cells and induce Tregs, is expressed virtually in all GBM and level of expression is associated with poor prognosis (70). In addition, GBM can induce apoptosis of activated T cells through expression of FasL (74) and PD-L1, the latter being expressed by GBM cells but also by TAMs (75, 76) and able to inhibit glioma-infiltrating lymphocytes, which commonly express PD-1 (77). Finally, hypoxia is associated with poor clinical outcome in GBM patients (78). All these parameters converge to attenuate spontaneous immune responses occurring in patients with brain tumors, leading to inefficient tumor control.

In addition to that, intrinsic differences between the brain and peripheral organs exist, which might lead to suboptimal immune activation against tumors located in the brain as compared to tumors located in peripheral organs (79). These differences certainly need to be considered when designing immunotherapeutic strategies for tumors in the brain.

Priming of Immune Responses to Brain Tumor Antigens

As described above, initiation of immune response to antigens located in the brain occurs, antigen presentation to naïve T cells occurring either *via* drainage of soluble antigen to LN or by transport *via* emigration of antigen-bearing DCs (62, 80). Immune response elicited by antigens that drain predominantly to the cervical LN were shown to be less effective than responses elicited to the same antigen reaching other lymph nodes (81), potentially due to induction of immunosuppressive myeloid cells. This might lead to suboptimal induction of immune response to tumors located in the brain as compared to other sites. Nonetheless, it cannot be entirely excluded that immune responses to brain tumors are elicited in the periphery in response to circulating tumor cells reaching secondary lymphoid organs; these having been reported in a significant number of patients with GBM (82, 83).

These issues need to be taken into consideration for therapeutic vaccination. A very important concern for tumor vaccines is the site of antigen injection to prime antitumor immune responses. In the many clinical trials of peptide and tumor vaccination performed in the last decades, several injection sites have been used, precluding evaluation of the efficacy of vaccination from different sites. However, a preclinical study comparing injection of a model antigen at different sites in glioma-bearing mice was able to demonstrate that vaccinating far away from the tumor was best to induce optimal CD8 effector function and brain infiltration (84). This was due to tumor-derived immunosuppressive factors reaching the LN and influencing the T cell response. These results are compatible with spontaneous antitumor immunity discussed previously (81).

Homing to the Brain

Tumor-specific T cells generated by vaccination or adoptive cell transfer need to reach the brain in order to exert their effector function. During a spontaneous antitumor immune response, homing of T cells to the tumor site is determined at the site of antigen capture by the APC, which will imprint T cells during

priming in the lymph nodes (62). Regarding the CNS, it was shown that T cell expression of α4β1 and CXCR3 facilitated infiltration of the brain (62, 63). It is therefore important to replicate this brain homing phenotype during therapeutic vaccination and adoptive cell transfer in order for sufficient cells to reach the brain. Indeed, it has been shown in animal models that adoptively transferred T cells are less efficient at infiltrating the brain than peripheral sites (85). Similarly, although adoptive transfer of TIL was shown to mediate regression of melanoma brain metastases (86), the latter have been shown to be less infiltrated by $CD3^+$ T cells than extracerebral metastatic sites, suggesting lower brain T cell homing (87). Therefore, for vaccine-induced or adoptively transferred cells to reach the brain, additional interventions need to be made. Brain homing has been shown to be enhanced by CXCL10, one of the CXCR3 ligands, secreted at the tumor environment (88), which can be promoted by injection of poly-ICLC (polyriboinosinic–polyribocytidylic acid stabilized with poly-L-lysine and carboxymethylcellulose), a TLR3 agonist. TLR3 is the most abundant TLR expressed by astrocytes and microglial cells and its activation has been shown to induce pro-inflammatory cytokines such as TNF-α, IL-6 and IFN-β and chemokines such as CCL2, CCL5, and CXCL10 (89). As a consequence, poly-ICLC has been extensively tested in patients with glioma, with the reported induction of robust vaccine-specific CD8 T cell responses associated with detection of CXCL10 in the circulation (90, 91). Regarding adoptive cell transfer, choosing culture conditions to generate cells with a tumor homing phenotype may be possible, although the exact conditions for this are not yet defined. In addition, transgenic expression of selected chemokine receptors could be envisaged in the case of TCR-transgenic and CAR T cells (92), although these strategies remain in the preclinical phase at present. Alternatively, it has been shown that increased brain migration of adoptively transferred CD8 T cells can be obtained by co-infusion of CD4 T cells specific for the same tumor antigen and bearing the Th1 phenotype (93).

Effector Function in the Brain Immunosuppressive Environment

Even if we know that immune response are able to occur in the brain, this organ nonetheless tightly regulates inflammation, mostly through TGF-β secretion. TGF-β2 is the most abundant TGF-β isoform detected in the adult brain and modulates response to brain lesions, including blocking of several pro-inflammatory cytokines and of MHC class II upregulation (94). In addition, the brain is one of the most densely vascularized organs in the body with VEGF being the main inducer of angiogenesis. As stated before, VEGF is also a strong inducer of immunosuppression by mediating accumulation of MDSC and Tregs and inhibiting the function and migration of T lymphocytes to the tumor (95). In consequence, tumor-specific T cells elicited by immunotherapy have to overcome, once they reach the brain, a series of obstacles before they can exert their effector function. As indicated before, CNS cells express FasL, which will induce apoptosis of incoming Fas^+ T cells (96). Surviving cells will have to cope with the immunosuppressive factors and cells described above and will further be inhibited by PD-L1 expression by tumor and myeloid cells. All these factors need to be considered to design efficient immunotherapies.

Efficacy of Immunotherapies

Recently, the distinction of T cell inflamed versus non-T cell inflamed tumors has allowed stratifying patients according to prognosis and response to immune checkpoint inhibitors (97). The current understanding is that, in T cell inflamed tumors, recruitment of tumor-specific CD8 T cells leads to secretion of pro-inflammatory (mostly IFN-γ) cytokines, which stimulates upregulation of PD-L1 and IDO and recruitment of Tregs (98, 99). In non-T cell inflamed tumors, T cell markers and chemokines involved in T cell recruitment are not detected, possibly due to lack of priming of the antitumor response or/and lack of migration at the tumor site. Importantly, T cell inflamed tumors have been shown to be associated with response to both therapeutic vaccines (100) and checkpoint blockade (98, 101, 102). In this regard, GBM can be considered as a poorly T cell inflamed tumor, as compared to tumors located in peripheral organs such as melanoma, renal cell carcinoma, breast, or ovarian cancers (103). Similarly, PCNSL are poorly infiltrated by immune cells as compared to their peripheral counterpart (104), suggesting that tumors located in the brain might be less prone to respond to immunotherapies, including ICB. Immunotherapeutic interventions should therefore include strategies to promote inflammation at the tumor site in the brain, possibly by inducing innate signaling to trigger antitumor adaptive immunity. One strategy to achieve this is tumor delivery of stimulator of interferon genes (STING) agonists, which have been shown in mouse models of glioma to promote infiltration by CD4 and CD8 T cells and prolong survival (18). Alternatively, type I IFN production can be induced by radiotherapy (105), and radiation of the tumor site has been shown to induce double strand DNA breaks and subsequent type I IFN activation *via* STING in mouse models of glioma (106). Finally, one study in mouse models, not yet explored for GBM, showed that treatment of non T cell inflamed tumors with LIGHT, a member of the tumor necrosis factor superfamily, led to secretion of pro-inflammatory chemokines and recruitment of T cells at the tumor site, which was associated with greater response to ICB (107).

Choice of Antigens

The choice of antigen for designing immunotherapeutic strategies is arguably even more important for tumors located in the brain as compared to those occurring in other sites. Indeed, whereas attack of healthy cells expressing the tumor antigen to some level, such as skin depigmentation observed due to the targeting of melanoma antigens shared by melanoma cells and melanocytes, can be tolerated in some organs, this is more critical for the brain. TAA recognized by T cells have been identified in glioma, although their number is fewer than for other malignances such as melanoma. They include, among others, IL13Rα2, EphA2, WT1, and survivin (108) and the antigens composing the IMA950 peptide cocktail (56), which were eluted from the surface of GBM cells and were shown to be expressed by the majority of patients with GBM (56). Equally, few TSA have been detected to date for GBM, but more will probably be identified in the future thanks to increased use of tumor sequencing and patient-specific

epitope identification (109). Until now, the most used TSA for GBM immunotherapy are EGFRvIII, a mutant antigen derived from the EGFR protein, which is expressed by 20–50% of GBM patients (110) and IDH1R132H, derived from the IDH1 protein and mainly expressed in grade II and III astrocytoma and patients with secondary GBM (111).

Altogether, tumors located in the brain have particular immunological features that will need to be taken into account for the design of immunotherapies. Among this, (i) the careful choice of antigen, (ii) the need to stimulate inflammation of the tumor site, (iii) to target the brain immunosuppressive milieu, (iv) to vaccinate far from the tumor site, and (v) to help cells home to the brain might be mandatory to address for brain tumor immunotherapy to be efficient.

ONGOING CLINICAL TRIALS FOR TUMORS IN THE BRAIN

Most immunotherapeutic approaches developed to date for tumors located in the brain have mostly targeted patients with glioma. PCNSL has not yet attracted much attention for vaccines and cell therapy and only one trial is investigating ICB in this malignancy. Most of the trials described below will therefore relate to glioma.

Peptide and Tumor Vaccines

Peptide vaccines (with or without DCs) for GBM have mostly used multipeptidic TAA vaccine formulations in adjuvant, incorporating the EphA2, IL-13Rα2, WT1, and survivin (90, 91, 112), or the IMA950 cocktail (113), although some peptides have been used alone (114–116). These trials have shown that vaccine-specific immune responses were elicited, which were not associated with autoimmunity, and clinical benefit was possibly observed for some individual patients. Following these results, additional studies are being conducted (**Table 1**), with single peptides (NCT02455557, NCT02049489), cocktails of minimal T cell epitopes (NCT01130077, NCT02358187, NCT02078648, NCT01920191, NCT02149225, NCT02709616), mixtures of overlapping peptides (NCT02332889), or DC-pulsed mRNA (NCT02649582, NCT02529072, NCT02465268, NCT02366728). One study is addressing efficacy of vaccination in pediatric patients with ependymoma (NCT01795313).

TABLE 1 | Currently ongoing peptide and tumor vaccine trials in tumors located in the brain.

Immunogen	Adjuvant	Additional drugs	Patient population	Diagnostic	Phase	Estimated enrollment	Country	NCT number
Peptide vaccines								
Peptide alone								
Tumor-associated antigens (TAAs)								
Single peptide								
Long peptide from survivin-KLH	GM-CSF + montanide			Newly diagnosed glioblastoma (GBM)	II	50	USA	NCT02455557
Multiple peptides								
HLA-A2-restricted peptides from EphA2, IL-13Rα2, and survivin	Poly-ICLC		Pediatric	HGG, DIPG, and recurrent LGG	Pilot	60	USA	NCT01130077
HLA-A2-restricted peptides from EphA2, IL-13Rα2, and survivin	Poly-ICLC		Pediatric	LGG	II	25	USA	NCT02358187
HLA-A2-restricted peptides from EphA2, IL-13Rα2, and survivin	Imiquimod		Pediatric	Recurrent ependymoma	na	24	USA	NCT01795313
SL-701 (HLA-A2-restricted peptides from EphA2, IL-13Rα2, and survivin)	Poly-ICLC	Bevacizumab	Adult	Recurrent GBM	I/II	76	USA	NCT02078648
IMA950 (10 HLA-A2-restricted peptides from BCAN, CSPG4, FABP7, IGF2BP3, MET, NLGN4X, NRCAM, PTPRZ1, TNC plus 2 MHC class II peptides from survivin and MET)	Poly-ICLC		Adult		I/II	16	Switzerland	NCT01920191

(*Continued*)

TABLE 1 | **Continued**

Immunogen	Adjuvant	Additional drugs	Patient population	Diagnostic	Phase	Estimated enrollment	Country	NCT number
Personalized overexpressed HLA-A2 or -A24-restricted peptides plus mutated peptides	GM-CSF + poly-ICLC		Adult	Newly diagnosed GBM	I	20	6 centers in Europe (GAPVAC)	NCT02149225
HSPPC-96	None	Bevacizumab	Adult	Recurrent GBM	II	165	USA	NCT01814813
Tumor-specific antigens (TSAs)								
Single peptide								
EGFRvIII peptide	GM-CSF	Bevacizumab	Adult	EGFRvIII$^+$ recurrent GBM	II	168	USA (ReACT)	NCT01498328
EGFRvIII peptide[a]	GM-CSF		Adult	EGFRvIII$^+$ recurrent GBM	III	700	Worldwide (ACT IV)	NCT01480479
IDH1R132H peptide	Montanide + imiquimod		Adult	IDH1R132H-mutated newly diagnosed HGG	I	39	Germany (NOA-16)	NCT02454634
IDH1R132H peptide	Montanide + GM-CSF + Td vaccine		Adult	IDH1R132H-mutated recurrent LGG	I	24	USA (RESIST)	NCT02193347
Mutated peptides	Poly-ICLC		Adult	Newly diagnosed GBM (UnMe MGMT)	I	20	USA	NCT02287428
Mutated long peptide	Poly-ICLC		Adult	Newly diagnosed GBM	Pilot	10	USA	NCT02510950
Personalized overexpressed HLA-A2 or -A24-restricted peptides plus mutated peptides	GM-CSF + poly-ICLC		Adult	Newly diagnosed GBM	I	20	6 centers in Europe (GAPVAC)	NCT02149225
DC + peptides/mRNA								
TAAs								
Single peptide								
ICT-121 (CD133 peptides)	None		Adult	Recurrent GBM	I	20	USA	NCT02049489
Multiple peptides								
Overlapping peptides from MAGE-A1, MAGE-A3, and NY-ESO-1	Poly-ICLC	Decitabine	Pediatric	HGG, PNET, and medulloblastoma	I/II	10	USA	NCT02332889
Personalized among preselected antigens	Imiquimod or Td vaccine		Adult	Newly diagnosed GBM	I/II	20	China (PERCELLVAC)	NCT02709616
mRNA								
WT1 mRNA	None		Adult	Newly diagnosed GBM	I/II	20	Belgium (ADDIT-GLIO)	NCT02649582
pp65 mRNA	None	Nivolumab	Adult	Recurrent HGG	I	66	USA (AVERT)	NCT02529072
pp65 mRNA	GM-CSF + Td vaccine		Adult	Newly diagnosed GBM	II	150	USA (ATTAC-II)	NCT02465268
pp65 mRNA	Td vaccine	Basiliximab	Adult	Newly diagnosed GBM	II	116	USA (ELEVATE)	NCT02366728

(*Continued*)

TABLE 1 | **Continued**

Immunogen	Adjuvant	Additional drugs	Patient population	Diagnostic	Phase	Estimated enrollment	Country	NCT number
Tumor vaccines								
Tumor alone								
Tumor lysate from GBM6 cell line	Imiquimod		Adult	LGG	Pilot	27	USA	NCT01678352
Tumor lysate from GBM6 cell line	Poly-ICLC		Adult	Recurrent LGG	Pilot	30	USA	NCT02549833
Tumor lysate from GBM6 cell line	Imiquimod		Pediatric	DIPG	Pilot	8	USA	NCT01400672
DC + tumor								
Tumor lysate	Imiquimod		Adult + pediatric	Recurrent LGG or HGG	I	20	USA	NCT01808820
Tumor lysate	Imiquimod		Pediatric	Recurrent HGG	I	20	USA	NCT01902771
Tumor lysate	Resiquimod + poly-ICLC		Adult	Newly diagnosed or recurrent HGG	II	60	USA	NCT01204684
Tumor lysate	None		Adult	Newly diagnosed or recurrent LGG	II	18	USA	NCT01635283
Tumor lysate from allogenic stem-like cell line	None	Bevacizumab	Adult	Newly diagnosed or recurrent GBM	I	40	USA	NCT02010606
Tumor lysate from autologous stem-like cells	None		Adult	Newly diagnosed GBM	II	100	China	NCT01567202

DIPG, diffuse intrinsic pontine glioma; HGG, high-grade (III or IV) glioma; LGG, low-grade (grade II) glioma; poly-ICLC, polyinosinic–polycytidylic acid stabilized with polylysine and carboxymethylcellulose; Td, tetanus diphtheria; UnMe MGMT, unmethylated MGMT promoter.

[a]*This study was recently discontinued after interim analysis due to absence of benefit as compared to control arm.*

Interestingly, some trials of personalized vaccination are ongoing (NCT01814813, NCT02709616, NCT02149225), one of which selects the peptides according to peptide elution from the patient's tumor, thus ensuring presence of the target at the tumor surface (NCT02149225).

Trials with TSA in glioma have mostly focused on the EGFRvIII mutation as a single peptide vaccine and two clinical trials in newly diagnosed (NCT01480479) or recurrent (NCT01498328) GBM are ongoing. However, whereas phase II studies had shown benefit for patients with recurrent or newly diagnosed GBM (7, 117, 118), the unique phase III trial assessing the benefit of EGFRvIII peptide vaccine in addition to standard treatment in newly diagnosed GBM patients (NCT01480479) was recently discontinued due to absence of improved overall survival in patients receiving the vaccine versus standard treatment.[1] Maybe this vaccine would profit from combination with immune checkpoint inhibitors to enhance vaccine efficacy or with other peptides to prevent immune escape (7). In addition to EGFRvIII, clinical trials targeting a long peptide spanning the IDH1R132H mutation occurring in grade II/III and secondary GBM are ongoing (NCT02454634, NCT02193347). Identification of the latter epitope, which is recognized by CD4 T cells, provides the opportunity to target both CD4 and CD8 T cells by generating a composite vaccine including the IMA950 antigens and the peptide spanning the IDH1R132H mutation. Finally, three trials are assessing efficacy of vaccination with neoantigens in GBM (NCT02287428, NCT02510950, NCT02149225), one trial importantly addressing the presence of the mutated peptide at the tumor cell surface (NCT02149225).

Although some studies inject peptide or DC/peptide vaccines alone, the majority of studies inject the peptides with an adjuvant, mostly the TLR3 ligand poly-ICLC, the TLR7 ligands imiquimod and resiquimod, GM-CSF, or Montanide. Given the critical importance of adjuvant choice for therapeutic cancer vaccination revealed in preclinical studies (119–121), this issue will eventually have to be addressed in a clinical context. Interestingly, subsequent to clinical and mice studies showing that preconditioning the tumor vaccine injection site by a recall response to tetanus/diphtheria improved lymph node homing of tumor

[1]http://www.celldex.com/pipeline/rindopepimut.php.

antigen-bearing DCs and magnitude of immune responses (19), four studies (NCT02193347, NCT02709616, NCT02465268, NCT02366728) use a Td recall vaccine as adjuvant, some testing as part of their clinical trial efficiency of DC migration to lymph nodes (19). Finally, one study is adding the anti-PD1 antibody nivolumab to a pp65CMV vaccine in recurrent grade III or IV glioma patients.

Vaccines using autologous tumor or allogenic GBM cell lines as source of tumor antigens are mostly employing pulsed DCs, although three pilot studies are injecting lysate from the allogenic GBM6 stem-like cell line (122) without DCs, in low-grade glioma (LGG, grade II, NCT01678352, NCT02549833) or diffuse intrinsic pontine glioma (DIPG, NCT01400672; **Table 1**). Trials using tumor lysate-pulsed DCs are using either autologous tumor (NCT01808820, NCT01204684, NCT01902771, NCT01635283), or stem-like cells (NCT01567202), or an allogenic stem-like cell line (NCT02010606). As for peptide vaccines, tumor cell vaccines are usually injected with one of the three above-mentioned TLR ligands, with one study combining poly-ICLC and resiquimod. As stated before, there is no trial of peptide or tumor vaccine ongoing for PCNSL.

Cell Therapy

At least one study of TIL infusion has been performed to date in brain tumor patients (123). With regard to peripheral blood-derived antigen-specific T cell transfer, only one study is being conducted, assessing the safety and efficacy of autologous CMV pp65-specific T cells to target GBM cells potentially expressing CMV (NCT02661282) (124). This might be due to the difficulty in detecting high levels of non-viral glioma-specific T cells in the peripheral blood of glioma patients and to the difficulty of amplifying them to great numbers for reinfusion. The latter phenomenon is probably related to the systemic defects in T cell function and proliferation observed in glioma patients, which are more pronounced than in other malignancies (125). Studies using TCR-transgenic T cells incorporating TCRs from glioma-specific T cells are similarly not yet being tested in the clinical setting, most probably due to the paucity of antigen-specific T cell clones characterized thus far for glioma. One study reporting generation of antigen-specific T cell clones from patients with GBM specific for different TAA (56) might be the first step toward development of this approach as it provides T cells with exploitable TCR sequences.

Studies with CARs have, in contrast, been quite extensively tested in preclinical glioma models and are in clinical trials (126). In the last 20 years of CAR development, initial experiments using first generation CARs bearing only the CD3ζ chain as signaling domain showed that such constructs were limited in efficacy. This led to the design of constructs incorporating CD28 or 4-1BB as costimulatory molecules (second generation CARs), which resulted in impressive success for the treatment of hematological malignances (127). Third generation CARs incorporating two costimulation molecules are being tested in B cell malignancies and neuroblastoma and a few clinical trials are even testing 4th generation CARS with additional CD27 costimulation. In brain tumors, CAR studies targeting six different antigens (EGFR, EGFRvIII, EphA2, Her2, IL13Rα, and MUC1) are ongoing (NCT02331693, NCT01454596, NCT02575261, NCT02442297, NCT02208362, NCT02617134), using second (CD28 or 41BB costimulation) or third (CD28 and 41BB costimulation) generation constructs (**Table 2**). Of note, the IL13Rα CAR, unlike the majority of CARs that use a single chain fragment variable part

TABLE 2 | Currently ongoing cell therapy trials in tumors located in the brain.

Specificity	Adjuvant	Additional drugs	Patient population	Diagnostic	Phase	Estimated enrollment	Country	NCT number
Naturally occurring T cells								
CMV-specific T cells			Adult	Newly diagnosed or recurrent HGG	I/II	54	USA	NCT02661282
CARs								
EGFR (CD28 costimulatory domain)		Cyclophosphamide fludarabine	Adult	Recurrent glioblastoma (GBM) with EGFR amplification	I	10	China	NCT02331693
EGFRvIII (CD28 and 41BB costimulatory domains)	IL-2	Cyclophosphamide fludarabine	Adult	EGFRvIII$^+$ recurrent GBM	I/II	107	USA	NCT01454596
EphA2 (CD28 costimulatory domain)			Adult	Newly diagnosed or recurrent HGG	na	60	China	NCT02575261
Her2 (CD28 costimulatory domain)			Adult	Her2$^+$ recurrent GBM	I	14	USA (iCAR)	NCT02442297
IL13Rα2 (41BB costimulatory domain)			Adult	Recurrent HGG	I	36	USA	NCT02208362
MUC1 (CD28 and 41BB costimulatory domains)	IL-12 in CAR construct	Cyclophosphamide fludarabine	Adult	MUC1$^+$ recurrent GBM	I/II	20	China	NCT02617134

HGG, high-grade (III or IV) glioma; na, not available.

(scFv) as the antigen-binding moiety, is composed of a modified IL-13 molecule (128). The safety profile of targeting some of the above-mentioned antigens is under scrutiny because of reported toxicity due to Her2 expression in heart and lung (129) and by expression of non-mutated EGFR in epithelial cells (130). Interestingly, two studies are injecting the CAR T cells in the brain, either intratumorally, in the resection cavity, or intraventricularly (NCT02442297, NCT02208362). Two other trials are using immunostimulatory cytokines, namely IL-2 with the 3rd generation EGFRvIII-specific CAR (NCT01454596) and IL-12 with the 3rd generation MUC1-specific CAR (NCT02617134, in the CAR construct itself), with the aim to enhance CAR T cell efficacy, although caution is warranted for IL-12 use (131). Finally, in an attempt to transfer CAR T cells that can best repopulate the T cell niche and generate long-term effector cells, a study targeting the IL13Rα protein is injecting central memory-enriched CAR T cells (132). Again, no cell therapy protocols are ongoing for PCNSL.

TARGETING THE TUMOR ENVIRONMENT

As discussed above, TGF-β is one of the main immunosuppressive molecules requiring targeting for tumors located in the brain. Accordingly, many trials using mRNA antisense oligonucleotides, soluble receptors, or antibodies to TGF-β and molecules inhibiting the kinase activity have been tested (133). Although reports from preclinical models were promising (66), clinical studies thus far have failed to demonstrate survival benefit associated with the use of TGF-β-targeting agents. The TGF-β mRNA antisense oligonucleotides trabedersen (AP12009) has not shown benefit in patients with grade III or IV glioma and is not being further tested (134). Galunisertib (LY2157299), a TGF-β receptor I kinase inhibitor, failed to demonstrate improved overall survival as compared to lomustine in patients with recurrent GBM (135) but is now being tested in combination with nivolumab in patients with GBM and recurrent pancreatic cancer and hepatocellular carcinoma (NCT02423343; **Table 3**). Similarly, fresolimumab, a pan-TGF-β antibody failed to show survival benefit in patients with glioma (136). Although these results are quite discouraging, it is important to pursue investigation of TGF-β targeting. One reason for the inefficiency of TGF-β blockade might be the activation of alternative pathways. We might therefore need to simultaneously target TGF-β and alternative pathways such as EGFR, PI3K/Akt, NF-κB, or JAK/signal transducer and activator of transcription (STAT), a strategy which has shown efficacy in preclinical studies of pancreatic tumors (137).

TABLE 3 | Currently ongoing trials targeting the tumor microenvironment.

Target	Molecule	Additional intervention	Patient population	Diagnostic	Phase	Estimated enrollment	Country	NCT number
TGF-β								
	Galunisertib (TGF-β receptor I kinase Inhibitor)	Nivolumab		Glioblastoma (GBM), recurrent NSCLC, and HCC	I/II	100	USA and Spain	NCT02423343
IDO								
	Indoximod (D-1MT)	Bevacizumab	Adult	Recurrent HGG	I/II	144	USA	NCT02052648
	Indoximod (D-1MT)		Pediatric	Newly diagnosed HGG, ependymoma, and medulloblastoma	I	66	USA	NCT02502708
	Epacadostat	Nivolumab	Adult	Advanced solid tumors including recurrent GBM	I/II	291	USA	NCT02327078
STAT3								
	WP1066		Adult	Recurrent HGG, melanoma brain metastases	I/II	33	USA	NCT01904123
MDSC								
	Capecitabine (prodrug of 5-flourouracil)	Bevacizumab	Adult	Recurrent GBM	I	12	USA	NCT02669173
	CSF1-R inhibitor (PLX3397)			Newly diagnosed GBM	I/II	65	USA	NCT01790503
	Anti-CSF1-R antibody (FPA008)	Nivolumab	Adult	Solid tumors including GBM	I	280	USA	NCT02526017
Tregs								
	Basiliximab (anti-CD25)	pp65 mRNA Td vaccine	Adult	Newly diagnosed GBM	II	116	USA (ELEVATE)	NCT02366728

D-1MT, 1-methyl-D-tryptophan; HGG, high-grade (III and IV) astrocytoma; nivolumab, fully human IgG4 anti-PD1; NSCLC, non-small cell lung cancer.

Vascular endothelial growth factor, due to its critical role in brain tumor angiogenesis, is being targeted using different approaches. The monoclonal antibody bevacizumab is approved as a single agent for the treatment of recurrent glioma (138, 139), but did not demonstrate survival benefit for patients with newly diagnosed glioma (140, 141). It is being used in trials of therapeutic vaccination in the setting of recurrent glioma (NCT02078648, NCT01814813, NCT01498328), but is not tested *per se* in combination with other interventions. Aflibercept (VEGF Trap), a recombinant fusion protein, which acts as scavenger molecule for VEGF, improved survival in preclinical models, possibly due to its high affinity for VEGF, but failed to demonstrate antitumor activity in patients (142). A number of small molecule inhibitors of the kinase activity of VEGF receptor are being tested in glioma (including cediranib, sunitinib, pazopanib, vandetanib, and sorafenib), but not in combination with immunotherapy for the time being.

A third pathway of investigation in brain tumors is the IDO pathway, IDO being detected in virtually all glioma samples, although not normally expressed in the brain (70, 143). Studies in mouse models of glioma using the IDO inhibitor 1 methyltryptophan (1MT) suggested that combination with other molecules might be required for antitumor activity to be seen (144); however, indoximod (D-1MT) is being tested as single agent in patients with newly diagnosed (NCT02502708, pediatric population) and recurrent glioma (NCT02052648). A more recent IDO inhibitor, epacadostat (INCB24360), selectively inhibits the enzymatic activity of IDO1 and is being tested in patients with advanced solid malignancies including recurrent GBM, in combination with the anti-PD1 antibody nivolumab (NCT02327078).

Another currently targeted protein in brain tumors is STAT3, a molecule that is downstream of several oncogenic signaling cascades in glioma, including EGFR and PDGF receptor. Constitutive STAT3 activation is detected in 50–60% of high-grade glioma (145) and mediates immune suppression at the tumor site (146). It is also been shown to be activated in PCNSL (147). A trial with WP1006, an inhibitor of the JAK2/STAT3 pathway, is currently ongoing in patients with recurrent GBM (NCT01904123).

Inhibiting MDSCs is under investigation, using several approaches that include induction of MDSC differentiation into DC, decreasing MDSC levels, and inhibiting MDSC function (148). One study in patients with recurrent GBM (NCT02669173) aims at targeting MDSC using low-dose capecitabine, a prodrug of 5-fluorouracil, which was shown to kill MDSC and restore antitumor T cell responses (149). Another way of MDSC depletion is the use of colony-stimulating factor 1 receptor (CSF1-R) inhibitors. CSF1-R is overexpressed by MDSC and TAMs in human glioma and its expression was shown to correlate with glioma grade (150, 151). It is involved in the recruitment of TAM and MDSC at the tumor site *via* interaction with CSF1 and is necessary for their survival. CSF1-R inhibition showed improved survival in a preclinical model of glioma, with reprograming of the TAM into pro-inflammatory cells (152). Use of the CSF1-R inhibitor PLX3397 as single agent in patients with recurrent GBM showed no improvement in survival (153), however, combination studies in preclinical models of melanoma demonstrated improvement of adoptive cell therapy, accompanied by reduction of tumor-infiltrating TAM and MDSC and augmentation of IFN-γ-secreting TILs (154), advocating for its use in combination therapies in humans. The same molecule is currently being tested in patients with newly diagnosed GBM (NCT01790503) and another trial using a CSF1-R antibody is ongoing in combination with the anti-PD1 antibody nivolumab in patients with advanced cancers including glioma (NCT02526017).

Finally, inhibition of Tregs is currently being investigated for tumors in the brain in one trial only, although initial studies using an anti-CD25 antibody to deplete Tregs in combination with an EGFRvIII peptide vaccine showed enhanced humoral response to the vaccine in patients receiving the antibody (155). In the ongoing trial, pp65 CMV mRNA-pulsed DCs are injected into a Td vaccine-pretreated site, with or without the anti-CD25 antibody basiliximab (NCT02366728).

At the moment, there are no trials targeting the tumor microenvironment in patients with PCNSL, although there is a rationale for their implementation (156).

IMMUNE CHECKPOINT BLOCKADE TRIALS

There are now numerous clinical trials testing the efficacy of ICB antibodies for tumors arising in the brain including glioma and PCNSL. An important issue related to the use of ICB antibodies is the mutation load of the targeted malignancies. GBM do not possess a high rate of mutations (around 2.5 mutations per megabase[2]) (157), except for a particular hypermutated rare subtype (158), lowering the probability of neoepitope-specific immune responses that can be amplified by ICB antibodies. Thus, the efficacy of immune checkpoint inhibitors might be less impressive as compared to other malignancies, as immune checkpoint inhibitors have been shown to work best in highly mutated tumors, with a threshold of 100 mutations per exome (3.3 mutations per megabase) (40, 41). As a consequence, trials in GBM might need to use these molecules not as single agents, but rather in combination with other immunotherapeutic strategies. Regarding PCNSL, recent studies revealed a median mutation load around 6.6 mutations per megabase (159), suggesting that this malignancy could be targeted with ICB antibodies as single agents. A further issue for the use of ICB antibodies from tumors located in the brain is whether efficacy is linked to penetration of antibodies to the tumor site in the CNS. Since, even in the condition where a tumor is present, blood–brain barrier breakdown is only partial, access of antibodies to tumors in the CNS will definitely be less efficient than for tumors located in peripheral organs. Anti-CTLA4, and to some extend anti-PD1, might exert their effect while seeing T cells in the periphery. Indeed, it has been shown that anti-PD1 treatment affected the phenotype of PD1-expressing Tregs in the peripheral blood

[2]http://icgc.org/.

of nivolumab-treated GBM patients (NCT02017717) (160). Considering anti-PD-L1 antibodies (such as durvalumab currently being tested in patients with GBM, see below), they will certainly need to access the tumor to reach PD-L1-expressing tumor cells, but an effect of anti-PD-L1 on circulating myeloid cells cannot be excluded. Studies in glioma mouse models have demonstrated the efficacy of anti-CTLA4 and anti-PD1 antibodies (161, 162) and studies demonstrating efficacy of anti-PD-L1 antibodies confirmed interest of these targets but do not provide the formal proof than these antibodies are able to enter the brain

TABLE 4 | Currently ongoing immune checkpoint trials.

Molecule	Additional intervention	Patient population	Diagnostic	Phase	Estimated enrollment	Country	NCT number
Anti-CTLA4							
Ipilimumab	±Nivolumab	Adult	Newly diagnosed glioblastoma (GBM)	I	42	USA	NCT02311920
Ipilimumab	Nivolumab	Adult	Recurrent GBM	III	440	Worldwide (checkmate 143)	NCT02017717
Anti-PD1							
Nivolumab	Gamma knife + valproate	Adult	Recurrent GBM	Pilot	17	USA	NCT02648633
Nivolumab	None and/or ipilimumab	Adult	Newly diagnosed GBM	I	42	USA	NCT02311920
Nivolumab	CSF1-R inhibitor	Adult	Solid tumors, GBM	I	270	USA	NCT02526017
Nivolumab	Galunisertib (TGFβ receptor I kinase inhibitor)	Adult	GBM, other solid tumors	I/II	100	USA and Spain	NCT02423343
Nivolumab		Adult	Newly diagnosed GBM (Me MGMT)	II randomized	320	Worldwide (checkmate 548)	NCT02667587
Nivolumab		Adult	Newly diagnosed GBM (UnMe MGMT)	III	550	Worldwide (checkmate 498)	NCT02617589
Nivolumab		Pediatric + adult	Newly diagnosed and recurrent GBM	II	29	Spain	NCT02550249
Nivolumab	CMV pp65-mRNA-pulsed dendritic cells	Adult	Recurrent HGG	II	66	USA	NCT02529072
Pembrolizumab		Adult	Recurrent HGG with hypermutant phenotype	Pilot	12	USA	NCT02658279
Pembrolizumab		Adult	Recurrent HGG	I	32	USA	NCT02313272
Pembrolizumab		Pediatric	Recurrent HGG/DPIG	I	70	USA	NCT02359565
Pembrolizumab		Adult	Newly diagnosed HGG	I/II	50	USA	NCT02530502
Pembrolizumab	MRI-guided laser ablation	Adult	Newly diagnosed HGG	I/II	52	USA	NCT02311582
Pembrolizumab		Adult	Recurrent GBM	II	20	USA	NCT02337686
Pembrolizumab		Adult	Recurrent GBM	II	81	USA	NCT02337491
Pembrolizumab		Adult	Recurrent PCNSL	II	21	Austria	NCT02779101
Pembrolizumab	Versus three PI3K/Akt pathways inhibitors	Adult	Recurrent GBM	IIb	58	Worldwide	NCT02430363
Pidilizumab		Pediatric	DPIG	I/II	50	Israel	NCT01952769
Anti-PD-L1							
Durvalumab	Bevacizumab	Adult	Newly diagnosed and recurrent GBM	II	108	USA and Australia	NCT02336165
Anti-LAG3							
Anti-LAG3	Pembrolizumab, urelumab	Adult	Recurrent GBM	I	68	USA	NCT02658981

Durvalumab, human IgG1 anti-PD-L1; HGG, high-grade (III and IV) astrocytoma; ipilimumab, humanized IgG1 anti-CTLA4; Me MGMT, methylated MGMT promoter; nivolumab, fully human IgG4 anti-PD1; NSCLC, non-small cell lung cancer; PCNSL, primary CNS lymphoma; pembrolizumab, humanized IgG4 anti-PD1; pidilizumab, humanized IgG1 anti-PD1; urelumab, fully human IgG4 anti-CD137; UnMe MGMT, unmethylated MGMT promoter.

(144, 163). Brain metastases from melanoma patients can be controlled by ICB antibodies, but with lower efficacy than metastases in extracerebral sites (164).

The number of clinical trials for GBM using anti-CTLA4, but mostly anti-PD1, has increased remarkably in the last 2 years. Indeed, the anti-CTLA4 antibody ipilimumab is being tested in combination with anti-PD1 in newly diagnosed (NCT02311920) and recurrent GBM patients (NCT02017717, in comparison with bevacizumab, **Table 4**). Rationale for investigating efficacy of multiple ICB antibodies originate from clinical studies in melanoma demonstrating higher efficacy of combination of anti-CTLA4 and anti-PD1 versus anti-CTLA-4 (165, 166) or either agent alone in PD-L1-negative patients (166), the limiting factor being however increased toxicity as treatments are combined. Preclinical studies also showed that only combination of ICB antibodies were able to induce regression of intracranial glioma (144, 161). Nevertheless, several trials using the anti-PD1 antibodies nivolumab (fully human IgG_4), pembrolizumab (humanized IgG_4), or pidilizumab (humanized IgG_1) are ongoing in the adult and pediatric populations in pilot, phase I, II, and III trials. Some trials are investigating anti-PD1 antibodies as single agents in newly diagnosed (NCT02667587, NCT02617589, NCT02550249, NCT02530502) or recurrent (NCT02550249, NCT02313272, NCT02359565, NCT02337686, NCT02337491) GBM patients, including children (NCT02550249, NCT02359565, NCT01952769). One trial is comparing the use of pembrolizumab in comparison to three suppressors of the PI3K/Akt pathways given together (NCT02430363). Rare hypermutated GBM tumors occurring in patients suffering from biallelic mismatch repair deficiency, which have been shown to respond to nivolumab treatment (158), are being targeted as well (NCT02658279). Two trials are addressing the efficacy of other ICB, the anti-PD-L1 antibody durvalumab (human IgG_1) in patients with newly diagnosed or recurrent GBM (NCT02336165) and an anti-LAG3 antibody compared to an anti-CD137 (urelumab, a fully human IgG4 antibody) combined or not with pembrolizumab (NCT02658981).

Currently, none of these trials are selecting patients according to the PD-L1 status. It has been shown in non-CNS malignancies that response to PD1 targeting was associated with PD-L1 expression (167–169) and one study demonstrated objective responses in patients whose tumors expressed PD-L1 only (169). However, in contrast to this, some studies observed treatment responses in PD-L1-negative patients, questioning the use of PD-L1 expression as a marker for patient selection. In that matter, one issue is the various protocols (including different antibodies, tumor sample size, cut-offs…) used for the assessment of PD-L1 expression that prevents direct comparison of studies (170). Regarding GBM, the same issue applies, but, regardless of the methodology used, the rate of PD-L1-positive tumors seems to be relatively high as compared to non-CNS malignancies (171). Expression in PCNSL samples, although less intensively assessed thus far, seems to be lower (172, 173). A careful assessment of PD-L1 expression in ongoing clinical trials of anti-PD1 and PD-L1 will be invaluable in helping define the role of PD-L1 expression as a marker of treatment efficacy in CNS malignancies.

As mentioned before, the relatively low mutation load of GBM might require using ICB antibodies in combination with antitumor vaccines or other therapeutic interventions. In that regard, other studies are combining anti-PD1 antibodies with (i) approaches to enhance tumor immunogenicity, (ii) therapeutic vaccines, or (iii) molecules targeting the tumor microenvironment. Enhancement of tumor immunogenicity is achieved through the concomitant use of gamma knife surgery to provide additional tumor antigens to the immune system and valproic acid, a histone deacetylase inhibitor shown to induce global DNA demethylation (NCT02648633). Others are using peritumoral MRI-guided laser ablation in order to breach the BBB and increase access of tumor antigens to the immune system (NCT02311582). At the moment, only one trial combining ICB antibodies with another immunotherapy is ongoing, using autologous DCs pulsed with pp65 CMV mRNA (NCT02529072). As mentioned above, elicitation of antitumor immune responses that reach the tumor is associated with adaptive immune resistance as tumor infiltration by IFN-γ-secreting cells lead to upregulation to PD-L1 in the tumor environment (37), a phenomenon that could be counteracted in a glioma mouse model of tumor-loaded DC vaccination by the concomitant use of anti-PD1 antibodies (162). Therefore, combining DC and other vaccines with ICB antibodies certainly merits further exploration. As already mentioned above, two trials are using ICB in the context of strategies aiming at targeting the tumor microenvironment, namely using a CSF1-R inhibitor (NCT02526017) or a TGFβ receptor I kinase inhibitor (NCT02423343). Regarding PCNSL, one trial is currently addressing the effect of anti-PD1 antibodies in recurrent PCNSL (NCT02779101).

CONCLUSION

Currently, ongoing trials for tumors located in the brain are principally designed on the same basis as for tumors located at other sites. Similarities between CNS and non-CNS tumors are the need for specificity, the need for T cell infiltration in the case of non-T cell inflamed organs, and the need to overcome local immunosuppression. The only feature that is unique to tumors located in the brain is the absence of metastases outside the CNS. This is an opportunity, as, if we can design immunotherapies that are efficient in getting functional antitumor T cell in the CNS, no other site needs to be targeted. Once we achieve this, the difference for tumors located in the brain will be determining the tolerated level for an inflammatory response to occur without damage to the brain. Integration of these parameters into future clinical trials will ultimately result in clinical benefit for the patient. In the interim, maximizing the biological information from existing trials may be highly informative. Finally, a notion that is also true for tumors located outside the brain, we should aim at investigating combination of vaccines, cell therapy, ICB antibodies, and molecules targeting the tumor environment, trying as well to exploit the beneficial effects of radio- and

chemotherapy. In this regard, selecting patients according to markers such as mutational load, tumor PD-L1 expression, and extent of T cell infiltration might help define combinations most beneficial for each patient. Understanding whether a non-inflamed tumor is the result of tumor escape or immune ignorance will help choose between helping existing T cells to efficiently exert their antitumor effect, providing exogenous T cells, and inducing tumor cell death to provide antigens to the immune system. With this, the dream of immunotherapy might come true, with long-lasting tumor remissions without significant toxicity to be seen for improvement of patient survival.

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All authors listed, have made substantial, direct and intellectual contribution to the work, and approved it for publication.

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REVIEW
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Immunosuppressive mechanisms of malignant gliomas: parallels at non-CNS sites

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The central nervous system (CNS) possesses powerful local and global immunosuppressive capabilities that modulate unwanted inflammatory reactions in nervous tissue. These same immune-modulatory mechanisms are also co-opted by malignant brain tumors and pose a formidable challenge to brain tumor immunotherapy. Routes by which malignant gliomas coordinate immunosuppression include the mechanical and functional barriers of the CNS; immunosuppressive cytokines and catabolites; immune checkpoint molecules; tumor-infiltrating immune cells; and suppressor immune cells. The challenges to overcoming tumor-induced immunosuppression, however, are not unique to the brain, and several analogous immunosuppressive mechanisms also exist for primary tumors outside of the CNS. Ultimately, the immune responses in the CNS are linked and complementary to immune processes in the periphery, and advances in tumor immunotherapy in peripheral sites may therefore illuminate novel approaches to brain tumor immunotherapy, and vice versa.

Keywords: glioblastoma, tumor immunotherapy, cancer immunotherapy, cancer immunosuppression, glioma, immune privilege

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Part I: Introduction

Contrary to common perceptions of central nervous system (CNS) "immune privilege," the brain can in fact elicit vigorous immune-stimulatory as well as immunosuppressive responses, the determinants of which are highly contextual. Understanding the determinants and mechanisms of both the stimulatory and suppressive responses may help elucidate novel immune-based strategies for brain tumor immunotherapy. In this review, we will discuss routes of glioma-mediated immunosuppression, including mechanical and functional barriers of the CNS, immunosuppressive cytokines, immune checkpoint molecules, tumor-infiltrating immune cells, and suppressor immune cells (**Table 1**). In addition, we will look to analogous immune-modulatory mechanisms observed in other sites of the body, as discoveries made at CNS and non-CNS sites are ultimately complementary and equally relevant to therapeutic development for tumors at all sites (1).

Part II: The CNS Immune Environment

The notion of "immune privilege" has long been ascribed to tissues wherein the immunological responsiveness is ostensibly blunted or modified (122). Early experimental observations that the brain lacked traditional lymphatic systems, contained few, if any, professional antigen-presenting cells (APCs), and mounted anemic immune responses against foreign antigens bolstered the theory that the brain was an "immunologically privileged" tissue. It is now apparent that the CNS is in fact capable of coordinating robust immune responses with the innate and adaptive immune systems,

TABLE 1 | Key examples of immune-modulatory mechanisms shared between malignant gliomas and non-CNS tumors.

	Malignant gliomas	Non-CNS tumor
TUMOR ANTIGEN PRESENTATION		
Antigen-presenting cells	Glioma-associated microglia and/or macrophages (2–4); DCs (5); B lymphocytes (6); possibly pericytes (7)	DCs (8–11); tumor-associated macrophages (12–14); B lymphocytes (6); possibly pericytes (7)
Location of antigen presentation	Brain parenchyma and/or tumor mass (2–4); tumor-draining lymph nodes (15–17)	Tumor-draining lymph nodes (18–20); tumor mass (21)
Routes of antigen egress from tumor	Fluid drainage (22, 23); migrating DCs less likely (24–27)	Migrating DCs (28–30) and/or fluid drainage (31)
IMMUNOSUPPRESSIVE CYTOKINES		
IL-10		
Sources	Glioma-associated macrophages and microglia (32)	Tumor cells (33, 34) and/or tumor-associated macrophages (35)
Actions	Immunosuppression (various) (36, 37); context-dependent pro-inflammatory and anti-tumor actions (38, 39)	Immunosuppression (various) (40); anti-angiogenesis (41), anti-metastatic (41), anti-tumor (42–44); anti-inflammatory (45)
TGF-β		
Sources	Glioma cells (46–48) and glioma-associated immune cells (49)	Tumor cells and tumor-associated immune cells (49–51)
Actions	Immunosuppression (various) (52); angiogenesis (53); maintains glioma stem cell populations (54); glioma cell autocrine proliferation (55); pro-invasion (56)	Immunosuppression (various) (50, 57, 58); tumor suppression (50, 57–59)
INDOLAMINE 2,3-DIOXYGENASE (IDO)		
Sources	Glioma cells and tumor-associated immune cells (60–62)	Tumor cells, tumor-associated immune cells, and endothelial cells (62–65)
Actions	Immunosuppression (various) (66); expansion of Treg population (67, 68)	Immunosuppression (various) (66); expansion of Treg population (67, 68)
REGULATORY T LYMPHOCYTES		
Predominant Treg type	nTreg (69) more than iTreg	nTreg more than iTreg (70)
Relevant Treg recruitment factors	CCL22 (71–73), CCL2 (71, 74, 75)	CCL2, CCL22 (76–81), CCL17 (81); CCL3, CCL4 (82); CCL5 (83)
TUMOR-ASSOCIATED MYELOID CELLS		
Types	Microglia (84, 85), macrophages (86), MDSCs (87)	Macrophages (88), MDSCs (89, 90)
Actions	Immunosuppression (various) (91–95); tumor invasion (96); tumor proliferation (97, 98)	Immunosuppression (various) (99); tumor invasion (100); tumor proliferation (100)
PD-1/PD-L1 IMMUNE CHECKPOINT		
Sources	Glioma cells (101); microglia (102); glioma-associated macrophages (103); neurons in tumor-adjacent brain tissue (104)	Tumor cells (105–109), tumor-associated macrophages (100, 110, 111); healthy tissue (112–114)
Relevant signaling pathways	PI3K/mTOR (101)	PI3K/mTOR (106, 108, 115, 116), MyD88/TRAF6 (117), MEK/ERK (117)
Actions	Immunosuppression, esp. via T cell suppression (118, 119); induction of glioma cell death (104)	Immunosuppression, esp. via T cell suppression (120, 121)

and that the immunological reactivity of the CNS is a mutable rather than an absolute state. Moreover, several of the structural and functional immunoregulatory features of the CNS that aid in dampening local immune responses are also reflected within other organs of the body. Therefore, the traditional notion of CNS "immune privilege" has become an imprecise characterization of the CNS immune environment, which is a more rightfully a highly contextual rather than an absolutely impregnable system.

Reframing the CNS Immune Environment

In recent decades, the consensus view of the CNS immune environment has shifted from one in which the blood–brain barrier (BBB) serves as a static barrier against the exchange of cells and soluble molecules into one in which egress and entry are dynamically regulated, often by mechanisms observed in other organ systems. During inflammation, immune cells migrate into the CNS parenchyma following dynamic gradients of chemotactic cues, including IFN-γ inducible cytokines (123, 124), α and β integrins (125), and matrix metalloproteinases (126), which also play key roles in leukocyte trafficking in peripheral tissues (127). Similarly, it has been postulated that soluble immune effectors, such as immunoglobulins (128), might also cross the BBB. One possibility is by way of carrier-mediated transporters (129, 130). For example, FcRn, a ubiquitous immunoglobulin receptor expressed by a wide variety of tissues, can mediate Ig transport across tissue barriers (131, 132). Although the routes by which Igs enter the CNS parenchyma is yet unknown, it has been postulated that FcRn, which is highly expressed on cerebral vessels (131), may play a key role in facilitating Ig entry into the CNS, as in other tissues (133).

Whereas the absence of traditional lymphatic systems was once heralded as evidence that the CNS was immunologically inert (134), it is now abundantly clear that soluble antigens routinely egress the CNS and reach the peripheral lymph nodes. *In vivo* tracer studies have demonstrated that CNS antigens drain via cerebrospinal fluid across the cribiform plate and into the nasal sub-mucosa (135). A separate pathway by which antigens travel to the cervical lymph nodes (CLNs) via the Virchow–Robin perivascular spaces within walls of the cerebral arteries has also been described (22, 23).

Indeed, during homeostatic conditions, antigens from the CNS are continuously sampled by DCs in the peripheral lymph nodes in the same fashion as antigens that arise from other sites (15). A more thorough discussion regarding antigen presentation in the CNS and peripheral tissues is provided in the next section of this review.

Lastly, although the entirety of CNS is often presumed to share the same immunological features, the relative absence of immune cells under homeostatic conditions is more accurately an attribute of the CNS parenchyma proper (127). At resting state, CSF-drained spaces, including the choroid plexus, leptomeninges, ventricles, and perivascular spaces, contain professional APCs and respond to foreign antigens in the same manner as organs do outside of the CNS (127, 136). By comparison, the parenchyma proper is generally devoid of peripheral immune cells and is maintained in a quiescent state by mechanical obstacles of the endothelial BBB (127). Obstacles against leukocyte entry include the CSF-drained Virchow–Robin perivascular space situated behind the endothelium, as well as the glia limitans, a wall of palisading astrocyte foot processes located between the perivascular space and CNS parenchyma (137). Aside from forming a second mechanical barrier against immune cells, the foot processes also express death ligand FasL/CD95L (138), which induces apoptosis in Fas-expressing T cells and arrests the inflammatory process. Accordingly, the vast majority of inflammatory cells that cross into the Virchow–Robin spaces during homeostatic states are retained in the perivascular space and never proceed past the glia limitans (127, 139). Inflammation and disease, however, can compromise the integrity of the BBB, thereby permitting circulating immune cells to infiltrate the parenchyma in significant numbers (136).

Hence, although the precise mechanisms underlying how and when the CNS coordinates immune responses remain to be clarified, there is accumulating evidence that several of the immunoregulatory features observed in the brain are shared by other tissues in the body as well. Baseline FasL expression, for example, is not unique to cerebral astrocytes but is also a feature in multiple peripheral tissues where immune homeostasis is favored, including lymphoid tissue, hepatocytes, testis, striated muscle, as well as certain glandular tissues (140–142). Blood–tissue barriers formed by intercellular tight junctions exist in the testis as they do in the CNS, and multiple organs, including the brain, liver, and gastrointestinal tract, secrete immune-modulatory cytokines that increase regulatory T cell expression and induce local immune tolerance (122). Therapeutic developments designed to overcome the immune-regulatory mechanisms of the BBB may therefore arise from discoveries made in the brain as well as findings made at other sites.

Part III: Tumor Antigen Presentation

Classically, extracellular antigens are captured at the cell surface, endocytosed, and presented on MHC class II molecules to CD4+ T-lymphocytes by specialized APCs (143). By comparison, endogenous antigens are processed in the rough endoplasmic reticulum of nearly all cell types and subsequently presented on MHC class I molecules to CD8+ T lymphocytes (144). Presentation of tumor antigens, however, is thought to involve a third process, termed "cross-presentation," whereby exogenous tumor antigens, scavenged from dying tumor cells, are presented on MHC Class I molecules to CD8+ T-lymphocytes, thereby directing the adaptive immune response toward malignant cells (145).

In peripheral sites, activation of tumor antigen-specific T cells is believed to take place within secondary lymphoid tissue, mediated by bone marrow-derived DCs via cross-presentation (145). Far less is known, however, regarding the process of priming T-cells against CNS tumor antigens (146). In particular, it remains unclear whether the anti-tumor immune response is initiated locally within the brain or peripherally in the body. The provenances of these processes have clear implications for brain tumor immunotherapies, such as dendritic cell-based vaccines (147, 148), that aim to exploit tumor antigen presentation to augment tumor immunity.

Tumor Antigen Presentation: CNS

Whether CNS tumor antigen presentation occurs within the brain or outside of it remains unclear, though the presence of APCs within the brain supports the hypothesis that presentation begins locally. Because of their strategic position behind the BBB and their essential role in CNS innate immunity, microglia are often charged with being the primary APCs for intracranial antigen presentation. The data show that microglia have the capacity to cross-present tumor antigens to CD8+ T cells via MHC class I *in vitro* (2–4) and *in vivo* (2, 4). Employing a murine model in which whole-body radiation was used to eliminate peripheral and CNS-associated APCs (e.g., DCs and macrophages in the perivascular spaces), Jarry et al. recently demonstrated that microglia could successfully cross-present intra-cerebrally injected OVA antigen to naïve CD8+ T cells *in vivo* (4), strengthening prior data (2).

Tumor-infiltrating DCs may also play a key role in glioma antigen presentation (146). The data indicate that DCs cross-present OVA antigen more efficiently than adult microglia, eliciting greater quantities of IL-2 and IFN-γ production from CD8+ T cells than microglia (2). Similarly, Jarry et al. reported that CD8+ T cell activation was more efficient in non-irradiated mice, which contained CNS-associated DCs along with microglia, than in irradiated mice, which contained solely microglia (4). Especially given that flow-cytometry (FACS) markers used to identify DCs lack the specificity necessary for distinguishing between DCs and activated microglia (5), APC activity may be falsely attributed to microglia in many cases.

Whether microglia and tumor-infiltrating DCs can successfully activate CD8+ T cells in the setting of malignant brain tumors, however, is uncertain. Current data suggest that microglia lose their capacity to express MHC molecules in the context of high-grade gliomas (3, 149, 150), likely due to the high levels of immunosuppressive cytokines, such as TGF-β, IL-10, and PGE2, within the glioma microenvironment (151, 152). Even after removing microglia from the glioma environment, the ability for the microglia to upregulate MHC expression following stimulation was substantially depressed compared to normal brain microglia (153). Moreover, in the presence of glioma cells, microglial production of pro-inflammatory cytokine TNF-α is suppressed by as

much as 50% compared to normal microglia, and activation of STAT3 transcription factor and secretion of immunosuppressive IL-10, both of which modulate immunosuppression, are greatly upregulated (154). Similarly, IL-10 has also been shown to inhibit DC maturation and maintain DCs in a tolerogenic state (155). These data suggest that malignant gliomas may skew APCs toward immunosuppressive phenotypes and hinder effective tumor antigen presentation within the brain. *In vivo* tumor models are needed to assess whether the APC capacities of microglia and DCs are in fact compromised in the glioma parenchyma and microenvironment.

Aside from microglia and DCs, tumor-associated macrophages (TAMs), B lymphocytes, and vascular pericytes may provide other cellular sources for CNS tumor antigen presentation. TAMs, which infiltrate gliomas in large numbers and possess cross-presentation capabilities (156), are thought to actually outnumber microglia (86) within the tumor mass. With regard to antigen-presentation capacity, data from a murine model of multiple sclerosis suggest that, compared to microglia, CNS-infiltrating macrophages are more highly activated and stimulate greater T cell proliferation *in vitro* (157). To our knowledge, however, no study to date has explicitly compared tumor antigen cross-presentation capacity of microglia to TAMs, likely due to limitations in reliably distinguishing microglia from TAMs within gliomas (158). Given that microglia and TAMs can both be polarized toward immunosuppressive M2-like phenotypes by the same sets of glioma-derived cytokines (159), it is possible that antigen-presenting capacity of microglia and TAMs are similarly impaired by the immunosuppressive glioma microenvironment.

B cells, which can function as efficient APCs outside of the CNS (6), are also believed to play a vital role in tumor antigen presentation in gliomas. Using a murine glioma model along with separate adenoviral vectors (Ad) encoding herpes simplex virus type I thymidine kinase (Ad-TK) and *fms*-like tyrosine kinase 3 ligand (Ad-Flt3L), which were used to kill tumor cells and recruit APCs to the microenvironment, Candolfi et al. showed that treatment with Ad-TK + Ad-Flt3L produced long-term survivors in 60% of WT mice but 0% in B-cell depleted mice (160). Moreover, when Ad-TK + Ad-Flt3L was administered to mice lacking transcriptional repressor Blimp-1, the absence of which causes arrest of terminal differentiation of B cells into antibody-producing plasma cells, Blimp-1-negative mice produced identical numbers of long-term survivors as WT mice, suggesting that tumor regression occurred irrespective of whether anti-tumor antibodies were generated (160). Lastly, in Ad-TK + Ad-flt3L-treated mice, the accumulation of antigen-bearing activated B cells within tumor-draining lymph nodes (TDLNs) along with evidence that the activated B cells were capable of stimulating CD8+ T cell proliferation *in vitro* were strong clues that B cells can cross-prime CD8+ T cells against glioma antigens and thereby orchestrate glioma regression (160).

Pericytes, which are perivascular cells that classically modulate blood flow, vessel permeability, and vessel remodeling at arterioles, venules, and capillaries, have also been shown to possess phagocyte and antigen-presentation capacity (7). Indeed, Peiper et al. recently reported that brain capillary pericytes, which are exquisitely sensitive to inflammatory cytokines, increase phagocytic activity and MHC class II expression when stimulated by TNF-α or IFN-γ (161). Key questions surrounding whether pericytes posses cross-presentation capacity and how the glioma microenvironment influences pericyte antigen-presentation ability remain to be answered. There are data from non-CNS tumor models, however, that suggest tumor-derived vascular pericytes may play an overall immunosuppressive role, and APC activity may therefore be impaired (162).

Interestingly, recent work by Thompson et al. illuminated that priming and differentiation of naïve CD8+ T cells can occur within tumor masses, irrespective of intratumoral APCs or TDLNs (21). It has been shown that prolonged TCR stimulation in the absence of CD28 co-stimulation might alone be sufficient for activating T cells (163), and high densities of tumor antigens within tumor masses may thereby provide a prolonged and powerful enough of a stimulus to activate T cells irrespective of APCs (21). Although these specific experiments involved melanoma tumors in non-CNS sites, there is also evidence that brain tumors themselves support terminal differentiation of CD8+ T cells (164). Therefore, the findings by Thompson et al. may yet find parallels in malignant brain tumors.

It is also possible, however, that presentation of brain tumor antigens occurs within peripheral lymphoid tissues outside of the CNS (16). Routes by which CNS antigens drain to the nasal mucosa and CLNs via CSF and/or perivascular spaces have been well described (22, 23), and recent evidence indicates that CNS antigens are continuously sampled in peripheral lymphoid tissue by DCs (15). Using intra-cerebral (IC) injections of fluorescent microspheres and OVA antigen in a mouse model, Walter et al. showed (1) that IC antigens preferentially accumulated in CLNs, and (2) that expansion of OVA-specific CD8+ T cells occurred within CLNs 2 days prior to their appearance in the brain, suggesting that cross-presentation occurs in the CLNs and not within the brain parenchyma (16). In a separate study, Okamoto et al. showed that 2 weeks following cerebral implantation of glioma tumors in rats, activated CD4+ and CD8+ T cells appeared exclusively within the CLNs, and their accumulation coincided temporally with T-cell infiltration into the tumor (17). Collectively, these data suggest that presentation of CNS tumor antigens may initiate in lymphoid tissue outside of the CNS.

Finally, it is also conceivable that priming the anti-tumor immune response involves processes both within and outside of the CNS. Transferring pre-activated tumor-specific CD8+ T cells into glioma-bearing mice, Masson et al. demonstrated that further phenotypic differentiation of tumor-specific CD8+ T cells occurs within the tumor mass (164). Compared to the pre-activated tumor-specific CD8+ T cells, tumor-infiltrating CD8+ T cells showed enhanced expression of IFN-γ, granzyme B, and $\alpha_E(CD103)\beta_7$ integrin, the latter of which was found to be important for T-cell retention within the brain (164). Further analysis of human glioma tissue revealed similar differentiation patterns, with 20–57% of tumor-infiltrating CD8+ T cells expressing $\alpha_E(CD103)\beta_7$ integrin compared to fewer than 5% of CD8+ T cells in peripheral blood (164). Consistent with that of murine tissue, approximately 60% of $\alpha_E(CD103)\beta_7$-expressing CD8+ T cells in human glioma tissue also co-expressed granzyme B (164). It has been hypothesized that

locally secreted TGF-β, which induces $\alpha_E(CD103)\beta_7$ expression in non-CNS sites (165), may also moderate $\alpha_E(CD103)\beta_7$ expression on T cells within gliomas (164). Further work is needed to evaluate how the glioma microenvironment initiates and/or shapes the effector immune response.

Tumor Antigen Presentation: Non-CNS Sites

In comparison to CNS tissues, there is a greater degree of clarity regarding the process of tumor antigen presentation in non-CNS tissues, though several aspects remain under contention. A preponderance of data indicate that presentation of tumor antigens occurs within the TDLNs, where resident DCs have been shown to play the key roles in priming naïve T cells (18–20). Additionally, several experiments have demonstrated that resident DCs within TDLNs can indeed cross-present tumor antigens to CD8+ T cells *in vivo* (8–10). Though macrophages are also endowed with cross-presentation capacities, they are substantially less efficient than DCs at priming CD8+ T cells (12–14). In the absence of convincing data supporting the primacy of alternative mechanisms, DCs have been presumed as the main APCs for cross-priming tumor-directed CTLs at non-CNS sites.

Further investigation, however, is needed to clarify the precise roles as well as cross-presentation capacities of DC subsets in tumor antigen presentation, as experimental models show that DC phenotypes can vary greatly depending on tissue and/or antigen type. DCs in murine lung tissue, for example, display CD103+ CD11b− and CD103− $CD11b^{hi}$ phenotypes while colonic DCs exhibit a predominately CD103−CD11b+ phenotype. Human liver harbors myeloid-derived CD1c+ DCs (166) while human renal tissue contains a greater portion of lymphoid-derived or plasmacytoid DCs (pDCs) than conventional myeloid-derived DCs (167). During CNS inflammation, the brain parenchyma is heavily infiltrated with DCs displaying CD11c+ phenotypes (18). Of note, a recent analysis of three resident DC subsets from human tonsil lymphoid tissue demonstrated that all subsets were capable of cross-priming CD8+ T cells with high efficiency (11).

Several animal studies have also illustrated that distinct DC subsets may mediate antigen presentation depending on type and location of antigen exposure (168–173). For example, whereas CD8α+ CD11b− DCs mediated cross-presentation of OVA antigen in the spleen, CD8α− CD11b+ DCs were responsible for OVA cross-presentation in the mesenteric lymph nodes (173). Analysis of circulating DCs in patients with NSCLC and breast cancer further revealed disparities in the proportion of pDCs to conventional myeloid-lineage DCs between the two malignancies, suggesting that tumor type may influence DC phenotypes (174). Further work is needed to evaluate the roles of phenotypic DC subsets in tumor antigen presentation as well as how tumors may influence phenotypic differentiation, as these are all important considerations for developing tailored immunotherapeutic interventions for various tumor sites (11).

As with the CNS, B lymphocytes and vascular pericytes may also participate in tumor antigen presentation at non-CNS sites. In fact, it has been shown that in mice that have been immunized against specific protein antigens, CD40 ligand-activated B lymphocytes traffic to secondary lymphoid organs and present peptide antigens to naïve T cells with comparable efficacy to DCs (175). Recently, B lymphocytes pre-loaded with specific tumor antigens were used successfully as a source of APCs for tumor eradication in an experimental model (176). As with the CNS, pericytes are potential sources for perivascular phagocyte activity at non-CNS sites (7). Further work is needed to determine whether pericytes associated with non-CNS tumors contribute to tumor antigen presentation and/or immune evasion.

The manner in which tumor antigens reach TDLNs at non-CNS sites also requires further clarification. Traditionally, it has been assumed that migrating DCs carry tumor antigens from the tumor site to TDLNs, where antigens are then transferred to resident DCs for subsequent T-cell priming (28–30). Evidence from viral models, wherein DCs carried antigens from the site of injection to draining lymph nodes for CTL activation, lent credence to the theory (29, 30, 177–179). The need for migrating DCs for antigen presentation in peripheral tissues was also a point of distinction between non-CNS and CNS tissues, where a preponderance of data suggested that intra-parenchymal DCs do not migrate to the CLNs in substantial numbers (24–27).

Recent evidence, however, has challenged the role of migrating DCs in tumor antigen presentation. Findings from several studies suggest that the immunosuppressive milieu of the tumor microenvironment may in fact hinder DC function and migration from peripheral tissues (180–184). IL-10, for example, which is produced by a number of tumors, prevents DC maturation and suppresses DC antigen-presenting capabilities (185). A recent study by McDonnell et al. reported that cross-presentation of tumor antigens within TDLNs was dependent on the continuous drainage of tumor antigens from the tumor site rather than DC migration (31), as is the case with CNS tissue. As previously discussed, Thompson et al., who described that priming of CTLs could occur within tumor masses themselves, raises the possibility that DCs altogether may be unnecessary for activating T cells (21). The high density of tumor antigens within the tumor parenchyma may provide sufficient stimulus for T-cell receptor (TCR) activation (21). Therefore, the cross-presentation of tumor antigens in peripheral tissues may in fact share commonalities with that of the CNS.

Antigen Presentation and Therapeutic Implications

In aggregate, these data show that much is still unknown regarding whether antigen-specific T cells, directed against CNS tumors, are primed locally in the CNS or peripherally in non-CNS sites. However, the data do speak strongly to the notion that priming tumor-specific T cells may, at least in part, occur within the body, emphasizing the need to evaluate anti-tumor immune responses directed at CNS tumors within a global context. Whether initial tumor antigen presentation occurs in the brain or in the body, for example, could have significant design implications for whether vaccine-based glioma therapies are designed for intracranial or peripheral administration.

Recent progress in evaluating tumor antigen presentation in the body has also identified shared features with the CNS. Similar to brain, peripheral tissues may also depend upon fluid drainage of tumor antigens to TDLNs rather than migrating DCs for the purpose of priming tumor-specific T-cells (31). Tumor-associated immunosuppressive cytokines, which will be discussed in further

detail in subsequent sections of this review, also present barriers to APC activity in CNS and non-CNS sites alike. Novel strategies aimed at augmenting anti-tumor immune responses at the level of tumor antigen presentation may therefore arise from discoveries made at both CNS and non-CNS sites. Notably, DC phenotypes can also vary greatly depending on tissue type, raising the possibility that DC-based therapies may ultimately also require tailored approaches that account for site-specific tumor biology.

Part IV: Immunosuppressive Cytokines – IL-10 and TGF-β

Cytokines with powerful immunosuppressive properties, including TGF-β and IL-10, are known mediators of tumor proliferation, invasion, and immune evasion. As such, targeted blockades of immunosuppressive cytokines are an attractive approach to tumor immunotherapy both in the brain and the body. A major challenge of cytokine-directed immunotherapy, however, lies in the pleiotropic and often paradoxical immune-regulatory functions of these cytokines. Neither TGF-β nor IL-10 is purely immunosuppressive and pro-tumorigenic in its effects. Therefore, developing successful immunotherapies that target immunosuppressive cytokines requires site-specific considerations that pay heed to micro-environmental context and tissue-specific biology.

Interleukin-10

Interleukin-10, arguably the most potent anti-inflammatory cytokine (185), is secreted by numerous cell types of the innate and adaptive immune system, including APCs and CD4+ T-helper cells, as well as malignant tumors of the brain and the body (186, 187). T-helper cells, monocytes, macrophages, and DCs are particularly important both as targets and actors of IL-10-mediated immunosuppression (155). Binding of IL-10 to its receptor (IL-10R) on DCs activates STAT3 transcription factor, which suppresses STAT-dependent signaling of inflammatory cytokines, IL-6, TNF-α, and IL-1B (188, 189); upregulates IL-10 secretion (190); and maintains DCs in an immature, tolerogenic state (155, 191). In macrophages, monocytes, and DCs, IL-10 also suppresses antigen-presenting capabilities by activating MARCH1, an E3 ligase that ubiquintinates cell-surface MHC Class II molecules for endocytosis and destruction (192, 193). IL-10 also hinders cytotoxic T-lymphocyte effector functions by inducing and sustaining FoxP3 transcription factor expression in immunosuppressive Treg cells (194, 195).

Paradoxically, IL-10 can also exert pro-inflammatory and anti-tumor effects (42). In fact, IL-10 gene was first isolated from T-cells that also secreted IFN-γ (196), illustrating the complex relationship between anti-inflammatory and pro-inflammatory response of IL-10. IL-10 is a potent stimulator of NK cells (197), mast cells, and B cells, and, often in combination with other cytokines, can potentiate cytotoxic activity of CD8+ T cells (198–202). IL-10 also exerts important anti-angiogenic effects by suppressing cytokine promoters of angiogenesis, which in certain pre-clinical tumor models has been shown to inhibit tumor growth (41, 203).

To date, investigations into the role of IL-10 in tumor growth has largely focused on its immunosuppressive actions. However, both immunosuppressive and anti-tumor effects appear to be active in tumors at all sites to varying degrees (185), which naturally presents challenges for IL-10-directed immunotherapy.

IL-10: Malignant Gliomas

Human gliomas have long been known to produce IL-10 *in vivo* (204). Among subclasses of human astrocyte tumors, the most aggressive tumors contained the highest levels of IL-10 mRNA, with glioblastoma tissue containing the most of any astrocyte tumor (204). Rather than secreting IL-10 directly, however, glioma cells produce soluble factors that induce tumor-associated macrophages (TAMs) and microglia to secrete the majority of the cytokine (32).

Consistent with its immunosuppressive actions elsewhere in the body, glioma-associated IL-10 down-regulates MHC class II expression on monocytes and inhibits IFN-γ and TNF-α production by immune cells (36, 37). IL-10 also upregulates checkpoint molecule B7-H1 (PD-L1) on both glioma-associated macrophages and circulating monocytes in peripheral blood (103). B7-H1 can bind and stimulate PD-1 receptor on activated T cells, producing T-cell anergy and apoptosis (118, 205). Furthermore, IL-10 has been shown to confer growth advantages to glioma tissues. *Ex vivo*, IL-10 both increases glioma proliferation (206) and confers invasive potential to glioma cells in a dose-dependent manner (207).

In conjunction with other cytokines, IL-10 can also facilitate anti-glioma immune responses. Mice implanted with gliomas expressing both IL-10 and IL-2 had significantly smaller (99% smaller) tumor sizes and increased T-cell infiltration at 14 days post-implantation compared to mice with IL-10$^-$/IL-2$^-$ tumors (38). Additionally, this reduction in tumor size could not be reproduced with either IL-10 or IL-2 expressing tumors alone (38).

More recently, Vleeschouwer et al. reported that persistent and elevated IL-10 production by T-cells was in fact required for T-cell suppression of glioma growth following stimulation with tumor lysate-loaded dendritic cells (39). Ectopic IL-10 delivery during the T-cell stimulation phase further increased the levels of IFN-γ production and hindered tumor growth (39). It has been postulated that the complex interplay between IL-10 and IFN-γ might regulate the immunosuppressive effect of indolamine 2,3-dioxygenase (IDO) tryptophan metabolism by glioma-associated APCs, resulting in a stronger anti-tumor immune response (208). The role of IDO in glioma-induced immunosuppression is discussed in subsequent sections of this review.

IL-10: Non-CNS Tumors

While IL-10 also plays a duplicitous role in tumor suppression and progression at tissues outside of the CNS, its biological actions in peripheral sites also differ in several important ways. IL-10 mRNA and protein have been isolated from a variety of human tumors, including ovarian (209), breast (203, 210), renal cell (211), lung (212), squamous and basal carcinomas (213), and metastatic melanoma (33, 214). Unlike gliomas, however, where the vast majority of IL-10 is produced by tumor-associated macrophages and microglia, several peripheral tumors produce IL-10 directly.

For example, metastatic melanoma (33) and bronchogenic carcinomas (34) produce IL-10 almost exclusively, with little or no secretion by TAMs.

At the same time, other peripheral tumors, similar to gliomas, may also rely upon TAMs to produce the majority of IL-10. HPV-16 associated carcinomas, for example, have been shown to recruit TAMs, which produce the majority of IL-10 (35). Whether or not similar soluble factors are utilized by gliomas and systemic tumors to induce TAMs to produce IL-10 is still unknown, but such knowledge would be therapeutically relevant for targeting IL-10 in these tumors.

In certain peripheral tumors, IL-10 also appears to have a particularly strong stimulatory effect on NK cells (197). In a murine B16 melanoma model, ectopic injection of IL-10 into the tumor mass reduced the numbers of infiltrating CD8+ and CD4+ T cells and macrophages (215), which is consistent with observations from gliomas; however, IL-10 also increased infiltration of NK cells in melanoma (215), which has not been reported in gliomas. Exogenous IL-10 was also shown to inhibit melanoma metastasis in mice that were deficient in B cells and T cells but with competent NK cells (41), suggesting that infiltrating NK cells may play a key role in suppressing metastatic spread.

The anti-angiogenic effects of IL-10 may also play an important part in inhibiting tumor growth and metastasis. IL-10 is known to suppress the macrophage production of pro-angiogenic cytokines, including IL-1, IL-6, IL-8, TNF-α, and MMP-9 (41, 216). Indeed, whereas the blood vessels were all but absent in the surrounding tissue of IL-10 secreting melanoma tumors, the tissue surrounding non-IL-10 producing tumors was highly vascularized (41). Whether IL-10 exerts similar anti-angiogenic and anti-metastatic effects in CNS tumors is yet unknown, although *in vitro* data suggest that the pro-proliferative effects of IL-10 in malignant gliomas may outweigh the inhibitory effects (206, 207).

Lastly, IL-10 serves a protective role in certain tissues of the body where chronic inflammation plays an etiological role in cancerogenesis. In these tissues, IL-10 is a key cytokine for maintaining anti-inflammatory T-regulatory cells and suppressing pro-inflammatory IL-17-expressing Th17 cells (217). Mice that were deficient in IL-10 spontaneously developed inflammatory bowel disease (IBD), which later progressed to colorectal carcinoma (43). Likewise, a small human study reported that IL-10 and IL-10R deficiencies, which has been linked to early onset IBD (45, 218), may also be associated with the development of malignant lymphomas (44). These pro-tumorogeneic associations become particularly important in the context of therapeutic approaches that may systemically deplete, or block the effects of, IL-10.

IL-10 in the Brain and Body: Therapeutic Implications

Taken together, these data illustrate the enigmatic role of IL-10-mediating tumor growth as well as suppression, the balance of which is greatly influenced by tumor biology and micro-environmental cues. It is particularly interesting that in the setting of malignant gliomas, IL-10 derived from TAMs exerts an overall tumorogenic and immunosuppressive effect, whereas IL-10 secreted in persistent and high levels by T-cells can produce pro-inflammatory and anti-tumor effects. These data indicate that cell of origin of IL-10 may determine, at least in part, its phenotypic actions in the tumor environment. Specific cell populations may therefore be selectively depleted to achieve the desired pro-inflammatory or anti-inflammatory effect.

From a therapeutic standpoint, it is also important to elucidate how IL-10 might interact with other cytokines in the microenvironment to generate an anti-tumor or pro-tumor response. IL-2, for example, appears to potentiate the anti-tumor response in malignant gliomas. In non-CNS tumors, IL-10 has been shown to augment CD8+ T-cell cytotoxicity in a manner that is dependent on its expression of IFN-γ and granzymes (219). Pegylated IL-10 (PEG-IL-10), which in pre-clinical tumor models was shown to expand tumor-resident CD8+ T cells and mediate tumor rejection (217), has entered human trials as monotherapy or in combination with chemotherapy for patients with advanced solid tumors, which include melanoma, NSCLC, renal cell, colorectal, ovarian, prostate, and pancreatic cancers (Clinical Trial NCT02009449) (Bauer 2014 ASCO). Whether PEG-IL-10 alone or in combination with IL-2 holds promise for treating malignant gliomas remains to be seen.

TGF-β

TGF-β is a 25-kDa cytokine that is produced by several cell types, including both immune cells and malignant tumors (220). TGF-β is formed as a pre–pro-polypeptide and is activated through a series of protealytic cleavage steps. The active isoforms of TGF-β, TGF-B1, TGF-B2, and TGF-B3, signal by bringing together two pairs of serine/threonine kinases known as type I and type II TGF-β receptors (57). Canonically, cross-phosphorylation of type I and II receptors leads to downstream phosphorylation of Smad family of transcription factors, which migrate to the nucleus and regulate transcription of various target genes (57).

TGF-β is highly pleiotropic, regulating a wide array of biological functions that include cell proliferation, migration, survival, angiogenesis, embryonic stem cell differentiation, and immune surveillance (220). Its role in cancer genesis is also manifold, serving as a suppressor of early-stage tumor proliferation but an abettor of late-stage tumor progression (58). Elevated expression of TGF-β and its receptors by several human cancers, both in the brain and the body, has been associated with higher tumor grade and/or poorer prognosis (221). These malignancies include prostate cancer, small cell lung carcinoma, pancreatic cancer, gastric cancer, transitional cell carcinoma of the bladder, as well as malignant gliomas (221).

TGF-β: Malignant Gliomas

TGF-β was, in fact, initially isolated from the serum of patients with malignant gliomas. Fittingly described as a soluble "humoral immunosuppressive" factor, glioma-derived TGF-β significantly depressed lymphocyte functions and induced systemic lymphopenia, particularly in CD4+ T helper cell populations (222). Subsequent decades of research have further elucidated that TGF-β actually depresses cytotoxic functions of all cells of the immune system, facilitating immune evasion and glioma growth (52). MHC class II expression on glioma cells, macrophages, and microglia, for example, are significantly depressed by TGF-β (223). Expression of NKG2D activating receptor on the surface of NK cells are likewise

reduced, as is production of CD8+ CTL cytolytic gene products perforin, granzyme A, granzyme B, FasL, and IFN-γ (224, 225). TGF-β also polarizes T-cells and monocyte-lineage cells toward immunosuppressive phenotypes, which further perpetuates a tolerogenic state that favors tumor growth (57). Moreover, TGF-β is believed to facilitate glioma growth and invasion by promoting angiogenesis (53), sustaining glioma stem cell populations (54), inducing the production of platelet-derived growth factor (PGDF), which serves as an autocrine proliferative signal for glioma cells (55), as well as increasing the synthesis of pro-invasive matrix metalloproteinases (56).

Strategies that block TGF-β signaling have been shown to restore anti-tumor immunity in pre-clinical glioma models. For example, *in vitro* silencing of TGF-β1 and TGF-β2 synthesis in human glioma cells using small interfering RNA (siRNA) techniques was shown to prevent NKG2D down-regulation on NK cells and enhance MICA expression on glioma cells (224). Furthermore, siRNA-silenced glioma cells displayed increased susceptibility to immune cell lysis (224). In a murine glioma model, inhibiting TGF-β1 receptor using SX-007, an oral serine/threonine kinase inhibitor, produced greater numbers of long-term survivors (33%) in the experimental group compared to control group (6%) (226). The treatment group receiving SX-007 also had higher levels of CD8+ T-cells in the CLNs than control groups, indicating TGF-β blockade can reverse its immunosuppressive effects (226). Taken together, these data illustrate that TGF-β confers predominately immunosuppressive and pro-invasive advantages to malignant gliomas, and blocking TGF-β signaling can reverse its malignant effects.

TGF-β: Therapies for Malignant Gliomas

In the brain, modulating TGF-β is particularly attractive. Radiation, a therapeutic cornerstone for malignant CNS tumors, has been shown to increase TGF-β expression both *in vitro* and *in vivo*. Neutralizing TGF-β might not only counteract the immunosuppressive and pro-invasive effects of TGF-β on the tumor but also attenuate the radiation-induced activation of TGF-β. Indeed, a small-molecule TGF-βR1 kinase inhibitor LY2109761 increased radio-sensitivity of GBM cell lines and stem cells *in vitro*. In combination with radiotherapy, LY2109761 reduced the tumor growth and prolonged the survival in ortho-topic intracranial murine glioma models compared to radiotherapy alone (227). Conceivably, this benefit might also extend to tumors at other sites that are frequently treated with radiotherapy, such as prostate adenocarcinoma or head-and-neck squamous cell carcinomas.

Several compounds targeting TGF-β signaling in malignant gliomas have entered clinical trials (220); their efficacy, however, remains inconclusive. One of the most promising compounds was Trabedersen, an anti-sense oligonucleotide against TGF-β2 mRNA that was shown to inhibit tumor proliferation and enhance anti-tumor immunity *in vitro* (228). In phase I/II trials, Trabedersen was associated with improved survival in patients with refractory high-grade gliomas compared to literature data (229). Although a subsequent randomized phase IIb clinical trial of Trabedersen reported improved tumor control and trended toward improved 2-year survival among patients with refractory anaplastic astrocytoma compared to chemotherapy (230), the results of the trial have been called into question based on several methodological weaknesses (231). The Phase III trial of Trabedersen, which was halted in 2012 due to patient recruitment issues, was recently terminated in light of advances in neurosurgical and first-line standard of care for glioblastoma (220). However, phase I and II trials of LY2157299, an oral TGF-β receptor kinase inhibitor, for newly diagnosed and recurrent glioblastomas have recently completed accrual, and efficacy data are expected in 2015 (232–235).

TGF-β: Non-CNS Tumors

Whereas TGF-β exerts predominately immunosuppressive and tumorigenic effects in the context of gliomas, its role in influencing tumor growth in other sites of the body is arguably more pleiotropic and context-dependent, which makes modulating TGF-β in systemic tumors exceedingly complex. Neutralizing TGF-β may indeed cause tumor regression at sites that depend on TGF-β for proliferation but, at the same time, may also inadvertently cause tumor growth in tissues where TGF-β serves as a tumor suppressor (50). TGF-β, for example, is a potent inhibitor of epithelial cell proliferation (236–238), and inactivating mutations of TGF-β receptors are implicated in the development of several human carcinomas (50). Neutralizing the protective effects of TGF-β could conceivably promote malignant transformation of epithelial tissue.

Even among tumors of the same tissue type, inactivating mutations of TGF-β and/or its receptor can lead to disparate effects. Mutations in TGF-β receptor are frequently found in colon cancer (239–242), and mouse models have shown that inactivating mutations in the TGF-β gene increases spontaneous formation of colorectal carcinoma (243). Yet, paradoxically, patients with a form of hereditary colorectal carcinoma, termed HNPCC, and who frequently have TGF-β receptor mutations actually have better prognoses than patients with sporadic colon cancer without TGF-βR mutations (239, 244). Lastly, similar to the potential off-target effects of IL-10, whether or not a tumor arises in a pro-inflammatory or anti-inflammatory environment also becomes a key consideration in modulating TGF-β. Gastric adenocarcinomas, for example, which can develop as a result of protracted tissue inflammation following *H. pylori* colonization, may flourish in the absence of TGF-β and other immunosuppressive cytokines.

Nevertheless, several strategies for targeting TGF-β in non-CNS tumors, including anti-sense oligonucleotides, monoclonal antibodies, vaccines, and small-molecule inhibitors, have shown moderate success in pre-clinical models of breast, colorectal, pancreatic, hepatocellular, and renal cell carcinomas, with some proceeding toward human trials (220). The efficacy as well as off-target effects of modulating this multi-faceted cytokine remain to be seen.

Part V: Indolamine 2,3-Dioxygenase 1 – Tryptophan Metabolism

Indolamine 2,3-dioxygenase 1 is a cytosolic enzyme produced by macrophages and dendritic cells, primarily in response to pro-inflammatory factors (such as IFN-γ, IFN-α, IFN-β, and LPS) (245, 246). IDO catalyzes the rate-limiting step of tryptophan

degradation, producing, among other Trp metabolites, kynurenine, which exerts several immunosuppressive effects that may help to regulate inflammation (66). Most notably, kynurenine facilitates expansion of T-reg populations and inhibition of T cell effector functions (67, 68). IDO, however, is also expressed by several human tumors in the brain and the body, including lung, prostate, colorectal, pancreatic, and endometrial cancers, as well as glioblastoma multiforme (60, 247). Moreover, level of IDO expression by malignant tumors has been correlated with poorer prognoses (248), indicating that IDO and the expansion of Treg populations may play a critical role in abetting tumors in evading host immunity.

IDO: Malignant Gliomas

Indolamine 2,3-dioxygenase 1 is not expressed in the brain under normal physiological conditions. IDO mRNA, however, is substantially elevated in human glioma tissues and correlates negatively with overall survival (60, 61). Similar to other tumor sites outside of the CNS, the malignant effect of IDO on glioma progression appears largely to result from IDO-mediated accumulation of thymus-derived nTreg cells, which subsequently exert immunosuppressive effects on effector cells in the tumor microenvironment (60). Specifically, production chemokine CCL22 by glioma cells is believed to play a key role in recruiting and trafficking peripheral nTregs into the glioma milieu, a subject that is discussed in more depth in subsequent sections of this review in Ref. (71, 72).

Recent data also indicate that glioma cells, rather than TAMs, microglia, and DCs, directly produce the majority of the IDO (60), which is distinct from tumors outside of the CNS where DCs account for the majority of tumor-derived IDO (62–65). In a murine glioma model where GL261 cells were injected intracranially into the brain of WT or IDO-deficient mice, peripheral expression of IDO had no impact on intratumoral T-cell accumulation or overall survival (60) between the two groups of mice. By comparison, implantation of IDO-producing GL261 tumor cells into the same set of mice resulted in significantly increased intratumoral Treg accumulation and reduced overall survival (60). Interestingly, when IDO-expressing and IDO-non-expressing glioma cells were implanted concurrently in separate cerebral hemispheres within the same mouse, any survival benefit normally attributed to IDO-deficient tumors was eliminated by the presence of IDO-expressing gliomas in the contralateral cerebral hemisphere (249). Taken together, these data illustrate that IDO, produced directly by glioma cells, globally suppresses the anti-glioma immune response by recruiting thymus-derived nTregs.

IDO: Site-Specific Considerations for Immunotherapy

Although tumors both in the brain and the body can exploit IDO-mediated immunosuppression to overcome host anti-tumor immune responses, molecular inhibition of IDO activity has produced different responses in different organs, which may reflect unique tissue-specific factors.

In a murine breast tumor model, 1-methyl-tryptophan (1-MT), a widely studied inhibitor of IDO, failed to inhibit tumor growth (250); however, in combination with cytotoxic chemotherapies, including paclitaxel, cisplatin, cyclophosphamide, and doxorubicin, 1-MT produced significant tumor regression (250). The synergic effects between cytotoxic chemotherapy and 1-MT have also been reported in melanoma models (251). In glioma models, however, it appears that, in sufficient doses, 1-MT alone can produce significant anti-tumor effects (249). Moreover, when co-administered with cytotoxic chemotherapy, 1-MT failed to improve survival over chemotherapy alone (249), suggesting that the synergism between IDO inhibition and chemotherapy may depend on differences in tissue biology between CNS and non-CNS tumors. It has been postulated, for example, that separate tryptophan-metabolizing enzymes, such as IDO2 or TDO, that are also known to mediate immunosuppression in gliomas, may provide compensatory kynurenine production under states of cellular stress (249).

Interestingly, administering 1-MT with anti-CTLA-4 and anti-PD-L1 monoclonal antibodies produced a 100% long-term survival rate in glioma-bearing mice (249), an improvement over the 90% long-term survival rate in anti-CTLA-4 and anti-PD-L1 therapies alone. By comparison, the same triple therapy regimen was dramatically less effective at extending survival in mice with intracranially implanted B16 melanoma tumors, illustrating that the utility of IDO modulation may differ substantially based on tumor type and environmental context (249).

Lastly, differential patterns of IDO expression among tumor types may also impact therapeutic efficacy of IDO modulation. Recent tissue analysis of 15 human tumor types showed that IDO expression was largely restricted to tumor cells, myeloid-lineage cells, and endothelial cells (62). The distribution of IDO expression among the three categories of cells, however, varied greatly from tumor to tumor. IDO expression within renal cell carcinomas tissue, for example, appeared to be largely restricted to the vasculature, whereas IDO expression within colorectal cancer tissue appeared to be limited to DCs (62). Whereas cervical tumor tended to express IDO on the outer edges of the parenchyma, IDO expression in endometrial tumors was more diffusely distributed throughout the parenchyma (62). The frequency of IDO expression also varied depending on tumor type. For example, cervical and endometrial carcinomas were found to be most frequently IDO+ (83 and 94% of all cervical and endometrial carcinoma tissue samples, respectively) while glioblastoma tissues were most frequently IDO− (only 8% of glioblastoma tissues were found to express IDO) (62). Further work is needed to characterize how variable expression patterns of IDO among different tissue types may affect IDO-targeted immune-modulation therapy.

Part VI: T-Regulatory Lymphocytes

Regulatory T lymphocytes (Tregs) are a highly diverse and plastic subset of CD4+ immunosuppressive helper T cells that play an essential role in promoting immunological tolerance (252, 253). As guardians against autoimmunity, Tregs can also hamper anti-tumor immune responses and facilitate tumor growth, an undesired consequence of that has long been recognized (254, 255). Malignancies both in the brain and body actively recruit and sustain Tregs into the tumor microenvironment and parenchyma, and numerous studies have correlated higher intratumoral Treg

density with higher tumor grades and poorer prognoses (256). Hence, Tregs are believed to play a pivotal role in tumor-mediated immunosuppression and subsequent immune escape, leading to the failure of immune therapies.

Natural and Adaptive Tregs

CD4+ Tregs comprise approximately 5–10% of circulating CD4+ T cell population, and, based on developmental origin, Tregs are classified as either thymus-derived natural Tregs (nTregs) or peripherally induced "adaptive" Tregs (iTregs) (257). Subsets of the CD8+ suppressive regulatory T-cells, for which less is known about their immunomodulatory roles in disease than CD4+ counterparts, also exist and are reviewed elsewhere (253).

nTregs develop in the thymus from CD4+ single-positive thymocytes via antigen presentation by thymic epithelial cells (257). nTregs characterized by stable and high-level expression of Forkhead Box P3 (FoxP3), key transcription factor and regulator for Treg development and immunosuppressive function (252). Mice and humans with rare FoxP3 gene dysfunctions suffer florid autoimmune attack on multiple organs and tissues, culminating in a fatal disorder known as immunodysregulation polyendocrinopathy enteropathy X-linked syndrome (IPEX) (258). More recently, the transcription factor Helios as well as neuropilin-1, a semaphorin III receptor, has also been identified as potential markers for nTregs (259–262). Although incompletely characterized, nTregs exert their immunosuppressive function in a contact-dependent, cytokine-independent mechanisms, which include the expression of surface molecules CTLA-4 and PD-L1, membrane-bound TGF-β, pericellular generation of adenosine, as well as through Granzyme B/Perforin and Fas/FasL pathways (49, 263–268).

By comparison, iTregs, which encompass several distinct CD4+ T cell types (257), differentiate in the periphery when antigens are presented to and recognized by naïve conventional CD4+ T cells (Tconv) under tolerogenic conditions. In contrast to nTregs, which display constitutive expression of FoxP3, iTreg FoxP3 expression is transient or even absent, and its induction appears dependent on IL-10 and/or TGF-β signaling (49, 252). iTregs also appear to exert their immunosuppressive effects by releasing soluble factors, such as IL-10 and TGF-β (49), instead of the cell-surface ligand molecules used by nTregs.

Ultimately, whether Tregs are thymically or peripherally derived, Tregs are capable of wholesale suppression of innate and adaptive effector immune cell function (253). From a therapeutic standpoint, however, it is valuable to understand the process by which tumor-infiltrating Tregs accumulate within various tumors, such that the targeted strategies might be developed to modulate specific Treg populations.

Treg Accumulation in Brain

Greater numbers of glioma-associated Tregs has been associated with higher tumor grade (269), and levels of tumor-infiltrating Tregs may prove to be an important prognostic indictor for survival (270), though the data are conflicting (271–273). Based on current methods for evaluating Treg phenotype, the data suggest that tumor-infiltrating Tregs in malignant gliomas are predominately nTregs, rather than iTregs (69). In a murine glioma model, levels of tumor-infiltrating Tregs were significantly diminished for mice that were thymectomized prior to tumor implantation compared to that of non-thymectomized mice (69). In addition, over 90% of Tregs within the tumor expressed Helios transcription factor (69), which is known to be highly expressed on thymus-derived nTregs but not iTegs in mice and humans (262), strengthening the claim that glioma-associated Tregs may be nTregs.

The precise mechanism by which gliomas recruit nTregs is still under investigation; however, it is becoming evident that gliomas produce several soluble factors (72) that aid in recruiting nTregs into the microenvironment and parenchyma. In particular, gliomas are known to produce CC chemokine ligand 22 (CCL22), which serves as a potent chemotactic factor for leukocytes expressing CCL22 receptor CC chemokine receptor 4 (CCR4). Glioma-infiltrating Tregs express particularly high levels of CCR4 compared to other tumor-infiltrating lymphocytes (73), and several *in vitro* migration studies have demonstrated the ability of glioma-derived CCL22 to induce Treg chemotaxis (71–73).

CC chemokine ligand 2, another chemokine produced by human gliomas (74) and a weaker ligand for CCR4, has also been implicated in glioma-mediated Treg chemotaxis (71, 75). *In vitro* administration of blocking antibodies to CCR4 as well as CCL2 receptor, CCR2, arrested Treg migration toward glioma supernatant (71). Whether CCL22 and/or CCL2 are significant Treg chemotactic factors *in vivo* is still a matter of contention (71, 72, 75); however, it is evident that other soluble factors within the glioma microenvironment also contribute to Treg chemotaxis, and these factors remain to be identified (72).

Interestingly, outside of the tumor parenchyma and microenvironment, circulating CCL22 appears to be depressed in the sera of patients with malignant gliomas (274). Additionally, in a serum analysis of 1,208 patients with glioma, one group recently reported that lower serum levels of CCL22 were a negative prognostic indictor for overall survival (274). Because gliomas are known to exert global immunosuppressive effects, the lower levels of CCL22 in sera seen in patients with higher grade gliomas compared to lower grade gliomas was thought to reflect glioma-mediated suppression of peripheral APCs, which are the predominant producers of CCL22 *in vitro* and *in vivo* (275). The precise mechanisms underlying this relationship, however, remain to be elucidated. Further clarification is needed regarding whether glioma-related production of CCL22 is related to levels of CCL22 in peripheral blood, as well as whether glioma-derived CCL22 is also associated with disease prognosis.

Though iTregs may likely play a lesser role in glioma immunoresistance (69), there is reason to believe that gliomas are also capable of converting Tconv into iTregs *in vivo*. TGF-β and IL-10, both of which are produced by gliomas *in vivo*, have been shown to induce Treg conversion *in vitro* (252, 276). Prostaglandin E2, which is also produced by gliomas via cyclo-oxygenase 2 (COX-2), can induce *de novo* Tconv to Treg conversion (277, 278).

Treg Accumulation at Non-CNS Sites

Whether tumor-infiltrating Tregs in peripheral sites are thymus-derived or peripherally induced Tregs is also controversial, especially in the absence of definitive markers for

distinguishing nTregs from iTregs (257). There is compelling evidence, however, that intratumoral Tregs from non-CNS sites also comprised predominately nTregs rather than iTregs, similar to the distribution in malignant gliomas. Using a mouse fibrosarcoma tumor model, Waight et al. recently demonstrated that intratumoral Tregs bore CpG hypomethylation at FoxP3 Treg-specific demethylated region (TSDR) (70), which is thought to be an epigenetic hallmark for nTregs (279). Moreover, epigenetic analysis of intratumoral Tregs from human NSCLC and ovarian tumors revealed demethylation at the FoxP3 TSDR similar to that observed in murine tumor models, suggesting that tumor-infiltrating Tregs in human tumors may also be nTregs (70). Whether similar distributions of Treg subtypes based on epigenetic markers are found in other peripheral tumors remains to be determined.

Similar to gliomas, several non-CNS tumors, including ovarian (76), breast (77), prostate (78), gastric (79), esophageal (80), as well as Hodgkin lymphoma (81) tumor cells can also elaborate CCL22 to help recruit Tregs into the tumor microenvironment. Notably, in one recent study of 417 cases of invasive breast cancer, high tumor expression of CCL22 was associated with higher histological grade and greater density of tumor-infiltrating Tregs (280). Furthermore, higher CCL22 expression was reported to be an adverse predictor of progression-free and overall survival (280). Higher ratio of stromal CCR4+ Tregs to CD8+ Tregs was also negatively associated with overall survival in human oral squamous cell carcinoma (OSCC) (281), indicating a potential relationship between CCL22 and overall survival in OSCC.

At the same time, Treg chemoattractant profiles can also vary greatly from tumor to tumor in peripheral sites. For instance, CCL17, another ligand to CCR4, does not appear to play a role in Treg chemotaxis in glioma or ovarian carcinoma but is a key mediator in Hodgkin's lymphoma and gastric adenocarcinoma (81). In colorectal carcinoma, TAMs secreting CCL20 attracted tumor-infiltrating Tregs that highly expressed CC chemokine receptor 6 (282). Likewise, in an experimental melanoma model, tumor-infiltrating Tregs expressed CCR5 and preferentially migrated toward its ligands CCL3, CCL4, and CCL5, which were elaborated by tumor-infiltrating myeloid-derived suppressor cells (MDSCs) (82). Similarly, CCR5–CCL5 signaling also appears to play a prominent role in Treg migration in both human and murine pancreatic adenocarcinoma (83).

Though, as with the brain, the most recent data suggest that the majority of tumor-infiltrating Tregs in peripheral sites are also nTregs, non-CNS tumors can also elaborate TGF-β, IL-10, and PGE2, which can induce peripheral iTreg conversion. Administration of anti-TGF-β antibody *in vitro* blocked conversion of Tconv to the Treg phenotype, and *in vivo* administration of anti-TGF-β antibody in mice implanted with renal cell carcinoma reduced tumor burden, decreased numbers of circulating FOXP3+ CD25+ CD4+ cells in peripheral blood, and removed the immunosuppressive capabilities of FOXP3+ CD25+ CD4+ T cells (283). This leaves open the possibility that iTregs play an important but poorly understood role in tumor immunoresistance. In fact, one murine sarcoma model illustrated that intratumoral nTregs and iTregs may collaborate to suppress different arms of the adaptive immune response, with nTregs preferentially suppressing CD8+ T cells and iTregs suppressing CD4+ T cells, respectively (284).

Therapeutic Implications

From a therapeutic standpoint, these findings are particularly important. Traditional approaches to depleting Tregs, such as anti-CD25 antibodies (285) and cyclophosphamide (286), are largely non-specific, and whether these strategies preferentially target nTreg or iTreg populations is currently unknown (257). However, with the current knowledge that nTregs may comprise the majority of tumor-infiltrating Tregs in the brain and the body, it may be possible to devise targeted depletion strategies for nTregs, thereby minimizing side effects associated with indiscriminate systemic Treg depletion (258). For example, nTregs are believed to exert their immunosuppressive effects predominately via contact-dependent, cytokine-independent mechanisms (49). These include co-stimulatory and co-inhibitory molecules CTLA-4 and PD-L1, membrane-bound TGF-β, pericellular generation of adenosine, and granzyme B/perforin and Fas/FasL pathways (49, 263–268). Therefore, it may be possible to modulate nTreg activity by blocking the interactions between these immunosuppressive cell-surface ligands and their receptors (287–289).

Blocking Treg recruitment may offer another route for reducing intratumor Treg burden in a specific manner. CCL22–CCR4, a shared chemokine pathway for Treg migration in several tumors of the brain and the body, may prove useful for reducing Treg burden in a targeted manner (290). Recently, Adeegbe et al. reported that using anti-CCR4 antibodies in human melanoma patients selectively depleted CCR4+ Tregs while sparing naïve Tregs (257). Other strategies that interfere with the CCL22–CCR4 axis have demonstrated moderate success in *in vitro* and pre-clinical *in vivo* experiments (290–292).

Finally, within tissues of the body where inflammation promotes carcinogenesis, such as with gastric cancers or colorectal carcinoma, greater numbers of Tregs suppress inflammation and may therefore have anti-tumor effects. Greater degree of Treg tumor infiltration has in fact been associated with better prognosis in colorectal cancers (293, 294). Therefore, immunotherapeutic strategies that target Treg depletion need to consider the environmental context within which tumorigenesis occurs.

Part VII: Tumor-Associated Myeloid Cells

The role of myeloid-lineage cells in promoting tumor growth and invasion has come into focus in recent years. At least five distinct subpopulations of tumor-associated myeloid cells (TAMCs) have been identified, including monocyte-derived tumor-associated macrophages (TAMs); angiogenic monocytes; immature, immunosuppressive myelomonocytic cells known as MDSCs; tumor-associated neutrophils (TANs); as well as microglia within the CNS (87). Their expansive roles in facilitating immunosuppression, angiogenesis, cellular proliferation, and tumor invasion in CNS and non-CNS sites have prompted investigations into new immunotherapeutic strategies aimed at neutralizing TAMCs. Representative classes of TAMCs as they relate to gliomas will be discussed below; an in-depth review of TAMCs can be referenced here (87).

TAMCs in Malignant Gliomas

Microglia and monocyte-derived macrophages (i.e., TAMs) together account for the majority of glioma-associated myeloid cells (87, 159). Microglia, the resident macrophages of the CNS, compose 5–20% of the total glial cell population (84, 85) and play an essential role in the innate defense system of the brain (91). Monocyte-derived macrophages, by comparison, are normally restricted to the perivascular, choroid, and meningeal locations of the CNS (see Part II: The CNS Immune Environment), gaining entry to the parenchyma only after disease and/or inflammation have disrupted the integrity of BBB. In the setting of glioma, TAMs and microglia can comprise upward of 30% of the total tumor mass, with reports indicating that high-grade gliomas tend to exhibit greater levels of TAMs and microglia accumulation than low-grade gliomas (87, 295, 296).

Similar to monocyte-derived macrophages, both microglia and TAMs can embody pro-inflammatory (M1) as well as immunosuppressive (M2) phenotypes depending on environmental cues (99, 297). In the presence of inflammatory signals, classically activated microglia and macrophages skew toward an M1-like phenotype, characterized by increased capacity to migrate, phagocytose, secrete cytotoxic factors, as well as express MHC class II and co-stimulatory molecules for T cell activation (91). In the setting of gliomas, however, the data suggest that microglia and TAMs polarize toward an M2-like phenotype (91–93), particularly in late stages of disease progression (298), and exhibit immunosuppressive, pro-invasive properties that facilitate tumor growth. It is important to note that the M1/M2 classification is useful for illustrating the dichotomous role of microglia and TAMs in tumorogenesis but is ultimately an oversimplification, as TAMs and microglia exhibit a continuum of phenotypes at any one time, and the functional outcome may ultimately hinge upon the balance of pro-inflammatory and anti-inflammatory TAMs and microglia in the tumor microenvironment (159).

Recent work has produced convincing evidence that microglia and TAMs represent distinct classes of mononuclear phagocytic cells based on developmental origin (299, 300); however, distinguishing between TAMs and microglia in glioma tissue has proved difficult. Historically, cell-surface markers, CD11b integrin and common leukocyte antigen CD45, have been used to parse the two cell populations, with microglia expressing $CD11b^{high}/CD45^{low}$ and TAMs expressing $CD11b^{high}/CD45^{high}$, but the reliability of these markers in practice remains controversial (87). Newer genetic techniques employing inducible gene reporters to identify unique developmental markers in non-diseased murine models have had success in distinguishing monocyte-derived macrophages from microglia *in vivo* (301–303); however, whether such techniques can accurately identify macrophages and microglia in the setting of glioma remains to be determined. Therefore, the subsequent discussion will refer collectively to both macrophage populations as glioma-associated microglia and macrophages (GAMs) (304).

Glioma-Associated Microglia and Macrophages

Gliomas recruit GAMs in significant numbers, with GAMs comprising as much as one-third of all tumor-associated inflammatory cells (305). GAMs are recruited via glioma-derived chemo-attractants, including CCL2 (306), CCL7 (307), CX3CL1 (308), and stromal-derived factor-1 (SDF-1) (309); GAMs are subsequently sustained within tissue via glioma-derived growth factors, such as CSF-1, G-CSF, and hepatocyte growth factor (310–312). In exchange for pro-growth factors, GAMs provide the tumor with matrix metalloproteinases, which facilitate tumor growth and invasion (96), as well as tumor proliferation promoting factors, such as epidermal growth factor (EGF) (97) and vascular endothelial growth factor (VEGF) (98). Under the influence of glioma-associated cytokines, GAMs further upregulate immunosuppressive programmed death ligand 1 (PD-L1) (94, 95), which promotes T-lymphocyte anergy, as well as FASL, which promotes T-lymphocyte apoptosis (313, 314). Moreover, gliomas induce GAMs to substantially decrease the expression of MHC molecules and pro-inflammatory cytokines (TNF-α) while increasing the secretion of transcription factor, STAT3, likely through S100B-receptor for advanced glycation end produces (RAGE) axis (315). GAM STAT3 activation promotes the secretion of immunosuppressive cytokines, IL-6 and IL-10, which are known to inhibit cytotoxic T lymphocyte function, among other immunosuppressive actions (316, 317).

Discoveries surrounding the role of GAMs in promoting tumor growth have been followed closely by strategies to modulate their immunosuppressive actions. Transcription factor STAT3, which is upregulated in glioma-associated microglia, is a promising target for molecular intervention. *In vitro* blockade of STAT3 using siRNA reduced the microglial expression of immunosuppressive cytokines, IL-6 and IL-10 (316). *In vivo* silencing of STAT3 in a murine glioma model promoted a pro-inflammatory microglia response that inhibited tumor growth (316). Corosolic and oleanolic acids, known inhibitors of STAT3, have also been shown to reduce the macrophage expression of CD163, a marker of the immunosuppressive M2 phenotype, as well as IL-10, suggesting that these molecules may hold potential for reversing M2-like polarization of microglia (318, 319). Other novel approaches include the use of antibodies to block microglia chemotaxis toward gliomas, analogous to efforts aimed at attenuating Treg cell recruitment. Anti-CCL2 therapy, for example, has shown success in prolonging survival in murine glioma models (320). Other novel strategies have been well reviewed here (321).

Myeloid-Derived Suppressor Cells in Malignant Gliomas

Compared to TAMs and/or microglia, relatively less is known about the role of MDSCs in gliomagenesis and progression. MDSCs represent a diverse population of immature and highly immunosuppressive myeloid cells that accumulate in the tumor, blood, lymph nodes, and bone marrow of tumor-bearing hosts in response to tumor-derived factors, such as IL-6, IL-10, PGE2, TGF-β2, and VEGF (89, 322, 323). Though controversial, MDSCs are most commonly classified as either monocytic or granulocytic MDSCs (also known as polymorphonuclear MDSCs), with granulocytic MDSCs exerting weaker immunosuppression compared to monocytic MDSCs on a per cell basis (89). A population of pro-myelocytic MDSCs, representing an even more immature lineage of myeloid suppressor cells that are negative for both monocytic and granulocytic markers, has also more recently been described (324).

It is thought that granulocytic MDSCs suppress antigen-specific CD8+ T cell activity via production of reactive oxygen species (ROS), which, for example, could trigger apoptosis in activated T cells by decreasing Bcl-2 expression (325), while monocytic MDSCs increase L-arginine metabolism via NO and ARG-1 pathways, causing micro-environmental arginine depletion, ultimately leading to downregulation of T cell receptor components as well as T cell cell cycle arrest (326–328). Additionally, MDSCs are also thought to interfere with T-cell trafficking, induce NK- and T-cell anergy, and enhance Treg activation and expansion (329). In a study by Raychuadhuri et al., T cells isolated from patients with GBM had significantly depressed IFN-γ production following stimulation. Subsequent depletion of MDSCs from peripheral blood using anti-CD33/CD15-coated beads significantly restored T cell IFN-γ production *in vitro* (330).

In gliomas, the majority of circulating MDSCs appear to be predominately granulocytic (329). Interestingly, Gielen et al. recently reported that while patients with GBM contain elevated levels of both granulocytic and monocytic MDSCs in peripheral blood when compared to healthy controls, glioma tissues contain almost exclusively granulocytic MDSCs (331), a finding that may have important implications for MDSC-targeted therapy. In addition, the authors reported that patients who had had longer courses of dexamethasone for cerebral edema displayed greater levels of both classes of MDSCs in peripheral blood, a finding that could merely reflect patient-level differences in tumor mass but also possibly dexamethasone-mediated alterations to myeloid cell phenotypes, warranting further investigation (331). There is also compelling data suggesting that circulating MDSCs may arise from glioma-associated monocytes. Chae et al. recently showed that mice that received transgenic green fluorescent protein (GFP)+ CD11b+ splenic monocytes along with GL261-Luc cells not only had shorter survival, faster tumor growth, and higher levels of intratumoral and circulating MDSCs compared to mice that received GL261-Luc cells alone but also their work showed that 30–50% of circulating MDSCs were GFP+, suggesting that MDSCs arose directly from GFP+ monocytes (332).

Myeloid-derived suppressor cell-targeted immunotherapy is an area of active research. For example, various murine glioma models have shown that depletion of MDSCs, either via COX-2 inhibition (278), antibody-mediated MDSC depletion (278), or CCL2 neutralization (320), can prolong the survival. Other strategies for modulating MDSCs have been highlighted here (329).

Tumor-Associated Myeloid Cells in Non-CNS Sites

Immunosuppressive myeloid cells are not unique to CNS tumors and are equally important facilitators of tumor growth and invasion in peripheral sites as well. Higher density of tumor-associated macrophages (TAMs) has been associated with poorer prognosis in several human cancers, including breast, prostate, bladder, colorectal, and gastric cancers (333). Increased levels of M2-polarized TAMs have been correlated with accelerated metastasis and reduced survival in pancreatic (334) and renal cell carcinoma (88) as well as certain lymphomas (335). Indeed, several glioma-derived chemokine mediators that are important in re-purposing microglia with immunosuppressive functions are also implicated in polarizing peripheral tumor-associated macrophages toward an M2 immunosuppressive phenotype (99).

Although the relative distribution of microglia and TAMs in gliomas has yet to be fully characterized (see above discussion), an intriguing observation that the majority of tumor-infiltrating mononuclear phagocytes in murine gliomas may represent monocyte-derived macrophages rather than native microglia suggests that monocyte-derived macrophages may play a significant role in coordinating glioma growth (86). Immunotherapy aimed at modulating macrophage populations in the CNS may therefore be highly pertinent to managing immunosuppressive macrophages within non-CNS tumors, and vice versa.

Myeloid-derived suppressor cells have also been implicated in facilitating local and systemic immunosuppression in the setting of non-CNS tumors, including breast, colon, lung, kidney cancer, and head-and-neck cancers (89, 90), making MDSC-targeted therapy relevant to tumor immunotherapy at all sites. The mechanisms by which MDSCs arise and confound anti-tumor immunity, however, may differ depending on tumor site. For example, tumor-conditioned media from certain non-CNS tumors has been shown to induce immunosuppressive phenotypes in myeloid cells (322, 336); however, *in vitro* data from Rodrigues et al. revealed that direct contact between monocytes and glioma cells was needed to induce an MDSC-like phenotype in monocytes (90). Moreover, Rodrigues et al. failed to find a correlation between serum levels of tumor-derived cytokines known to stimulate MDSC proliferation in patients with gliomas compared to healthy counterparts (90), suggesting that the elevated levels of circulating MDSCs in patients with gliomas may arise from direct contact between tumor-infiltrating macrophages and/or monocytes and glioma cells rather than via systemic cytokine-induced conversion. Recent work from Chae et al., who showed GFP+ monocytes co-injected with GL261 cells into murine brains, led to increased levels of GFP+ MDSCs lends credence to the theory (332).

The relative proportions of circulating granulocytic, monocytic, and lineage-negative MDSCs may also vary depending on tumor type. Compared to patients with melanoma, renal cell carcinoma, and bladder carcinoma, patients with GBM had the greatest levels of granulocytic MDSCs (330). While the relative distribution of granulocytic, monocytic, and lineage-negative MDSCs in the peripheral blood of patients with renal cell carcinoma and bladder cancer appear consistent with that of GBM (i.e., granulocytic > lineage-negative > monocytic MDSCs), patients with melanoma have nearly equal percentages of granulocytic and lineage-negative MDSCs (330). The exact clinical relevance of differing proportions of MDSCs in different tumor types has yet to be elucidated; however, given that different subclasses of MDSCs may utilize different mechanisms of immunosuppression, MDSC-targeted immunotherapy may ultimately need to account for the predominant subsets of MDSCs associated with various tumor types.

Lastly, in keeping with the theme of other immunotherapeutic targets discussed in this review, targeting MDSCs may ultimately be highly contextual and tumor-dependent. Although depletion of MDSCs in certain glioma models has led to survival benefits

(278, 320, 337), eliminating MDSCs may produce opposite effect in other tumor models. For example, Kerkar et al. reported that IL-12 immunotherapy in a B16 murine melanoma model "reprogramed" MDSCs, which in turn actually potentiated the anti-tumor effects of CD8+ T cells (338). By comparison, IL-12 immunotherapy prolonged the survival in a GL261 murine glioma model regardless of whether MDSCs were depleted (339), indicating that MDSCs may play a different supporting role in IL-12 immunotherapy in melanomas versus gliomas. Further work is needed to ascertain the functional outcome of depleting MDSCs in different tumor models. Simultaneously, it will also be prudent to assess the viability of "reprograming" MDSCs into mature myeloid cells that promote tumor elimination, similar to what has been accomplished with using all-trans retinoic acid in the treatment of acute pro-myelocytic anemia.

Part VIII: Immune Checkpoints Molecules

Therapeutic modulation of co-stimulatory and co-inhibitory receptors of the immune system, often referred to as "immune checkpoint molecules," has erupted in recent years following the seminal work in blockading cytotoxic T-lymphocyte antigen 4 (CTLA-4), a co-inhibitory molecule expressed on activated T-cells and Treg cells. Ipililumab, an mAb directed against CTLA-4, was the first therapy to procure survival benefits for patients with metastatic melanoma (340), providing proof-of-concept that disrupting checkpoint molecules could alone reverse tumor immunoresistance and lead to immune-mediated tumor eradication.

Numerous immune checkpoint molecules have subsequently been identified (341) and hold substantial promise as targets for tumor immunotherapy in the brain and the body. In this regard, characterizing immune checkpoint molecule, programmed cell death protein-1 ligand (PD-L1), its role in immune regulation, and opportunities for therapeutic intervention in the CNS and other sites is particularly instructive.

Programmed Cell Death Protein-1 Ligand

Programmed cell death protein-1 ligand (B7-H1, CD274) is a trans-membrane glycoprotein of the B7 family of co-stimulatory molecules with potent immune-regulatory properties (120, 342). Under normal physiological states, PD-L1 is largely restricted to myeloid-lineage cells, including DCs and macrophages (343), and binds its receptor, programmed cell death protein-1 (PD-1, CD279), which is predominately expressed on activated T-cells (342, 344). Activation of PD-1 suppresses proliferation and lytic functions of effector T cells while expanding immunosuppressive Treg cells (341). Under inflammatory states, PD-L1/PD-1 signaling protects against rampant T cell activation and autoimmunity. Numerous tumors in the brain and the body, however, also express PD-L1, which can suppress tumor-directed cytotoxic T-cells that would otherwise destroy it (341). PD-L1 expression in several tumors, including renal cell carcinoma (105, 106), lung carcinoma (107, 108), breast carcinoma (109), and glioblastoma (119), has been correlated with higher tumor grade and poorer prognosis (120).

PD-L1: Malignant Gliomas

Aside from endothelial cells of the BBB, PD-L1 is usually not expressed in the CNS (345–347), and PD-L1 expression on glial cells and/or neurons is typically signs of pathological states. In gliomas, PD-L1 expression is positively correlated with malignancy grade (205) and is likely driven by genetic alterations that also potentiate oncogenesis. Loss of PTEN tumor suppressor gene enhances the expression of PD-L1 on glioma cells, suggesting that activation of PI(3)K-Akt-mammalian target of rapamycin (mTOR) pathway may modulate PD-L1 translation (101). Concurrently, greater degrees of PD-1 expression on peripheral CD4+ and CD8+ T cells are also observed in gliomas of higher malignant grade (119), and co-culturing alloreactive CD4+ and CD8+ T cells with PD-L1-expressing glioma cells significantly depresses the production of inflammatory cytokines, such as IFN-γ and IL-2 (118). Accordingly, the PD-1/PD-L1 axis has become an attractive target for glioma immunotherapy. Blocking PD-L1 on glioma cells with mAbs in combination with radiotherapy has yielded particularly potent survival benefits in pre-clinical models (348).

In addition to glioma cells, TAMCs provide another source of PD-L1. Microglia are known to upregulate PD-L1 expression under inflammatory states (94), and microglial expression of PD-L1 has indeed been reported in human glioblastoma tissue (102). Recently, Parsa et al. reported that gliomas also induce PD-L1 expression on tumor-infiltrating macrophages, which may further contribute to PD-1-mediated T-cell suppression (103). This finding is particularly compelling, as it provides a cellular basis by which gliomas may induce global immunosuppression via monocyte-derived macrophages. Whether the benefits observed from anti-PD-L1 mAbs are due primarily to blocking PD-L1 expressed by gliomas, TAMs, microglia, or combinations thereof, remains to be determined.

Notably, in the first study that extensively characterized the role of neurons in the GBM microenvironment in inhibiting tumor growth, Liu et al. reported that high levels of neuronal PD-L1 expression in tumor-adjacent brain tissue (TABT) corresponded favorably with overall patient survival, and low TABT PD-L1 expression and high GBM PD-L1 expression portended poorer patient survival (104). Mechanistic analysis revealed neurons expressing PD-L1-induced caspase-mediated apoptosis of GBM cells (104). Neuronal expression of IFN-β, which enhances neuronal expression of PD-L1, was also postulated to suppress glioma growth via its tumor-suppressor functions (104). These findings illuminate the potential drawbacks of indiscriminate administration of PD-L1 neutralizing antibodies, which might limit native host neuron defenses against gliomagenesis.

PD-L1: Non-CNS Tumors and Implications for Therapy

The biological pathways implicated in enhancing PD-L1 expression in gliomas are equally important to the development of immunoresistance in tumors outside of the CNS. Expression of PD-L1 on colorectal carcinomas, for example, has also been linked to loss of PTEN tumor suppressor (349). Similarly, PI3K/mTOR pathway activation is also associated with PD-L1 expression in

breast (115), lung (108), renal cell (106), and prostate carcinomas (116). Molecular therapies targeting these pathways are therefore relevant to all of these tumors.

At the same time, other malignancies also utilize distinct signaling pathways to enhance PD-L1 expression, requiring that targeted molecular suppression of PD-L1 be tailored toward each site based on tumor-specific biology. For example, MyD88/TRAF6 and MEK/ERK pathways enhance PD-L1 expression in multiple myeloma (117), while constitutive activation of anaplastic lymphoma kinase (ALK) drives PD-L1 expression in certain lymphomas and lung carcinomas (341, 350).

Targeting the PD-L1/PD-1 axis by indirectly modulating PD-L1-bearing TAMs may also prove to be relevant strategy for CNS and non-CNS tumors alike (100, 110). Hepatocellular carcinomas, like gliomas, also recruit high numbers of PD-L1-expressing TAMs into the tumor microenvironment, corresponding with poor prognosis (111).

Lastly, the astonishing discovery that neuronal expression of PD-L1 in the tumor microenvironment protects against gliomagenesis (104) may also find parallels in the body. Pancreatic, heart, endothelial, small intestine (112), and placental tissues (113) also express PD-L1 (114). Whether tissue expression of PD-L1 at sites outside of the CNS similarly protects against local tumorogenesis remains an open question.

Beyond PD-L1

Beyond PD-L1, effector immune cells express myriad checkpoint molecules that also contribute tumor immunoresistance both in brain and body. Targeting cytotoxic T-lymphocyte antigen 4 (CTLA-4), which modulates early stages of T-lymphocyte activation, has proved useful in reversing immunoresistance in gliomas, as it has in non-CNS tumors (351–355). In an experimental glioma model, anti-CTLA-4 mAb procured an 80% long-term survival rate, concurrent with enhanced proliferation of CD4+ CD25− T cells and resistance to suppression by Treg cells (351). Glucocorticoid-induced tumor necrosis factor receptor-related gene (GITR) has emerged as an important checkpoint molecule in Treg cells (356). Additionally, Tim-3 (357) and 4-1BB (CD137) (358) are other key immune checkpoint molecules that will also require site-specific considerations, especially as immunotherapeutic strategies develop to target these ligands individually and in combination (68).

Part IX: Concluding Remarks

A major challenge for the field of brain tumor immunology lies in elucidating key determinants and constituents of the pro-inflammatory and anti-inflammatory responses in the CNS such that they might be augmented for therapeutic gain. Contrary to the historical view of the CNS as immunologically sequestered from the rest of the body, the immune responses in the CNS are linked and complementary to immune processes in the periphery. The phenotype of the immune response often hinges upon cytokines and cellular mediators that exert highly pleiotropic and sometimes paradoxical actions depending on the specific tumor and environmental context. In evaluating these processes, however, it will be helpful to recognize that routes by which the CNS coordinates immune modulation are not without precedent: analogous immunological mechanisms exist at sites outside of the CNS, and advances in tumor immunotherapy in peripheral sites may therefore illuminate novel approaches to brain tumor immunotherapy, and vice versa (1). Therefore, this suggests that the intricacies of the brain immune environment need to be examined within the context of the entire body.

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OPINION
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Systemic Immunotherapy for the Treatment of Brain Metastases

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BACKGROUND

Significant progress has been made in the treatment of selected malignancies with immune-modulating antibodies. Phase III trials of anti-CTLA-4 in melanoma and anti-PD-1 in melanoma, renal cell carcinoma (RCC), and non-small cell lung cancer (NSCLC) showed improved overall survival (OS) compared to standard therapies (1–5). As a result, immune checkpoint inhibitors are now approved for the treatment of these diseases. Blockade of CTLA-4 (ipilumimab and tremelimumab), PD-1 (nivolumab, pembrolizumab, pidilizumab and others), and PD-L1 [BMS 936559 (6), durvalimumab (7), and atezolizumabes (8–11)] can produce durable responses in patients with metastatic cancer. Clinical trials with these agents, alone and in combination, are ongoing. Moreover, additional immune checkpoint modulators are in pre-clinical and clinical development. Other approved immunotherapies include high-dose bolus interleukin-2 (IL-2), interferon alpha-2b, and Sipuleucel-T. There are limited data, however, on the impact of immunotherapy in patients with measurable metastatic disease to the brain. Registration trials of immune therapies excluded patients with active brain metastases based on a historical poor prognosis in this patient population coupled with uncertainty about the ability of the drugs to cross the blood brain barrier (BBB). These active therapies might however have benefited patients with microscopic brain deposits.

Brain metastases were historically managed with whole brain radiation therapy (WBRT) or surgical resection, depending on the size, number, histology, symptoms, and location. The availability of high-resolution magnetic resonance imaging (MRI) and stereotactic radiosurgery (SRS) to small, emerging lesions has improved local lesional control. These modalities allow higher doses of radiation. In many institutions, WBRT is reserved for patients with multiple or larger lesions not amenable to SRS (12, 13). These treatments are not without limitations and consequences. For example, WBRT has been associated with cognitive decline, while SRS can result in radiation necrosis, cerebral edema, and delayed tumor hemorrhage (14, 15). More often, however, focal therapies are limited in efficacy due to distant cerebral relapse and lack of treatment of microscopic tumor foci not evident on imaging. As new systemic treatments, particularly immune-modulating agents, show prolonged survival of patients with aggressive extra-cerebral disease, these drugs need to be assessed for efficacy in active brain metastases. There are a number of ongoing investigations to determine if these antibodies cross the leaky BBB found in tumors despite their size (16, 17). Alternatively, although brain metastases might contain pre-existing tumor infiltrating lymphocytes (TILs), immune modulation induced by these agents may allow cytotoxic T cells into the tumor microenvironment in the brain, resulting in antitumor immunity. Several lines of evidence suggest that T cells within the tumor microenvironment are responsible for the responses seen with these therapies (18, 19). To date, there have been no published pharmacokinetic or pharmacodynamic studies in on-treatment brain tissue to allow determination of drug penetration into the tumor, primarily due to the difficulty accruing patients to trials requiring brain biopsies, particularly from patients who are responding to therapy. Although animal studies have been done, drug distribution and T cell activation might not reflect that of humans.

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Metastatic melanoma is the solid tumor with the highest propensity for dissemination to the brain (20). The only chemotherapy widely used for melanoma known to definitively cross BBB is temozolamide, which induced responses in 7% of melanoma brain metastasis patients (21). Other anti-neoplastic drugs that cross the BBB include fotemustine, etoposide, cisplatin, vinblastine, and motoxantrone and can be used depending on tumor cell sensitivity (22–26). Targeted therapies such as erlotinib, afatinib, and lapatinib have also shown evidence of ability to cross the BBB (27–29).

PRECLINICAL DATA

The ability of immune-modulating antibodies to cross the BBB and control brain metastases is the subject of ongoing investigations. In primary CNS tumors, preclinical data with immune-modulating antibodies have shown promise. In mice with SMA-650 intracranial tumors, anti-CTLA-4 was tolerated well (30). An increase in CD4+ cells and decrease in T regs prolonged survival in these animals. Similarly, PD-1 blockade combined with radiation was tested in mice with GL261 intracranial tumors and showed improved survival (31). The combination of PD-1 and CTLA-4 inhibitors similarly showed improved survival in animal models (32). These examples suggest that BBB drug penetration in tumors might be obtainable, for primary CNS tumors and for metastatic tumors, although this remains to be verified in humans with each drug and tumor type.

CLINICAL DATA

High-dose IL-2 was one of the first immune-modulating agents to demonstrate activity in melanoma and RCC. There have not been any formal trials of IL-2 specifically for patients with brain metastases. A retrospective series reported a response rate in active brain metastases lower than expected for extra-cerebral disease, however without excessive toxicities (33). One of the first studies to investigate the effect of immunotherapy on brain metastases in patients with metastatic melanoma was a retrospective analysis of the phase II trial with ipilimumab, which reported 5 of 12 patients were responders (34, 35). Following this observation, a phase II trial of ipilimumab specifically for patients with brain metastases from melanoma opened (36). Results of 72 patients accrued showed prolonged OS, particularly notable in asymptomatic patients. These findings were confirmed in an expanded access protocol of ipilimumab with a 20% 1-year OS in patients with stable, asymptomatic brain metastases (37). Based on these promising results, the Italian Network for Tumor Biotherapy (NIBIT) designed a phase II trial of ipilimumab in combination with fotemustine (NIBIT-M1) with twenty asymptomatic patients with brain metastases. Stable disease or partial response was seen in 25% and another 25% had complete response in the brain (38, 39).

A follow-up randomized trial (NIBIT-M2) was subsequently initiated for patients with untreated melanoma brain metastases comparing fotemustine monotherapy, fotemustine plus ipilimumab 10 mg/kg and ipilimumab 3 mg/kg + nivolumab 10 mg/kg (NCT02460068). Objectives include OS, safety, disease control rate (intra and extra-cerebral) objective response rate, duration of response, and progression-free survival. This study will also examine quality of life. Various groups are studying the effect of immune-modulating agents alone and in combination with other therapies for the treatment of brain metastases from melanoma. For example, ipilimumab and nivolumab or nivolumab monotherapy is being studied in a large multi-arm phase II trial (NCT02320058 and NCT02374242) and combinations of ipilimumab with various forms and schedules of radiation are being investigated (NCT01703507, NCT01950195 and NCT02097732). Results of these trials are pending.

A phase II trial of pembrolizumab for patients with metastatic melanoma or NSCLC and untreated brain metastases is ongoing. Preliminary results from this trial were presented at ASCO 2015 (NCT02085070) (40, 41). In this two-arm study, patients are eligible if they have at least 1 untreated or progressive brain metastasis (5–20 mm), not requiring steroids and are without neurological symptoms. Patients in the melanoma arm require brain metastasis biopsy or resection of metastatic brain lesion prior to starting therapy or availability of previously resected brain lesions for correlative studies. Patients in the NSCLC arm are required to have PD-L1 positive tumors. In the NSCLC arm, 11 patients were evaluable for response as of June 2015. Brain metastasis response rate was 45%, and systemic response rate was 45%. Only one patient with a systemic response had disease progression in the brain, and two patients with disease progression as their best systemic response were unevaluable in the brain due to rapid systemic progression. The duration of response in the brain was at least 12 weeks for four of five responders, and all responses were ongoing at the time of data analysis (40). In the melanoma arm, 18 patients were accrued at the time of analysis. Four patients were unevaluable due to rapid extra-cerebral progression or hemorrhage, and one was too early for response evaluation. Four patients achieved partial response, three had stable disease, and seven had disease progression (two with mixed response and one with histologically demonstrated pseudoprogression). Response in the body was largely concordant with brain response, although in some cases brain response occurred after extracerebral response. Response in the brain was ongoing at 4+, 6+, 6+, and 11+ months (41).

Studies completed to date suggest that immune checkpoint inhibitors have activity in the brain that might be similar to that of extra-cerebral sites (42). In the phase II study of ipilimumab brain metastases activity in asymptomatic patients was similar to that of patients without brain metastases with a disease control rate of 24 and 27%, respectively. The 1- and 2-year progression-free survival were 31 and 26%, respectively (36, 43). The NIBIT-M1 study described above confirmed these findings with an immune-related disease control rate for patients with brain metastases of 50% compared with 46.5% of the entire treated population. Interim data from our phase II trial of pembrolizumab in patients with metastatic melanoma and NSCLC with untreated brain metastases showed that all responses in the melanoma arm were concordant, while three or four in the NSCLC arm were concordant (40, 41). Results suggest that immune-modulating agents may have similar durable responses in the brain as seen systemically, and support use of systemic therapy alone or in combination with

focal therapy (SRS or surgery) in the treatment of brain metastases from immune therapy responsive diseases such as melanoma and lung cancer.

There are data to suggest that responses might be further improved by combining immune checkpoint inhibitors with radiation. Several studies have evaluated the combination in other disease sites (44–47). A number of mechanisms have been described explaining the combined effect; radiation upregulates inflammatory cytokines (i.e., TNFα, IFN-γ, and CXCL16), promoting tumor detection and facilitating T cell infiltration (48, 49). Radiation can upregulate PD-L1 (50). The abscopal effect, in which local radiation is thought to cause a systemic response resulting in shrinkage at distant sites, further supports the use of radiation combined with immune-modulating agents (51). Knisely et al. published a series of patients with metastatic melanoma with brain metastases who achieved a median survival of 21.3 months if they received ipilimumab and SRS versus 4.9 months if they underwent SRS but did not receive ipilimumab (44). Mathew et al. looked at a similar population with 25 patients receiving both ipilimumab and SRS versus 33 patients receiving SRS alone (46). The analysis did not show a significant benefit in 6-month OS between the two groups, although this was not a randomized trial and the groups were not balanced. Lastly, Silk et al. reported improved OS in patients receiving ipilimumab and SRS (47). Exploratory analysis within the same study showed no increase in OS with the addition of ipilimumab to WBRT. The timing of administration of concurrent immune checkpoint inhibitors and radiation has not yet been determined. Kiess et al. found increased rates of progression if patients were treated with SRS before or during ipilimumab compared with those who received SRS after systemic therapy (52). Future studies will provide insight into the optimal timing for combining radiation and immune-modulating therapies, such as NCT02097732, which is investigating SRS to brain metastases before or in the middle of ipilimumab induction.

Toxicities unique to central nervous system metastases, such as vasogenic edema and tumor necrosis represent an additional challenge. Early recognition of potential symptoms is essential. One of the challenges in treating brain metastasis patients with immune therapy is management of neurological symptoms, which might be from perilesional edema, intralesional hemorrhage, necrosis most commonly seen in previously irradiated lesions, or tumor growth due to treatment failure. Examples of perilesional edema seen on FLAIR images before and on therapy in two patients receiving pembrolizumab are shown in **Figure 1**. Both patients responded well to transient steroids and remain on pembrolizumab with good disease control for over a year. Depending on the size and location of the brain metastasis, patients might require surgical intervention due to neurologic symptoms. Moreover, it is sometimes impossible to determine whether lesions enlarge on study due to inflammation, necrosis, or tumor growth, and current imaging modalities can be inadequate (53). Our institutional experience suggests that despite the indisputable benefit of systemic immune therapy in some tumor types, radiation necrosis occurs with greater frequency in patients treated with immunotherapy than other types of systemic therapy. We, and others, have used bevacizumab to control perilesional edema and worsening radiation necrosis, with variable success, and surgical intervention or laser interstitial thermacoagulation therapy is sometimes needed although caution must be taken with histologies more prone to hemorrhage (54–59). Furthermore, the incidence of seizures from perilesional edema might be decreased with use of prophylactic anti-epileptic medications.

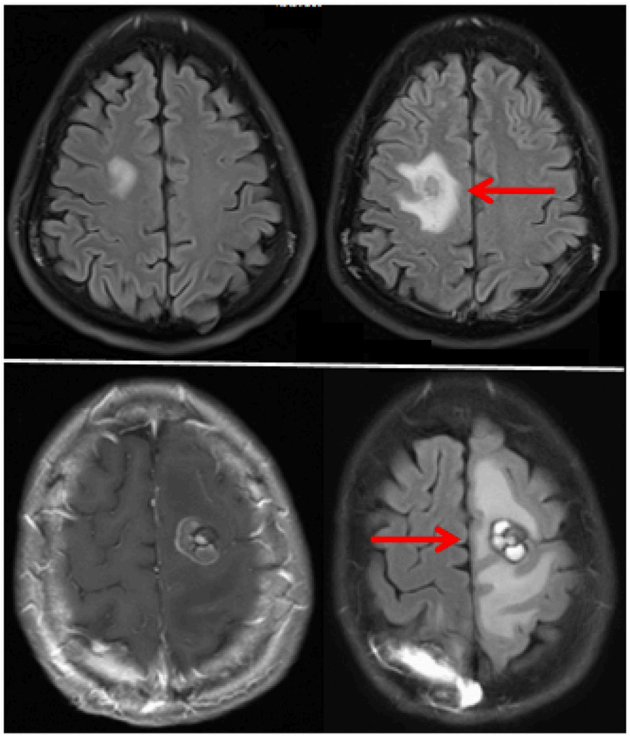

FIGURE 1 | MRI FLAIR images of two patients with perilesional while receiving pembrolizumab. The top and bottom frames represent the two separate patients. Images prior to therapy are on the left and after therapy on the right.

FUTURE DIRECTIONS AND CONCLUSION

Use of immune therapy for non-irradiated brain metastases has shown promise in a small number of clinical trials, and requires validation in larger studies and in different tumor types. Experience to date suggests that activity of immune checkpoint inhibitors in brain metastases is similar to that of extracerebral metastases, and exclusion of patients with brain metastases from clinical investigations is no longer justified, although separate studies or separate cohorts for patients with untreated brain metastases might be required. Challenges with treating this patient population include drug-related toxicities such as perilesional edema and tumor-related confounding factors such as necrosis in previously irradiated lesions and intralesional hemorrhage, both of which might require intervention with local or systemic modalities such as surgery, radiation, anticonvulsants, steroids, or VEGF inhibitors. Efficacy of immune checkpoint inhibitors might be further enhanced by combining more than one inhibitor

or with combinations with chemotherapy, targeted therapy, or radiation therapy. As the breadth of immunotherapies available for investigation and use expands, predictive biomarkers will also need to be studied and validated. This can be particularly challenging in patients with brain metastases due to the morbidity associated with biopsy; however, if concordance of response is persistently observed as newer drugs are studied in this patient population, extra-cerebral biopsies might suffice. Clinical trials designed specifically for this patient population addressing the effects of multi-modality therapy, particularly combinations of immune checkpoint inhibitors and radiation, are necessary for improving outcomes among individuals with brain metastases.

AUTHOR CONTRIBUTIONS

JC and HK wrote this article together.

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OPINION
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Is the concept of central nervous system immune privilege irrelevant in the setting of acute infection?

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Keywords: central nervous system, infection, host immunity, viruses, bacteria

While historically viewed as an immune-privileged area fully isolated from the immune system, the central nervous system (CNS) is now appreciated to maintain dynamic bi-directional communication with the immune system across the blood–brain barrier (BBB) (1, 2). In no setting can this communication be more urgent that acute CNS infection – a dampened or delayed host response could allow an invading virus or bacterium to gain a foothold within the brain, while over-exuberant or protracted inflammation might cause substantial collateral damage to sensitive and non-renewable cells such as neurons. In this opinion piece, we compare host immunity against one prototype virus and one prototype bacterium known to cause disease either outside or within the CNS. Allowing for some variability in disease pathogenesis, and leaving aside complex issues related to chronic intrauterine or neonatal infections, we argue that antimicrobial host responses in both CNS and non-CNS tissue compartments of adult hosts who acquire these infections exhibit many more similarities than differences. In this setting, the concept of CNS immune privilege seems antiquated.

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Immune Surveillance of the Normal CNS and Mobilization of Host Responses During Acute CNS Infection

It is now accepted that there is a need for constant immune surveillance of the adult CNS as part of normal host defense, acknowledging that simultaneous mechanisms must keep local CNS inflammation strongly in check (1). Indeed, blockade of normal lymphocyte homing through the CNS can occasionally trigger local virus recrudescence (3), and low numbers of lymphocytes and antigen-presenting dendritic cells (DC) are found in perivascular spaces of the normal brain (4). For infectious particles that unexpectedly gain access to the CNS, some pathogen clearance occurs via bulk flow along paravenous routes by means of a process that depends on astrocytic water channels (4). This so-called "glymphatic system" of the brain ultimately carries antigenic material toward a specific group of large-caliber veins that drain to deep cervical lymph nodes (CLN) (5, 6). The CLN are increasingly appreciated as an important site where antigen-specific immune responses bound for the CNS are generated (7). Blood-borne pathogens, on the other hand, are mostly carried to the spleen where adaptive immunity occurs. Immune cells then migrate back to the CNS under the influence of chemokine gradients induced by infection, and bind and traverse the BBB via the actions of specific adhesion receptors and degradative enzymes.

Infections in the Periphery or the CNS Caused by the Same Pathogen – How Much do Host Responses Differ?

Streptococcus pneumoniae is an important pathogen because it is the main cause of both community-acquired pneumonia and meningitis induced by a bacterial pathogen in otherwise healthy older

TABLE 1 | Pathogenesis and host responses elicited during a prototype bacterial (*Streptococcus pneumoniae*) or viral (lymphocytic choriomeningitis virus) infection of adult hosts when localized in either the periphery or the CNS [data adapted from Ref. (10–15)][a].

	Streptococcus pneumoniae		Lymphocytic choriomeningitis virus	
	Pneumonia	**Meningitis**	**Visceral infection (liver, spleen)**	**Meningitis**
Natural routes of infection (humans)	Inhalation Local spread from nasopharyngeal colonization	Inhalation Local spread from an infected sinus or inner ear	Inhalation Direct contact with infected rodents Direct inoculation via infected solid organ transplant	Inhalation Direct contact with infected rodents Direct inoculation via infected solid organ transplant
Experimental routes of infection (mice)	Intranasal	Intranasal Intracisternal	Intravenous Intraperitoneal	Intracranial
Innate immune receptors activated	TLR2, TLR4, TLR9, NOD2, NRLP3	Unknown	TLR2, PKR, RLR, TLR7, MDA5	TLR2, CXCR3
Early innate immune mediators induced	IL-1β, TNF-α, IL-6	IL-1β, TNF-α, IL-6	IFN-α/β, TNF-α, IL-6, IL-10, CCL2, CCL5, CXCL10	IFN-α/β, CCL2, CCL3, CCL5, CXCL10
Site of main adaptive immune priming	Hilar/mediastinal lymph nodes Spleen	Cervical lymph nodes Spleen	Spleen Mesenteric lymph nodes	Spleen Cervical lymph nodes
Principal effector cells activated and mobilized	Neutrophils Monocytes Dendritic cells Lymphocytes	Neutrophils Monocytes Dendritic cells Lymphocytes	CD8+ CTL NK cells Dendritic cells	CD8+ CTL Monocytes
Time to mobilize immune cells to target tissue	Hours	Hours	Hours–days	Hours–days
Soluble immune mediators involved in pathogen containment and/or clearance	IL-1β, TNF-α, NO, complement C1, IL-10	TNF-α, ROS, NO	IFN-α/β	IFN-α/β, CXCL10, IFN-γ
Mechanisms of pathogen clearance	Phagocytosis Neutrophil oxidative burst Complement activation	Phagocytosis Neutrophil oxidative burst Complement activation	Virus-specific CTL	Virus-specific CTL
Other relevant immune features	Disease severity and complications higher in asplenic individuals (humans) IκB and IL-10 polymorphisms raise susceptibility (humans)	Intracranial complications more common in asplenic individuals (humans)	Vicerotropic viral strains may cause chronic infection and immunosuppression via CTL exhaustion (mice)	No evidence of chronic CNS infection (humans)
Potential for target tissue immunopathology (humans)	Moderate (10% overall mortality)	High (75% develop intracranial complications, 25% mortality)	Low (healthy adults) High (immunocompromised organ transplant recipients)	Low (healthy adults) High (immunocompromised organ transplant recipients)
Potential for target tissue immunopathology (Mice)	High (most models cause lethal disease with extensive lung damage)	High	Moderate (adult mice)	High (adult mice infected with naturally occurring Armstrong strain)
Effectors of target tissue immunopathology	Lipocalin-2, NO, malondialdehyde, IL-1β, TNF-α	IFN-γ, TNF-α, glutamate, NO, ROS, caspase-9/3, myeloperoxidase	Virus-specific CTL, perforin	Virus-specific CTL, perforin
Role of immunotherapy in improved disease outcome	No proven role to date (humans) IVIG, MALP-2, and pneumococcal P4 peptide all improve survival (mice)	Corticosteroids of limited benefit to prevent hearing loss (humans) Inhibitors of caspases, ROS, IDO, kynurenine pathway improve cognitive outcomes (mice)	No proven role to date (humans)	No proven role to date (humans) Virus-specific CTL plus virus-specific CD4+ T cells can clear persistent infection following adoptive transfer (mice)

[a] *Includes studies where peripheral and CNS responses were not directly compared, and therefore differences may reflect how the individual studies were conducted and what parameters were examined.*

CCL, C–C motif ligand; CTL, cytotoxic T lymphocyte; CXCL, C–X–C motif ligand; CXCR, C–X–C motif receptor; IDO, indoleamine 2,3-dioxygenase; IFN, interferon; IL, interleukin; IVIG, intravenous immune globulin; MALP, macrophage-activating lipopeptide; MDA, melanoma differentiation-associated protein; NO, nitric oxide; NOD, nucleotide-binding oligomerization domain-containing protein; NRLP, NOD-like receptor family, pyrin domain-containing; PKR, protein kinase R; RLR, RIG-I-like receptor; ROS, reactive oxygen species; TLR, Toll-like receptor; TNF, tumor necrosis factor.

children and adults. Much has also been learned about host immune responses elicited by pneumococcal pneumonia or meningitis using mouse models (8, 9). These infections develop in both mice and humans following pathogen inhalation and subsequent local tissue invasion (**Table 1**). Tissue-resident innate immune pathways are activated and host immunity is mobilized within hours. Outcome is determined over days to a few weeks. Morbidity and mortality, even in previously healthy hosts, is substantial, as many of the same mediators that have antibacterial activities also cause direct cellular damage (proteins, membrane lipids, DNA). Current polyvalent vaccines are effective in preventing both forms of invasive disease.

Lymphocytic choriomeningitis virus (LCMV) is an arenavirus to which both mice and humans are susceptible and that causes varying combinations of visceral (hepatitis, pancreatitis, myocarditis) and/or CNS (meningitis, encephalitis) involvement in adult hosts. Murine LCMV infection has served as a prototype experimental system to study immunity to viruses for many years (10). This pathogen gets inhaled or directly inoculated into susceptible murine and human recipients (**Table 1**). Both innate and adaptive immune pathways are mobilized and disease can last several weeks. Healthy adults generally recover reasonably well, although the disease is more fulminant in immunocompromised hosts. In mice, the same virus-specific cytotoxic T cell (CTL) response that clears virus from infected tissues also causes tissue immunopathology. For acute CNS infection (choriomeningitis) in the setting of impaired CTL activity, survival is the trade-off for poor viral clearance.

The host responses provoked by these two naturally occurring pathogens are used here to compare how the immune system recognizes and responds to the same challenge in the periphery and the CNS (**Table 1**). The availability of mouse models for both pathogens that can be manipulated to cause infection either outside or within the CNS allows for the identification of immune determinants that directly influence disease outcome. It seems notable that many more similarities than differences in host immunity are seen, and that there is little evidence suggesting immune recognition is delayed or immune responses dampened for those infections localized primarily to the CNS compartment. Outcome differences for infections occurring at the two sites are likely driven by mechanisms independent of the host response.

Conclusion

Both the afferent and efferent arms of the immune system are efficiently activated in the context of either pneumococcal or LCMV infection, and the kinetics, magnitude, and composition of immune responses elicited in adult hosts appear remarkably similar when either a visceral organ or the CNS is the main target of disease (**Table 1**). Acute CNS infections generally result in less favorable outcomes than those localized to the periphery, but this is more likely explained by the exquisite sensitivity of the brain to cellular damage rather than any delayed immune recognition behind the BBB. This comparison leads us to suggest that the concept of CNS immune privilege in the adult host seems somewhat obsolete in the setting of acute CNS infection.

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Immune responses to non-tumor antigens in the central nervous system

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The central nervous system (CNS), once viewed as an immune-privileged site protected by the blood–brain barrier (BBB), is now known to be a dynamic immunological environment through which immune cells migrate to prevent and respond to events such as localized infection. During these responses, endogenous glial cells, including astrocytes and microglia, become highly reactive and may secrete inflammatory mediators that regulate BBB permeability and recruit additional circulating immune cells. Here, we discuss the various roles played by astrocytes, microglia, and infiltrating immune cells during host immunity to non-tumor antigens in the CNS, focusing first on bacterial and viral infections, and then turning to responses directed against self-antigens in the setting of CNS autoimmunity.

Keywords: neuroimmunology, non-tumor antigens, glial cells, CNS autoimmunity, blood–brain barrier, CNS infections

INTRODUCTION

The central nervous system (CNS) was previously viewed as an immune-privileged area, fully isolated from the immune system by the blood–brain barrier (BBB). In early studies, Ehrlich reported that while various organs were strongly stained following intravenous, intra-arterial, or subcutaneous injection of intravital dyes, the brain was only weakly stained or not at all (1). Other studies found that tissue grafts were not rejected when implanted into the brains of test animals (2), leading to the idea that the CNS was fully "immune-privileged." This viewpoint had to be altered, however, after it was discovered that a graft within the CNS could be rejected if a second graft was placed subcutaneously into the same animal (3). This finding clearly demonstrated that foreign antigens are recognized in the CNS if peripheral priming occurs (3). It is now accepted that the BBB is a dynamic, interactive, and regulatory tissue interface that allows bi-directional communication between the CNS and the immune system (4, 5).

The BBB, formed by complex interactions between capillary endothelial cells (ECs), astrocyte end-feet, pericytes, and microglia (6, 7), is the largest and most stringent barrier that impedes the paracellular movement of ions, solutes, proteins, water, and leukocytes into the CNS (8). However, the BBB can also be influenced by peripheral immune events, creating what has now come to be known as the neuro-immune axis (4, 9, 10). The neuro-immune axis is not only responsible for establishing the blood–CNS barrier at baseline, but it also regulates communication between the CNS and the immune system during pathological conditions such as viral or bacterial infections, ischemia, or inflammatory autoimmune disorders such as multiple sclerosis (MS) (11). It achieves this state by responding to secreted factors from both immune and CNS cells, as well as by regulating the exchange of chemokines, cytokines, and immune cells between the blood and the CNS (4, 9, 10). Therefore, the original concept of the BBB being a purely anatomical barrier has now shifted to one where the BBB is considered a highly reactive interface controlled by signals from ECs, glial cells, pericytes, and neurons in the CNS, as well as from immune responses in the periphery (12–21).

STRUCTURAL CHARACTERISTICS OF THE BBB

The BBB is composed of capillary ECs ensheathed by astrocyte end-feet, pericytes, and microglia (6, 7). Astrocyte end-feet completely surround the abluminal surface of brain capillaries forming a layer known as the glial limitans, but direct contact with EC is inhibited by a dense basement membrane (22). While astrocytes are necessary to maintain BBB integrity by secreting factors that alter barrier permeability (6, 23), they are not actually required to form the BBB, which develops even before these astrocytic processes are present (24–26). Astrocytes control blood flow to the CNS by regulating vascular tone through fluctuating calcium currents (27). Pericytes are essential to barrier formation, as the BBB is compromised in pericyte-deficient mice (28, 29). These cells regulate gene expression in EC and induce the polarization of astrocyte end-feet (28). Microglia play a role at the BBB by regulating substrate transport across EC and by linking the brain to systemic immune activity (30).

Blood–brain barrier EC forms a highly sophisticated barrier via a network of tight junctions (TJ) and adherens junctions (AJ) (8, 31, 32). The EC of the CNS are unique in that the TJ restrict the paracellular passage of solutes, have no pinocytic activity, and have few if any fenestrations (33–39). This causes the BBB to have high endothelial electrical resistance (40, 41), some 50–100 times higher than peripheral microvessels (42–44). The TJ are composed of a parallel network of intramembranous protein strands, composed of claudins, occludin, and zonula occludin (ZO) proteins (37). Claudins, specifically claudin-3, -4, and -12, compose the TJ backbone (45–47). Occludin is not required for TJ formation (48);

instead, it plays a role in "sealing" the junction thereby increasing electrical resistance (49, 50). CNS microvessel TJ are also abundant in ZO-2, and to a lesser extent, ZO-1, that are cytoplasmic accessory proteins that serve to anchor the transmembrane proteins of the TJ to the actin cytoskeleton of the ECs (51, 52).

The choroid plexus (CP) is a villous structure located on the roof of the four cerebral ventricles where cerebrospinal fluid (CSF) is actively secreted. The CP is highly vascular and contains the blood–CSF barrier (BCSFB) (51). Unlike the BBB, however, the BCSFB arises from cuboidal choroid plexus epithelial cells (CPE) with a very different TJ structure. The CPE express ZO-1 and ZO-2 in different amounts (51), and have a different claudin signature, expressing claudin-1, -2, -3, and -11 (51, 53, 54). Furthermore, capillaries within the CP villi are fenestrated (51, 55, 56), reflected by a much lower endothelial electrical resistance than the BBB (57). For these reasons, the BBB is considered more of an absolute barrier, while the BCSFB may be where most normal immune surveillance of the CNS occurs (58).

IMMUNE SURVEILLANCE AND INFILTRATION OF THE CNS

It is now accepted there is a constant need for immune surveillance of the normal CNS as part of host defense (11, 59, 60), with mechanisms present that simultaneously keep excessive inflammation in check (61). To assist in maintaining this control, the healthy CNS is relatively devoid of antigen-presenting cells (APC), lacks constitutive human leukocyte antigen (HLA) class I and II protein expression on parenchymal cells, and does not maintain typical lymphatic vessels (11). CD4+ T cells, having first encountered antigens in deep cervical lymph nodes (62), carry out routine surveillance of the CNS by searching for their cognate antigens presented by macrophages in the CSF (11, 63). Resting lymphocytes fail to enter the CNS (64), while activated T cells of all specificities can traverse the BBB and/or BCSFB (65). Those cells that do not encounter their cognate antigen within a few hours then circulate out of the CNS (66, 67).

The first steps of pathogenic neuroinflammation involve changes at the BBB, including increased production of chemokines and up-regulation of adhesion molecules by the EC resulting in leukocytes traversing the BBB and accumulating in the perivascular space of post-capillary venules (11, 68). Even during these early events, however, cellular recruitment remains tightly controlled as parenchymal lymphocytes express a unique adhesion molecule profile, different from peripheral T cells (69–71). Once in the perivascular space, T cells encounter the glial limitans as well as astrocytes that express and release factors that induce apoptosis (72), inhibit proliferation (72), induce differentiation into a regulatory (Treg) phenotype (73). Microglia and neurons also assist in controlling neuroinflammation. Microglia do so by expressing a homolog of the co-stimulatory molecule B7, programed death protein (PD)-1, which negatively regulates T cell activation and cytokine production (74). Neurons secrete transforming growth factor (TGF)-β, exert cell contact-dependent effects that support the conversion of CD4 T cells to Tregs, and can be induced to express the PD-1 ligand, PD-L1 (75). Thus, while the BBB is not the impenetrable barrier it was once thought to be, CD4+ T cell surveillance of the CNS is still a tightly controlled process.

HOST IMMUNE RESPONSES TO BACTERIAL INFECTIONS OF THE CNS

Bacterial infections of the CNS are rare, but often life threatening, events (76). Excluding direct inoculation following CNS trauma, bacteria typically gain CNS entry following hematogenous dissemination from distant sites (lungs and heart valves) or by direct extension from parameningeal foci of infection (inner ear and sinuses). Penetration of the BBB may occur via three potential mechanisms: (1) direct destruction of capillary ECs (77, 78), (2) disruption of intercellular TJ and migration in between ECs (79), and (3) transcytosis via intracellular vesicles directly through ECs (80). Once inside, numerous innate immune receptors and pathways are activated (**Figure 1**).

MICROGLIA

Analogous to peripheral tissues, resident CNS immune cells known as microglia bear a wide range of innate immune receptors. Common bacterial motifs, referred to as pathogen associated molecular patterns (PAMP), are recognized by cognate pattern recognition receptors (PRR), including Toll-like receptors (TLR), on the surface and in the cytoplasm of microglia, and to a lesser extent, on astrocytes (81–83). Microglial activation, triggered either by intact bacteria or bacterial cell wall antigens (84, 85), results in rapid changes in cellular morphology *in vivo* (86). Similar to tissue resident macrophages found in the periphery, microglia can phagocytize bacteria and present bacterial antigens via HLA to infiltrating CD4 T cells *in vivo* (84, 87, 88). These cells also rapidly produce pro-inflammatory cytokines and chemokines that recruit peripheral leukocytes to the area of infection and activate astrocytes. For example, during both experimental *Streptococcus pneumoniae* and *Staphylococcus aureus* infections of the CNS, microglia produce tumor necrosis factor (TNF)-α, interleukin (IL)-6, IL-12, C-X-C motif ligand (CXCL)1, CXCL2, C-C motif ligand (CCL)2, CCL3, and CCL5 *ex vivo*, mediators that recruit neutrophils (CXCL1 and CXCL2), monocytes (CCL2 and CCL3), and T cells (CCL5) (84, 85, 89–91). These activated microglia also secrete matrix metalloproteinases (MMP) that enhance BBB breakdown and facilitate additional leukocyte extravasation into the CNS (92). Finally, microglia can have direct bactericidal activity, being capable of producing reactive oxygen species (ROS), reactive nitrogen intermediates, and other proteases that kill bacteria *in vivo* (93–96).

ASTROCYTES

Microglia partner with astrocytes to eliminate infection as quickly as possible in order to minimize neuronal damage (86, 97, 98). In the normal CNS, astrocytes contribute to gap junction stability of the BBB (99). Their release of pro-inflammatory mediators such as IL-1β (100, 101), nitric oxide (102), TGF-β (103), and MMPs (92) *in vitro* suggest these cells may compromise BBB integrity in the setting of bacterial infection. Astrocytes are activated by bacterial PAMP or mediators produced by microglia; this changes their morphology and further triggers their release of innate inflammatory mediators both *in vitro* and *in vivo*. These mediators can include complement proteins, IL-1β, IL-6, and the chemokines, CCL2, CCL3, CXCL1, and CXCL10 (104–111), which further help recruit neutrophils, monocytes, and T cells. In response to interferon (IFN)-γ, TNF-α, and/or IL-1β, astrocytes

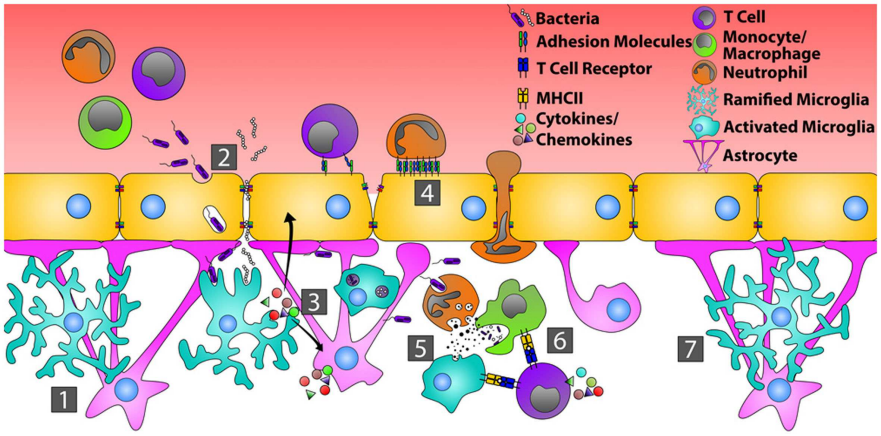

FIGURE 1 | Orchestration of the immune response during bacterial infection of the CNS. In the quiescent CNS (1), bacteria typically gain entry by transcytosis across the endothelial cells of the BBB, or by passing in between endothelial cells where tight junctions have been disrupted (2). Common bacterial motifs (PAMPs) are recognized by pattern recognition receptors (PRRs) on microglia and astrocytes resulting in their activation. This causes changes in glial cell morphology, enhanced production of inflammatory mediators that recruit neutrophils, monocytes, and T cells, and increased endothelial cell expression of adhesion molecules, including ICAM-1 and VCAM-1 (3). Circulating neutrophils, monocytes, and T cells then bind and extravasate into the infected CNS (4). Neutrophils directly phagocytize and kill bacteria through the release of defensins, lytic enzymes, and anti-microbial peptides (5). Neutrophils also produce MMPs, IL-6, IL-8, CXCL9, and CXCL10 that further open the BBB and shift the chemotactic profile toward the recruitment of T cells. Bacterial antigens are presented to T cells by microglia and/or infiltrating monocytes, transitioning from innate immunity toward an adaptive immune response (6). Resolution of bacterial infection returns tight junctions to normal and microglia and astrocytes to a resting state (7).

also up-regulate the cell surface adhesion molecules, intercellular adhesion molecule (ICAM)-1, and vascular cell adhesion molecule (VCAM)-1 *in vitro* (112–116), which would enhance the infiltration of monocytes and T cells into the CNS *in vivo*.

NEUTROPHILS

As in the periphery (117, 118), neutrophils are one of the primary lines of host defense during CNS bacterial infections (112, 119, 120). Studies in knockout mice show that the main chemokines driving neutrophil recruitment to the CNS are the C-X-C motif receptor (CXCR)-2 ligands, CXCL1 and CXCL2 (121). Furthermore, CSF samples from patients with bacterial meningitis show elevated levels of neutrophil attracting chemokines compared to controls (122, 123). Neutrophils, like microglia, respond to PAMP through various TLR, and are activated by cytokines such as TNF-α and IFN-γ *in vitro* (124). Neutrophils activated in the periphery up-regulate adhesion molecules that enhance their migration into tissues (125), while BBB EC express E-selectin and P-selectin during CNS bacterial infection (126), suggesting a mechanism that allows for the migration of neutrophils during these infections. Once neutrophils recognize a bacterial pathogen, they can directly phagocytize these organisms (127), as well as release MMP, defensins, lytic enzymes, and anti-microbial peptides that aid in clearing the infection (128). The inflammatory cytokine, TNF-α, induces neutrophils to produce IL-6, IL-8, CXCL9, and CXCL10 *in vivo* (129, 130), thereby shifting the chemotactic profile toward the recruitment of T cells and driving the adaptive immune response.

T CELLS

Adaptive immune responses are important in fighting CNS bacterial infections (131). During bacterial meningitis, T cell production of IFN-γ leads to the generation of chemokines that preferentially recruit monocytes and more T cells (132), supporting the transition from an innate to an adaptive immune response. Furthermore, IFN-γ, potentially made locally by T cells, increases the antigen-presenting capacity of microglial cells *in vitro* via up-regulation of HLA class I and II molecules, the co-stimulatory molecules, B7-1 and B7-2, and CD40 (133, 134). Bacterial antigen presentation by microglia activates T cells (135), driving further T cell proliferation and greater production of IFN-γ.

HOST IMMUNE RESPONSES TO VIRAL INFECTIONS OF THE CNS

Viruses use a variety of mechanisms to gain entry into the CNS. In the case of alphaherpesviruses (i.e., herpes simplex virus and varicella-zoster virus) and rabies virus, infection of peripheral nerves allows viral particles to travel by anterograde axonal transport into the CNS. Human immunodeficiency virus and human T cell leukemia virus-I enter the CNS parenchyma by infecting host immune cells in the periphery, and using them as "Trojan horses" to carry viral particles across the BBB. Finally, Epstein–Barr virus and West Nile virus directly infect the ECs of the BBB, resulting in barrier disruption and enhanced migration of immune cells into the parenchyma (136).

Because viruses can infect microglia, astrocytes, oligodendrocytes, as well as terminally differentiated and non-renewable cells such as neurons, the ensuing immune response within the CNS must avoid extensive cytolytic damage of virus-infected target cells (137). In general, innate anti-viral immunity such as the generation of type-I IFN occurs very rapidly, while the adaptive immune response is slower because it must first develop in the periphery (138). Important components of adaptive anti-viral immunity involve IFN-γ production by T cells as well as the

expansion and migration of virus-specific antibody secreting cells (ASC) (138, 139) (**Figure 2**).

MICROGLIA, ASTROCYTES, AND OLIGODENDROCYTES

During CNS viral infections, virus-specific PAMP activate individual TLR present on microglia, astrocytes, and oligodendrocytes. The former two cell populations, in particular, respond by producing anti-viral and pro-inflammatory mediators. During experimental mouse hepatitis virus (MHV) infection, astrocytes and microglia produce both type-I IFN (IFN-α and IFN-β), as well as IL-6, TNF-α, IL-12, IL-1α, and IL-1β *in vivo* (140–142). Furthermore, MHV infection triggers MMP-3 and MMP-12 release from astrocytes and oligodendrocytes (142), which along with IL-6 and the up-regulation of adhesion molecules on cerebrovascular endothelium, enhance cellular migration across the BBB (143). Astrocytes produce CXCL10, CXCL11, and CCL5 *in vivo* that recruit virus-specific CD4+ and CD8+ T cells (144–146), as well as ASC (147, 148), to the CNS to promote viral clearance. CXCL9 production from microglia is dependent on IFN-γ, while CXCL10 and CXCL11 are up-regulated by type-I IFN and TNF-α (149–152).

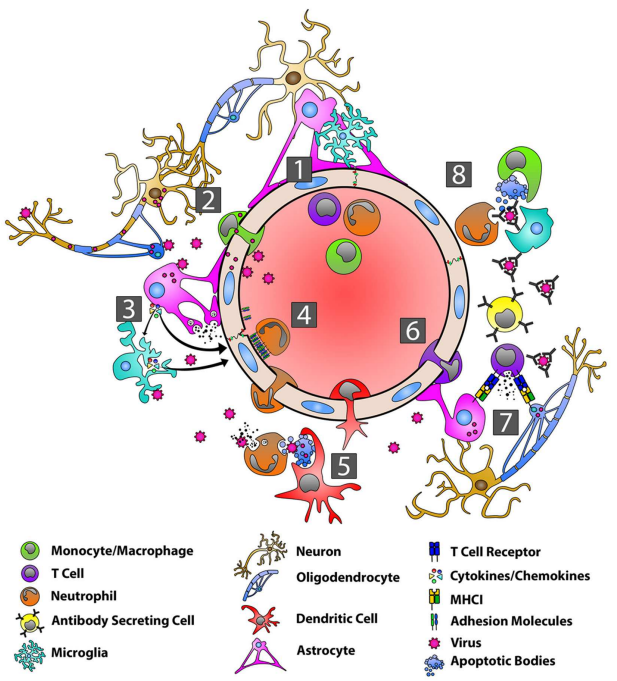

FIGURE 2 | Orchestration of the immune response during viral infection of the CNS. With the BBB in a resting state (1), viruses can gain entry into the CNS by infecting peripheral nerves and traveling by anterograde axonal transport into the CNS, by infecting host immune cells in the periphery and using these cells as "Trojan horses" to carry them across the BBB, or by directly infecting BBB endothelial cells (2). Viral PAMPs then activate microglia, astrocytes, and oligodendrocytes (3). Microglia and astrocytes produce a range of anti-viral/pro-inflammatory cytokines, including type-I IFNs, IL-6, TNF-α, IL-12, IL-1α, and IL-1β (3). Astrocytes also produce MMP-3 and MMP-12 resulting in the up-regulation of adhesion molecules on endothelial cells (3). Interactions between adhesion molecules and neutrophils contribute to BBB breakdown via the production of MMP-9 and the disassembly of the tight junctions (4). DCs are seen in the CNS within several days and migrate to draining lymph nodes where they activate and expand virus-specific T cells (5). Chemokines produced by astrocytes are responsible for recruiting virus-specific CD4+ and CD8+ T cells as well as ASCs to the CNS (6). CD8+ T cells produce IFN-γ and lytic molecules, including granzyme B and perforin, to eliminate virus from astrocytes, while IFN-γ controls viral replication in oligodendrocytes (7). Virus-specific antibodies control virus replication in cells such as neurons via complement–independent, non-cytolytic mechanisms. These antibodies inhibit virus budding and replication, viral RNA transcription, and cell-to-cell virus spread.

MYELOID LINEAGE CELLS

Neutrophils and macrophages are recruited to the CNS following viral infection (153, 154). Thus far, macrophages appear to have more limited anti-viral activity in the CNS (155), but neutrophils contribute to the breakdown of the BBB by interacting with EC via adhesion molecules to promote the disassembly of tight junction complexes (156). Neutrophils also secrete MMP-9 that degrades the extracellular matrix and basal lamina of the BBB and further opens the BBB (157). This has been most clearly demonstrated in the MHV model, where depletion of MMP-9 inhibited lymphocyte infiltration into the CNS (157, 158). Dendritic cells (DC) are seen in the CNS within a few days after CNS viral infection. These cells rely on the chemokine CCL3 to migrate to cervical lymph nodes draining the CNS, where they prime virus-specific T cells (159).

T CELLS

In the MHV model, virus-specific CD8+ T cells are detected in local lymph nodes prior to CNS infiltration and then accumulate in the CNS (160). Both CD4+ and CD8+ T cells are in part recruited to the CNS by the chemokines, CXCL9 and CXCL10, acting through their cognate receptor, CXCR3 (161–163). T cell expression of CCR2 and CCR5 likely contribute to CNS recruitment as well (164, 165). The role of CD4+ T cells in this setting is to support CD8+ T cell function via the production of IFN-γ (166). CD8+ T cells are the main anti-viral effector cells in the CNS during infection and are essential for clearing virus from glial cells (142, 167, 168). CD8+ T cells produce IFN-γ and lytic molecules, including granzyme B and perforin (169). These lytic molecules eliminate virally infected astrocytes (170), while IFN-γ serves to control viral replication in oligodendrocytes (171, 172). In both the MHV and Sindbis virus (SINV) encephalitis models, T cells promote B cell proliferation and differentiation (173, 174), in part by secreting the cytokines, IL-10 and IL-21 (175–177).

B CELLS AND ANTI-VIRAL ANTIBODIES

Virus-specific ASC help control viruses in the CNS through potent complement-independent, non-cytolytic mechanisms (141, 178–183). These ASC arise either from ectopic lymphoid follicle-like structures within the CNS (152) or migrate from cervical lymph nodes where they have expanded and up-regulated CXCR3 and CXCR4 on their surface prior to entering the CNS (184). ASC recruitment to the CNS has been most extensively studied in the SINV encephalitis model. The initial ASC entering the CNS have an HLA class II positive, plasmablast-like phenotype, but these cells gradually lose HLA class II expression and acquire a more plasma cell-like phenotype (139, 141). Virus-specific antibodies function to neutralize both extracellular virus as well as virus particles budding from infected cell membranes. During SINV infection,

antibodies that bind the E2 viral envelope glycoprotein inhibit virus replication (185) and prevent viral budding from infected neurons without actually killing target cells (182, 186). Similarly, during rabies virus infection, antibodies against the RV glycoprotein inhibit viral RNA transcription and prevent cell-to-cell viral spread (187). Antibodies can also trigger natural killer (NK) cells and macrophages to induce antibody dependent cell-mediated cytolysis of virally infected cells (152). Finally, in exchange for non-cytolytic viral clearance in the acute setting, virus-specific ASC must persist in the CNS long term to prevent viral reactivation at a later date since viral RNA is never fully eradicated from target tissues (139).

HOST IMMUNE RESPONSES TO SELF (MYELIN) ANTIGENS IN THE CNS

MULTIPLE SCLEROSIS

Multiple sclerosis, an autoimmune disease characterized by infiltrating immune cells targeting myelin antigens in the CNS, is the most common cause of neurologic disability in persons younger than 40 years of age (188). Pathologically, MS lesions are characterized by focal inflammation, demyelination, and axonal damage (189). MS is a complex disease whose occurrence and progression are influenced by both genetic (190–192) and environmental (193, 194) risk factors. Evidence derived from both human genetic studies and a related mouse model, experimental autoimmune encephalomyelitis (EAE), suggest that encephalitogenic CD4+ T cells are primary initiators of disease. Genome-wide association studies show that MS risk alleles are confined to immune related genes governing antigen presentation as well as the proliferation and survival of T cells, including HLA class II (HLA-DRB1*1501), IL-2R, and IL-7R (190–192). Moreover, EAE in mice is induced by immunizing animals with various myelin peptides (195), or via the adoptive transfer of myelin-specific CD4+ T cells, resulting in a disease having some clinical and pathological similarities to human MS (196, 197). In MS patients, CD4+ T cells localize within CNS lesions present in the brain (198) and spinal cord (199), and elevated frequencies of myelin-reactive CD4+ T cells can be found in circulating the blood (200, 201). Although not described in detail here due to space constraints, many MS lesions also contain abundant CD8+ T cells whose specificity and role in disease pathogenesis remain poorly understood. Likewise, therapies targeted specifically at B cells have proven highly effective in MS patients, highlighting an emerging role for this cell type in both relapsing and progressive forms of disease.

ROLE OF CD4+ T CELLS IN AUTOIMMUNE INFLAMMATION OF THE CNS

During both MS and EAE, self-reactive T cells are likely activated in the periphery (189), where they undergo initial differentiation and expansion (124). Upon entry into the CNS, these cells are reactivated by myelin epitopes presented by an as of yet unidentified local DC (202, 203). Production of cytokines such as IFN-γ and TNF-α from activated CD4+ T cells results in local activation of resting microglia, leading to the up-regulation of HLA class I and II as well as co-stimulatory molecules (B7-1, B7-2, and CD40) on the surface of these cells (133, 134, 204, 205). These activated microglia are capable of serving as APC for infiltrating myelin-specific CD4+ T cells *in vivo* thus sustaining this pathogenic local T cell response (97). Production of cytokines, chemokines, and MMPs by microglia (206) facilitate local inflammation by causing BBB breakdown and recruiting more immune cells into the CNS. These include circulating monocytes capable of differentiating into inflammatory DC and macrophages upon tissue entry (207), culminating in demyelination (124). Furthermore, microglial production of IL-23 and IL-1β promotes granulocyte macrophage colony-stimulating factor (GM-CSF) secretion by CD4+ T cells (208). GM-CSF has been shown in EAE to promote CNS inflammation by mobilizing Ly6C^{hi} monocytes from the bone marrow into the periphery, thereby increasing the number of circulating monocytes available for recruitment to the CNS (207). GM-CSF can also increase HLA class II expression and pro-inflammatory cytokine production by microglia, macrophages, and DC *in vitro* (209, 210). IL-17 producing T cells have been detected within CNS lesions during both EAE and MS (211, 212). IL-17 promotes brain inflammation, inducing the production of pro-inflammatory cytokines, TNF-α, IL-6, and IL-1β most probably from astrocytes, microglia, or macrophages. It also stimulates the release of chemokines responsible for recruiting neutrophils to the CNS, particularly CXCL1 and CXCL2 (213, 214). Finally, IL-17 can disrupt TJ in the BBB, allowing further migration of CD4+ T cells to the CNS (212, 215).

ROLE OF GLIAL CELLS IN AUTOIMMUNE INFLAMMATION OF THE CNS

Microglia

Microglia play important roles in augmenting CNS inflammation, demyelination, and neuronal damage in both EAE and MS (67, 216–218). Activation of microglia occurs rapidly following the induction of EAE and results in the release of cytokines, chemokines, ROS, and tissue-degrading MMP (206). One mediator, TNF-like weak inducer of apoptosis (TWEAK), triggers proliferation, angiogenesis, inflammation, is associated with extensive myelin loss, and induces astrocyte cell death during MS (219). IL-17 produced by microglia (220) worsens brain inflammation by stimulating GM-CSF production, as well as increasing IL-6, inflammatory proteins, nitric oxide, and adhesion molecule expression by macrophages. Moreover, expression of myeloperoxidase (MPO) and ROS by microglia results in direct myelin degradation and neuronal damage (216, 218). Paradoxically, microglia also can play a neuroprotective role during CNS autoimmunity. These cells can promote remyelination, protect neurons, and suppress the adaptive immune response within the CNS (221, 222). Within MS lesions, microglia and macrophages express the neurotrophic factors, nerve growth factor (NGF), and brain-derived neurotrophic factor (BDNF), supporting neuronal survival (220, 223, 224). Furthermore, microglia secrete the anti-inflammatory cytokines, IL-10 and TGF-β, and express the inhibitory receptor, PD-L1, responsible for inhibiting T cell proliferation and cytokine production (74, 225).

Astrocytes

Astrocytes are a major source of CCL2 and CXCL10 in the CNS, regulating the migration of monocytes into the brain (CCL2) and microglia into the lesion site (CXCL10) (111, 226–228). One study suggested these cells play a more prominent role in regulating the recruitment of peripheral monocytes into the CNS (229). CXCL12,

a chemokine that induces the expression of CXCL8 and CCL2, is also expressed by astrocytes in MS lesions (230). CXCL12 can be cleaved by MMP-2, also expressed by astrocytes in MS and EAE, into a neurotoxic peptide that causes further neuronal damage (231). Similar to microglia, astrocytes also play a protective role during MS and EAE. Homeostatic astrocyte functions include buffering potassium, removing extracellular glutamate that can accumulate to toxic levels, adjusting water balance, and controlling synaptic activity and blood flow in the CNS (8). These cells are also able to produce neurotrophins and the anti-inflammatory cytokine, IL-10 (232).

CONCLUSION

The vast complexity of cellular interconnections within the CNS, and the non-renewable nature of many neural cells, mandate that some local immune responses be tightly controlled while others (i.e., cytolytic ones) be excluded to the fullest extent possible. The BBB is a dynamic and highly regulated tissue interface that helps make the CNS a unique immunological environment. It responds to signals from both neurons and glial cells on one side while simultaneously being able to sample immunological events passing through intravascular compartments. Immune cells perform normal surveillance of the CNS by searching for antigens previously encountered in extraneural sites such as the deep cervical lymph nodes. Pathological conditions such as infections caused by viruses or bacteria elicit changes at the BBB, including the up-regulation of a unique subset of adhesion molecules as well as heightened release of chemokines by ECs. Mediators produced by astrocytes and microglia further increase BBB permeability and recruit additional circulating leukocytes into the CNS. The ensuing immune response must then be tightly controlled in order to avoid collateral tissue damage. As such, astrocytes and microglia maintain mechanisms to dampen inflammatory responses. In some settings, immune cells such as ASC persist long term within the CNS to prevent viral reactivation. When normal control mechanisms fail, neuroinflammatory diseases such as MS can result. For this reason alone, it is imperative that the complexity of immune reactions within the CNS be better understood.

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Immunotherapeutic advancements for glioblastoma

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Immunotherapy seeks to improve the body's immune response to a tumor. Currently, the principal mechanisms employed are: (1) to improve an aspect of the immune response (e.g., T cell activation) and (2) to encourage the targeting of particular antigens. The latter is typically achieved by exposing the immune system to the antigen in question, *in vivo*, or *in vitro* followed by re-introduction of the primed cells to the body. The clinical relevance of these approaches has already been demonstrated for solid tumors such as melanoma and prostate cancer. The central nervous system was previously thought to be immune privileged. However, we know now that the immune system is highly active in the brain and interacts with brain tumors. Thus, harnessing and exploiting this interaction represents an important approach for treating malignant brain tumors. We present a summary of progress in this area, focusing particularly on immune-checkpoint inhibition, vaccines, and T cell engineering.

Keywords: immunotherapy, glioblastoma, vaccines, antibodies, monoclonal, checkpoint modulators, T cell engineering

INTRODUCTION

Patients with cancer are typically immunosuppressed. This appears to be a survival strategy of the more aggressive tumors and is in excess of that which would be expected by external factors such as chemotherapy, malnutrition and steroid use. When discussing immunotherapy for tumors affecting the nervous system, the prototype remains glioblastoma (GB, grade IV glioma). This is the most common malignant primary central nervous system (CNS) malignancy (1). Aside from developments in the treatment of systemic metastases to the brain, the use of immunotherapy of other CNS tumors is at a relatively less developed stage.

An early observation germane to this field was that tumors may (rarely) resolve following an infection. This phenomenon has been documented, for example, in locally advanced pancreatic cancer (2). Therapeutic applications of this observation began with William Coley in 1891, when he injected inactivated *Streptococcus Pyogenes* and *Serratia Marcescen* into patients with sarcoma (3). By inducing systemic immune activation, it was hoped that the immune system would also increase its activity against the tumor. Indeed, the vaccine *did* cause tumor regression in some patients (4). Another relatively non-specific approach, which has proven to be of clinical value, has been the use of the Bacillus Calmette–Guérin (BCG) vaccine in those affected by bladder cancer (5).

These early, non-specific approaches suffered from unpredictable clinical responses. The use of genetically modified live bacteria remains under active investigation, principally *Salmonella* (6). In the case of GB, the addition of live bacteria to surgical wounds in the hopes of triggering local inflammation has proved controversial (7).

More tumor-specific therapies have been developed, which do not rely on a generalized immune response. Such approaches have already proven advantageous in highly immunogenic malignancies such as melanoma (4). Tumor-infiltrating lymphocytes are well recognized in GB. Studies to date have yielded conflicting data on the significance of these in relation to patient outcomes (8, 9). Nonetheless, their very presence makes enhancing their activity and specificity an attractive goal.

The gravity of GB has been a motivator for novel approaches. The median survival remains around 15 months and recurrence/progression is almost inevitable (10). Current treatment modalities include surgery, radiation, chemotherapy (temozolomide, bevacizumab, nitrosoureas), and electrical field treatment. This latter, known as NovoTTF-100A®, uses alternating electric fields to inhibit cell growth and has almost no side effects apart from local irritation of skin (11). The use of "targeted" chemotherapy, usually a single-agent specifically aimed at a particular cell-signaling pathway, has thus far been disappointing.

We focus on two emerging methods of harnessing the immune system in the treatment of GB:

- preventing the tumor from evading the immune system.
- exposing the immune system to antigens expressed by the tumor, thus stimulating it to attack the tumor.

To further illustrate these two points, we provide data from recently published clinical trials and from abstracts presented at the 2014 American Society of Clinical Oncology Annual Meeting (ASCO).

CNS IMMUNOLOGY

The CNS was previously considered as a relatively 'immune-privileged' site. This was thought to reflect, in part, the protective nature of the blood–brain barrier (BBB). However, we now know that the CNS has an active and tightly regulated immune system (12). The circumventricular organs, which lack a BBB, have the ability to detect infection in the peripheral bloodstream. Areas with high vascularity, such as the leptomeninges and the

choroid plexus, may also lead to microglial activation upon detection of pathogen-associated molecular patterns (PAMPs) in the bloodstream (13).

Microglia (phagocytic in function) are part of the evolutionarily older *innate* immune system. They are concentrated in the brain's gray matter and are less numerous in white matter (the tracts of which may be used by GB to move to new locations) (14). Aside from the production of pro-inflammatory factors in the presence of infection, microglia are believed to play a role in removing neurotoxic debris (e.g., preventing the amyloid-β accumulation noted in Alzheimer's disease).

The *adaptive* arm of the immune system (responsible for immunologic memory) was thought to be limited in the CNS due to the lack of lymphatic channels. Instead, cellular waste from the interstitial fluid is transferred to the CSF for removal via the glymphatic system. Circulating lymphocytes may be found within the CNS in their activated form but naïve T cells are essentially absent (15–17).

However infiltration of lymphocytes, especially T cells, is increased in patients harboring GB as the BBB becomes disrupted, suggesting an important interaction between the immune system and the tumor (18, 19). The tumor responds with a number of strategies to counteract the immune system. These include down regulation of major histocompatibility complex (MHC, responsible for presenting antigens) (20), an increase in cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) and programed cell death protein 1 (PD-1) (21, 22), IL-10 (23), TGF-β (24), and by damping immune activity by recruiting regulatory T cells (T_{Regs}) (25).

In addition to the BBB, the blood–tumor barrier must be overcome. The formation of new blood vessels by the tumor is often disorganized, with abnormal flow dynamics and immature pericytes, making recruitment of lymphocytes challenging. Experiments in mice and clinical observation support the view that immunotherapy is likely to be much less effective as the vasculature becomes more chaotic (26).

IMMUNE-CHECKPOINTS

Immune-checkpoints prevent excessive immune activation, which may lead to collateral damage in healthy tissue. GB makes use of this apparatus to impair nearby T cell functionality. GB induces a state of chronic antigen exposure, which gradually increases the expression of immune-checkpoint proteins and culminates in lymphocytic exhaustion or anergy (27). By overcoming this habituation, it is hoped that immune-mediated cytotoxicity may be recovered.

While many proteins involved in this process have been identified, we focus here on two for which clinical applications have been developed: CTLA-4 and PD-1. Both are responsible for the down regulation of T cell activity (28). CTLA-4 is located on cytotoxic (CD8+) and the two major subsets of helper (CD4+) T cells. This protein restricts the activity of the T cell (29, 30). The ligand for CTLA-4 is similar to that of the co-stimulatory receptor CD28, (a complex of CD80 and CD86). It is thought to be a competitive agonist at this site (31, 32). T cell activation is inhibited by reducing both the production of IL-2 and the expression of its receptor, as well as arresting lymphocytes in the G1 phase of the cell cycle (33). Additionally, this immune-checkpoint protein has been shown to enhance the suppressive function of T_{Reg} cells (34, 35).

Ipilimumab is an antibody, which inactivates CTLA-4. This was the first agent focusing on immune-checkpoint blockade to receive approval from the FDA (36). It is used for patients with melanoma and has proven to be effective for those with brain metastases (37). In GB, a similar approach has been hampered by safety concerns. One review of 10 patients demonstrated that treatment was devoid of significant toxicities in all but 1 patient (38). However, in a subsequent study with five patients, all experienced auto-immune-related adverse effects (39). This typically consisted of a rash with colitis and hypothyroidism; there was also one case each of encephalitis and partial status epilepticus.

PD-1 expression is induced upon activation of a T cell; it serves to limit the potentially deleterious activity of lymphocytes in peripheral tissues. PD-1 has been shown to be expressed by T_{regs} and activation of its receptor appears to aid in their proliferation (40). PD-1 is also expressed by B cells and NK cells (41).

Nivolumab is a therapeutic antibody against PD-1. Is has proven to be effective when used with ipilimumab in patients with melanoma (42). There is an ongoing phase III trial comparing its efficacy with bevacizumab in patients with recurrent glioblastoma (NCT02017717). Pembrolizumab is another such antibody. Its activity in patients with metastatic melanoma depends on the presence of pre-existing cytotoxic T cells, which are thought to be deactivated by the tumor (43).

PD-1 binds to a ligand, PD-L1. This latter is up-regulated in numerous types of cancer (44). However, the use of PD-L1 as a biomarker for response to therapeutic checkpoint blockade is complicated by its heterogeneous expression in tumors, complex signaling networks, and the normal expression found on lymphocytes and other cells within the tumor microenvironment. In GB, expression of PD-L1 has been linked to the loss of the tumor suppressor PTEN (phosphatase and tensin homolog) and consequently the PI3K–Akt signaling pathway (phosphatidylinositol 3-kinase – protein kinase B a.k.a. Akt) (45). An antibody blocking PD-L1, MPDL3280A, has shown efficacy in the setting of metastatic bladder cancer in a phase I trial (46). This approach appears most effective in those patients in whom pre-existing immunity is suppressed by PD-L1, as evidenced by high levels of PD-L1 and CTLA-4 expression (47).

A more radical approach to recovery of immune function is that of bone-marrow transplant. Autologous progenitor cells have been used in GB to facilitate higher doses of cytotoxic chemotherapy. However, given the mortality with a complete marrow transplant, this has not been the subject of a trial. Experience with other tumor types suggests that this process "resets" the immune system and thus allows for recovery of cytotoxicity (48).

VACCINES

Current approaches to immunotherapy may be classified as active or passive (49). "Passive" refers to antibodies to tumor antigens, or immune-conjugates aimed at targeted drug delivery (50). "Active" vaccines are intended to stimulate the patient's own immune

response. They may be cell-based (e.g., pulsed dendritic cells) or non-cell based (i.e., heat-shock protein-based vaccines).

PEPTIDE VACCINES

Exposing short protein sequences to the immune system is usually done with peptides that are presented by HLA-A2 (human leukocyte antigen). This is the most common of the HLA subtypes but is found in only 50% of Caucasians and 30% of African-Americans. To overcome this limitation, antigens binding other class I HLAs have been developed, bringing population coverage to around 70%. Promising proteins from this line of investigation include: PTPRZ1 (receptor-type tyrosine-protein phosphatase zeta; function unclear but implicated in directional outgrowth of glioma cells), SEC61G (Protein transport protein Sec61 subunit gamma; involved in protein translocation across the endoplasmic reticulum for degradation), TNC (tenascin C; an extracellular glycoprotein typically expressed in development/differentiation and following injury), and EGFR (51).

EGFRvIII is a constitutively active mutant form of the epidermal growth factor receptor, which is present in approximately 33% of GB (52). Its presence is an independent negative prognostic indicator for survival in patients who manage to survive at least 1 year after initial diagnosis (53). A phase II trial was conducted in order to determine the immunogenicity, progression-free survival (PFS), and overall survival (OS) in patients who received a peptide-based vaccine (PEPvIII) targeted at EGFRvIII-expressing GB (54). Eligibility criteria included: gross total resection, Karnofsky performance status (KPS) $\geq$80%, and no evidence of progression after initial chemo-radiation. Immune reactivity after vaccination was monitored by observation of a delayed-type hypersensitivity (DTH) reaction to intradermal injections of PEPvIII and recall antigens. Eighteen patients were enrolled. Median PFS and OS were 14.2 and 26 months for those vaccinated vs. 6.3 and 15 months for controls. The skin test was performed in 17 patients; all showed no response prior to vaccination and all but 3 after vaccination. Of 14 patients tested, 6 demonstrated a positive humoral response against PEPvIII. The toxicity profile was deemed safe with most adverse reactions consisting of cutaneous reactions at the injection sites. (One patient had a severe allergic reaction). A phase III trial to confirm these results is ongoing.

HEAT-SHOCK PROTEIN VACCINES

Heat-shock proteins (HSP) are molecular chaperones; they provide protein stability by facilitating folding and aid in intra-cellular localization (55). Their activation is induced by adverse environments such as hypoxia, inflammation, and oxidative stress (56). Neoplastic cells are constantly exposed to such stressors; they rely on the HSP for survival.

A vaccine that includes HSP has proved safe and tolerable in a Phase I study of 12 patients with recurrent GB (57). After vaccination, peripheral leukocytes generally showed a response to HSP-96-bound peptides, as demonstrated by IFN-γ production (via real-time PCR). Lymphocytic infiltrates expressing IFN-γ were identified in those undergoing biopsy. Those showing an immune response to the vaccine showed an increase in median OS to 47 weeks vs. 16 in those with no response.

In the subsequent phase II trial, 41 patients with gross total resection of recurrent GB were vaccinated with HSPPC-96 (58). The median PFS of this cohort was 19.1 weeks with a median OS of 42.6 weeks. In both studies, the treatment appeared safe and tolerable.

AUTOLOGOUS VACCINES

These techniques rely on *ex vivo* modification of the patient's immune system or of the tumor itself, followed by re-introduction of the altered cells. The immune system, particularly cytotoxic T lymphocytes, may be stimulated with tumor antigens. Neoplastic cells may be irradiated, or altered with viruses, in the hopes of increasing their immunogenicity and lowering their propensity for evasion of the immune system (49, 59).

Newcastle disease virus (NDV) combined with autologous tumor has been used as a vaccine. This virus has been shown to replicate selectively in neoplastic cells and to possess immunogenic properties (60). Twenty-three patients had their tumor surgically resected and incubated with hemagglutinating units of avirulent NDV. Concurrently, a control group was established, which comprised patients receiving standard care with a KPS of $\geq$60. An improvement in median PFS and OS was seen by comparison with controls: 40 weeks vs. 26 and 100 weeks vs. 49, respectively. Significant DTH skin reactions were noted when vaccinated patients were tested against irradiated tumor cells, both virus-modified and unmodified (61).

Autologous formalin-fixed tumor vaccines (AFTV) use fixed tissue to sensitize T cells to tumor antigens. In a Phase I/IIa trial, 22 newly diagnosed patients with resected GB received AFTV with concomitant fractionated radiotherapy (62–65). Median PFS and OS were promising at 7.6 and 19.8 months. Again, the treatment combination was well tolerated and adverse events were mostly limited to cutaneous reactions induced by the injection (66).

DENDRITIC-CELL-BASED VACCINES

This process involves obtaining dendritic cells from a patient and pulsing them with glioma antigens derived from a resection. A major advantage is that multiple antigens may thus be presented (49, 67). This is of particular relevance to GB, which is known to display high intra-tumoral heterogeneity. Evidence of efficacy has already been established for metastatic prostate cancer with sipuleucel-T, although those with nervous system metastases were excluded from the pivotal trials (68).

DCVax-L® is another such dendritic-cell-based vaccine. In a phase I clinical trial, 23 patients with resected GB had an immunogenic lysate prepared from their tumor plus dendritic-cells derived from peripheral blood mononuclear cells (PBMC). The dendritic cells were supplemented with granulocyte-macrophage colony-stimulating factor (GM-CSF) and IL-4 before exposure to the lysate. The treatment was safe, tolerable, and without evidence of dose-limiting toxicity (69). The median PFS and OS were 15.9 and 31.4 months, respectively. A randomized phase III trial is ongoing (NCT00045968).

This approach is also being explored as a way to target glioma stem cells, which represent a radioresistant and chemoresistant subpopulation of cells within a patient's tumor. In a phase I trial,

Table 1 | Immunotherapy-based clinical trials for glioblastoma, which are currently recruiting.

Trial name	Phase	Target accrual	Therapy	Primary outcome	Identifier
PEPTIDE-BASED					
Phase I/II trial of IMA950 multi-peptide vaccine plus poly-ICLC in glioblastoma	I/II	16	IMA950 multipeptide based vaccine/poly-ICLC/temozolomide/ radiotherapy	Safety, tolerability	NCT01920191
Safety and efficacy study of SL-701, a glioma-associated antigen vaccine to treat recurrent glioblastoma multiforme	I/II	100	SL-701/imiquimod cream 5%/sargramostim 150 mg	Safety, tolerability, OS, ORR	NCT02078648
GAPVAC Phase I trial in newly diagnosed glioblastoma patients	I	20	APVAC1 vaccine/poly-ICLC/ GM-CSF APVAC2 vaccine/poly-ICLC/ GM-CSF	Safety, feasibility, biological activity	NCT02149225
Phase I study of safety and immunogenicity of ADU-623	I	38	ADU-623	Safety, tolerability, immunogenicity	NCT01967758
IMMUNE CHECKPOINT BASED					
A randomized study of nivolumab vs. bevacizumab and a safety study of nivolumab in adult subjects with recurrent glioblastoma (GBM) (CheckMate 143)	III	260	Nivolumab, bevacizumab, ipilimumab	Safety, tolerability, efficacy	NCT02017717
HEAT-SHOCK PROTEIN BASED					
Research for immunotherapy of glioblastoma with autologous heat-shock protein gp96	I	20	gp96	Safety, efficacy	NCT02122822
AUTOLOGOUS-BASED					
Randomized phase II multicentre study to investigate efficacy of autologous lymphoid effector cells specific against tumor-cells (ALECSAT) in patients with glioblastoma multiform measured compared to avastin/irinotecan	II	175	ALECSAT/bevacizumab/irinotecan	PFS	NCT02060955
Pilot study of autologous t cells redirected to EGFRVIII-With a chimeric antigen receptor in patients with EGFRVIII + glioblastoma	I	12	CART-EGFRvIII T cells	Safety, feasibility	NCT02209376
DENDRITIC-CELL BASED					
Study of a drug [DCVax®-L] to treat newly diagnosed GBM brain cancer	III	300	DCVax®-L	Efficacy, PFS	NCT00045968
A study of ICT-121 dendritic cell vaccine in recurrent glioblastoma	I	20	ICT-121 DC vaccine	Safety, tolerability	NCT02049489
Phase I study of a dendritic cell vaccine for patients with either newly or recurrent glioblastoma	I	40	[a]Dendritic cell vaccination/ temozolomide/radiotherapy [a]Dendritic cell vaccination ± bevacizumab (for patients previously treated with bevacizumab)	Safety, tolerability	NCT02010606
Dendritic cell vaccine for patients with brain tumors	II	60	Autologous tumor lysate-pulsed DC vaccination ± (0.2% resiquimod or adjuvant poly-ICLC)	Efficacy	NCT01204684
Basiliximab in treating patients with newly diagnosed glioblastoma multiforme undergoing targeted immunotherapy and temozolomide-caused lymphopenia (REGULATe)	I	18	RNA-loaded dendritic cell vaccine (basiliximab)	Safety, efficacy	NCT00626483

(Continued)

Table 1 | Continued

Trial name	Phase	Target accrual	Therapy	Primary outcome	Identifier
Vaccine therapy with or without sirolimus in treating patients with NY-ESO-1 expressing solid tumors	I	30	DEC-205-NY-ESO-1 ± sirolimus	Safety, tolerability	NCT01522820 *(not glioma-specific)*
Ph I personalized neoantigen cancer vaccine with radiotherapy for patients with MGMT unmethylated, newly diagnosed glioblastoma	I	20	Radiotherapy, personalized NeoAntigen Vaccine (NeoVax)	Safety, efficacy	NCT02287428
Dendritic cell vaccine for malignant glioma and glioblastoma multiforme in adult and pediatric subjects	I	20	DC vaccination/tumor lysate/ imiquimod	Safety, efficacy	NCT01808820
Vaccine therapy and temozolomide in treating patients with newly diagnosed glioblastoma	I	10	DC vaccination/temozolomide	Safety	NCT01957956
Dendritic cell vaccine therapy with *in situ* maturation in pediatric brain tumors	I	20	DC vaccination/tumor lysate, imiquimod	Safety	NCT01902771
T-CELL BASED THERAPY					
CAR T cell receptor immunotherapy targeting EGFRvIII for patients with malignant gliomas expressing EGFRvIII	I/II	160	Anti-EGFRvIII CAR transduced PBL/aldesleukin/fludarabine/ cyclophosphamide	Safety, PFS	NCT01454596
Cellular immunotherapy study for brain cancer (alloCTL)	I	15	Alloreactive CTL	Safety, efficacy	NCT01144247
CMV-specific cytotoxic T lymphocytes expressing CAR targeting HER2 in patients with GBM (HERT–GBM)	I	18	HER2.CAR CMV-specific CTLs	Safety	NCT01109095

Therapy: Poly ICLC, an immunostimulant and ligand for the toll-like receptor; composed of carboxymethylcellulose, polyInosinic-polyCytidylic acid, and poly-l-lysine double-stranded RNA; Sargramostim, recombinant granulocyte–monocyte colony-stimulating factor; GM-CSF, granulocyte–monocyte colony-stimulating factor; APVAC, activated personalized vaccination; DC, dendritic cell; PBL, peripheral blood lymphocytes; CAR, chimeric antigen receptor; Aldesleukin, recombinant IL-2; CMV, cytomegalovirus; CTL, cytotoxic T lymphocyte.

Outcomes: OS, overall survival; PFS, progression free survival; ORR, objective response rate.

Retrieved from https:// clinicaltrials.gov/ on 12/18/2014.

17 patients with newly diagnosed GB were given a dendritic-cell-based vaccine with a combination of glioma stem cell antigens. This approach (the ICT-107 vaccine) reported a promising median PFS and OS of 16.9 and 38.4 months, respectively. Interestingly, five patients who underwent a subsequent resection had a decrease or absence of cells positive for CD133, a glioma stem cell marker (70). A phase II trial was initiated with the same vaccine but despite currently unpublished data demonstrating a significant increase in PFS, there was no increase in OS (49). A phase III trial is planned nonetheless. A similar concept has been applied in the production of a vaccine (ICT-121) that targets CD133-positive glioma cells (CD 133 is an enrichment marker for cancer stem cells). A phase I trial involving this vaccine is underway (NCT02049489).

VIRAL PROTEIN-BASED VACCINES

A variety of studies have identified human cytomegalovirus (CMV) proteins and nucleic acids in approximately 90–100% of primary GBs (71–73). Although the role of CMV in the pathogenesis and progression of GB is not fully understood, the prevalence of these antigens in tumor cells and relative absence in normal surrounding tissue provides an important opportunity to develop targeted immunotherapeutics (74). Interestingly, one patient receiving DCVax-L developed a specific anti-CMV (anti-pp65) cytotoxic T cell response (75).

To date, immunotherapeutic targeting of CMV has been tried in a limited number of patients with high-grade gliomas. One case study describes a patient with recurrent GB who received adoptive transfer of CMV-specific T cells concurrently with temozolomide, which resulted in 17 months without disease progression (76). Recently, a trial involving patients with GB demonstrated that the transfer of expanded CMV-specific T cells lead to a median OS of 403 days (vs. historical median OS of 180 days) and 4/10 patients who completed the treatment remained progression-free during the study period (77). Ongoing trials are assessing the use of CMV-specific dendritic-cell vaccines (NCT00639639) and CMV-specific T cells following drug-induced lymphopenia in GB (NCT00693095). Direct targeting of CMV with valganciclovir has been the subject of some controversy and is not currently recommended outside the context of a clinical trial (78).

T CELL ENGINEERING

Adoptive cell transfer using genetically engineered T cells represents another attractive immunotherapeutic approach to treating GB. T cells that recognize specific tumor-associated antigens (TAAs) can be generated by fusing an extracellular binding domain

(usually derived from a TAA-specific monoclonal antibody) to the intra-cellular signaling domain of the T cell receptor (TCR) to form a chimeric antigen receptor (CAR) (79). CAR T cell activation is MHC-independent and therefore circumvents issues involving down regulation of HLA class I molecules and defects in antigen processing that tumors use to evade T cell recognition (80). These engineered cells are also potentially more useful than antibody-based immunotherapies because they have the ability to migrate through blood vessel walls, penetrate solid tumor, and recruit addition components of the immune response (81). CARs have been developed for glioma-specific antigens, including HER2, IL-13Rα2, and EGFRvIII, and have demonstrated potent antitumor activity with *in vivo* models (81, 82).

Interestingly, the CARs generated against HER2 in GB patients, also recognized the CD133+ stem cell populations, that are thought to contribute to tumor recurrence (80). Mounting evidence that this has led to a number of clinical trials exploring the safety and effectiveness of CARs against HER2 (NCT01109095), IL-13Rα2 (NCT00730613; NCT01082926), and EGFRvIII (NCT01454596).

WHAT HAVE WE LEARNED?

Although immunotherapy has been with us for over a century, we are still in the preliminary stages of refining this therapeutic approach. Thus far, immune-based treatments have proven to be relatively safe with minimal toxicities, especially by comparison with traditional cytotoxic chemotherapy. Currently, it is estimated that <20% of patients with GB enroll in clinical trials, so increasing participation would appear to be a clear priority. Given the variety of methods receiving attention, much of the field is anticipated to be in phase I and II trials for some years (**Table 1**). Hence, the usual caveats apply regarding lack of power, lack of randomization, and the use of historical controls. In spite of this, the preliminary survival data have, on the whole, been encouraging.

Using peripheral immune reactivity as a surrogate marker for disease activity (and thus outcomes) is attractive, in that it may allow for more rapid development of active agents. In practice, it has thus far led to mixed results. While some trials link immune reactivity with a better prognosis, others show no such association (83). It is hoped that greater standardization and more refined methods will overcome these difficulties.

Trials to date have studied the effects of immune-checkpoint inhibitors and vaccines separately. As our knowledge of these treatments increases, we can begin to consider combining both. Such an approach has already been shown to be efficacious in a murine model of glioma (84).

Approaches targeting specifically just one antigen have the drawback that evolution of resistance appears almost inevitable in those with GB. Such difficulties are well recognized in solid tumors to which "targeted" approaches have been applied: at least two such agents are thought to be necessary (to inhibit tumor growth) and preferably three (85). Those which aim to simulate the immune system or expose it to a broad range of antigens thus hold greater promise. As data on the safety of single-agent approaches accrues and as patents expire, rational multi-agent combinations are likely to become the norm for most patients.

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REVIEW
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Brain tumor immunotherapy: what have we learned so far?

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High grade glioma is a rare brain cancer, incurable in spite of modern neurosurgery, radiotherapy, and chemotherapy. Novel approaches are in research, and immunotherapy emerges as a promising strategy. Clinical experiences with active specific immunotherapy demonstrate feasibility, safety and most importantly, but incompletely understood, prolonged long-term survival in a fraction of the patients. In relapsed patients, we developed an immunotherapy schedule and we categorized patients into clinically defined risk profiles. We learned how to combine immunotherapy with standard multimodal treatment strategies for newly diagnosed glioblastoma multiforme patients. The developmental program allows further improvements related to newest scientific insights. Finally, we developed a mode of care within academic centers to organize cell-based therapies for experimental clinical trials in a large number of patients.

Keywords: immunotherapy, malignant glioma, dendritic cell vaccines, immunomodulation, galectin-1, oncolytic viruses

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Introduction

High grade gliomas (HGG) are brain tumors occurring in adults and children. The WHO grade IV HGG, called glioblastoma multiforme (GBM), is the most frequent brain cancer in adults with an incidence of 3–4 per 100,000 adults per year (1) and 2 per million children (2). The treatment for these patients consists primarily of maximal safe surgery in order to debulk the tumoral mass for symptomatic relief and to obtain tissue for histological diagnosis, followed by radiochemotherapy and maintenance chemotherapy to induce optimal local tumor control. In spite of improved surgery and radiotherapy, and the addition of temozolomide (TMZ) to the multimodal treatment strategy, the prognosis of patients with GBM remains poor: the median overall survival (OS) is about 15 months, with 88% of patients dying within 3 years (3, 4). Relapse is universal and is believed to be due to the extensive spread of tumor cells into surrounding regions of the brain (5, 6). At the time of relapse, the prognosis is particularly poor, with reports of 100% mortality within 18 months (7). A recent review pointed to the progression-free survival (PFS) at 6 month and median OS as most useful and accessible end points, the latter ranging between 5 and 13 months for relapsed GBM patients (8). The prognosis upon recurrence might be improving with the initiation of new multimodal treatment strategies (9–11). Most reports are not yet focusing on long-term survival. In spite of being an orphan disease, the tumor still causes the highest number of years of life lost due to cancer (12). One of the particular challenges with classical chemotherapeutic strategies is overcoming the blood–brain barrier. Therefore, preclinical research is focused on alternate approaches, such as targeted therapy (13) including anti-angiogenesis strategies (14), and especially immunotherapy. Treating cancer by means of immunotherapy (e.g., cancer vaccines, adoptive cell transfer, and checkpoint blockade) has slowly evolved over decades in a nowadays

clinically applicable treatment in a number of cancer types (e.g., metastatic melanoma, renal cell carcinoma, non-small cell lung cancer, prostate cancer. . .).

Active specific immunotherapy with autologous mature dendritic cells (DCm) loaded with autologous tumor cell lysate (DCm-HGG-L) is an emerging and innovative treatment approach for patients with HGG. The development of DC therapy in HGG has started in 1999 in our center. Since then, we established a complete translational research program from bench to bed (**Figure 1**) including *in vitro* experiments (15, 16), *in vivo* experiments in the GL261 model (17–19), early clinical phase I/II clinical trials as part of the HGG-IMMUNO-2003 cohort comparison trial for relapsed HGG patients (20–26), a phase I/II clinical trial HGG-2006 for patients with newly diagnosed GBM (EudraCT 2006-002881-20) (27, 28), and the recently finished phase IIb prospective placebo-controlled double-blind randomized clinical trial (RCT) HGG-2010 (EudraCT 2009-018228-14). In parallel to this clinical program, advanced MRI studies have been performed on HGG, in particular to characterize immunotherapy-related changes (29–32). In this program, insights from preclinical research were translated into the HGG-IMMUNO-2003 cohort (A–D) comparison trial. Data from these cohorts were then used for integration into the multimodal treatment of patients with primary diagnosis of GBM. As such, the vaccination technology from cohort C was used for the HGG-2006 trial, while the technology from cohort D is now used for the RCT HGG-2010. In parallel, according to the evolving legislation, the preparation for the clinical applications was embedded into a Good Manufacturing Practice (GMP) facility within the University Hospitals Leuven. The translation back from bed to bench has been realized by samplings of tumor tissue and blood samples taken at defined vaccination time points. The new preclinical research perspectives in 2014 include galectin-1 targeting as a strategy for immunomodulation and oncolytic virus therapy.

The preclinical and clinical results, together with clinical results obtained independently by other research teams provide a strong rationale to continue exploration of immunotherapy in patients with HGG. We summarized our insights in several reviews and commentary papers (33–39). The emerging field of immunotherapy for HGG has been extensively reviewed by other researchers as well (40–43). A first meta-analysis on the available results in the literature show clear benefit of immunotherapy for OS (44). In this review, it is our intention to focus on our own experience.

Rationale for Active Specific Immunotherapy Against HGG

Theoretical Concept of Dendritic Cell Vaccination

Dendritic cells (DCs) are a subset of white blood cells, critical to most aspects of adaptive immunity due to their central role as specialized antigen-presenting cells (APCs) in the initiation phase of T cell responses (45). Typically DCs reside as immature cells in almost every organ and tissue at the interface of potential pathogen entry sites. Danger-triggered DCs start to mature: they up-regulate chemokine receptors, which guide them to draining lymph nodes. There, the mature DCs are capable of inducing primary T cell responses due to their high levels of major histocompatibility complex (MHC), adhesion and costimulatory molecule expression. As opposed to the other APC, DCs are able to present and cross-present the antigenic peptides in the context of both MHC Class II and Class I molecules, respectively (46, 47).

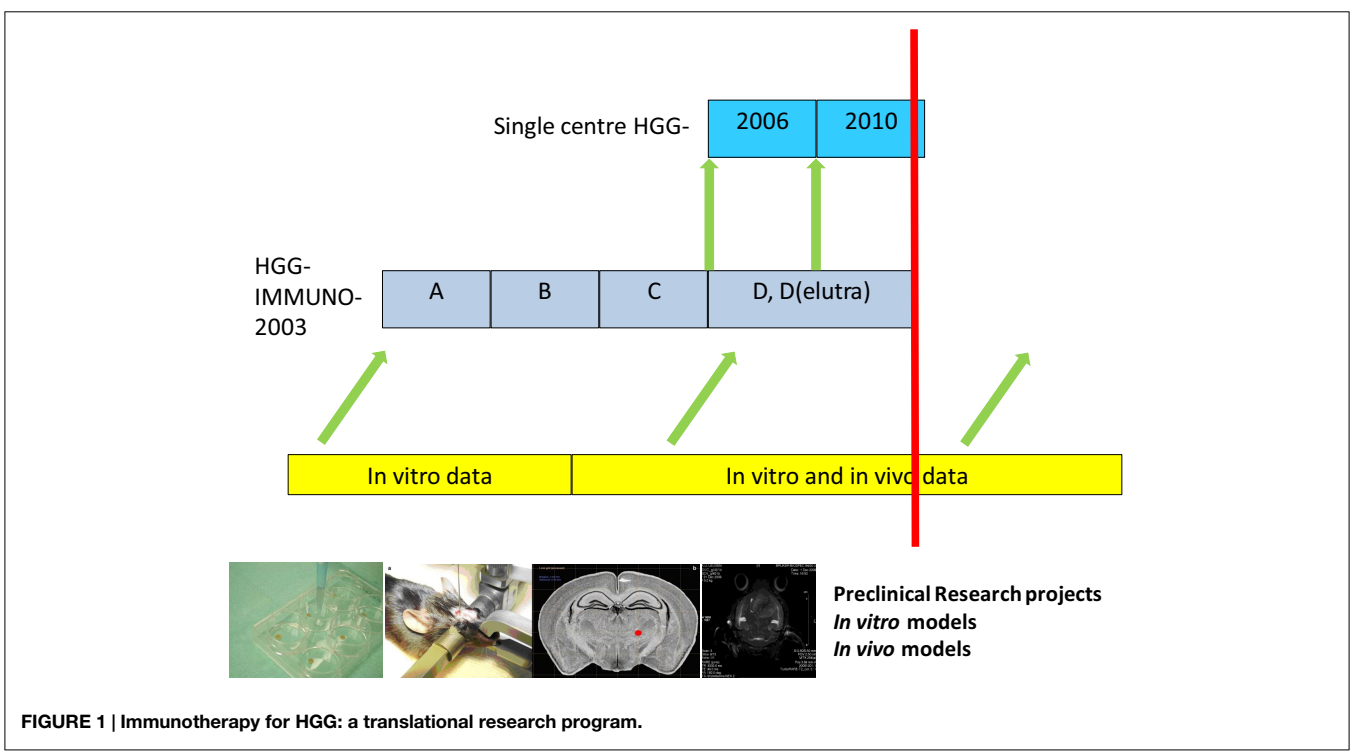

FIGURE 1 | Immunotherapy for HGG: a translational research program.

In this way, they can prime not only CD4+ T helper cells, but also CD8+ cytotoxic T cells (CTLs) (48). Both effector cell types are believed to be necessary to induce an effective cell-mediated immune response (49).

Dendritic cells are not only sentinels in the adaptive immune response, but have also been shown to be strong activators of NK cells and NKT cells (50), thus linking the innate and adaptive immune responses. In this way, both tumor cells with and without expression of MHC class I molecules can theoretically be killed (51). All these particular characteristics make DCs a perfect adjuvant in active specific immunotherapeutic strategies, in which one aims to induce a specific immune response *in vivo* (52–55).

Justification of the Use of Dendritic Cell Technology in Glioma Therapy

Gliomas have been shown to express an impressive collection of glioma-associated antigens (GAAs) (56). Till today, antigen search is a field of interest (57) including even tumor-driving mechanisms (58). Up till now, however, identification of a universally expressed GAA with a critical downstream cell survival-related function has not been identified. Therefore, just targeting the known GAA using individual peptides would inherently lead to immune escape because of the positive clonal selection of antigen-loss variants (59, 60): those tumor cell clones that do not express the particular, targeted GAA (anymore), will escape from the immune rejection and thus have an important proliferation advantage as compared to the cell clones that do express the targeted GAA. That heterogeneity in GAA expression in gliomas represents the main reason to use whole tumor cell lysates as a source of GAAs to load the DC. In case, the GAAs are expressed not only exclusively on the tumor cells but also on normal healthy cells, tolerance and induction of auto-immunity are possible, both being theoretical hurdles to a beneficial immune response: in the former case, an antitumoral immune response cannot be induced because the GAA is considered a self-antigen and in the latter case, a pathological immune response against normal tissues is mounted.

In general, tumor vaccination strategies are not entirely new anymore (52). Especially for the spontaneously more immunogenic tumors like malignant melanoma (61), renal cell carcinoma (62), mesothelioma (63), leukemia (64), gynecological tumors (65–67) and prostate carcinoma (68), several vaccination strategies have been used in the past. Large-scale production of clinical grade DCs became possible (69), including the development of several closed culture systems to obtain large amounts of DCs for clinical use (70–72). DC vaccination for prostate cancer reached full marketing authorization (Provenge®).

The brain, once considered as immune privileged site (73), is a dynamic immunological environment. Astrocytes, microglia and infiltrating immune cells play a major role in the brain during host immunity to antigens (74). The question of immune privilege in the context of malignant glioma is fading (56, 75). Proof of the principle of immunotherapy has been demonstrated in *in vitro* experiments (15, 16) and in several rodent models (37). In these models, induction of protective immunity and immunological memory against syngeneic orthotopic gliomas have been shown after vaccination with DCs loaded with GAAs of different antigen sources.

Immunotherapy for Patients with Relapsed HGG

Overview of Different Cohorts

We started in 2001 to implement preclinical insights into clinical practice after obtaining approval of the local Ethics Committee. Since 2003, we initiated the HGG-IMMUNO-2003 study protocols consisting of sequential therapy-optimalization protocols in consecutive cohorts for patients with relapsed HGG. It is aimed to prove the feasibility and explore the efficacy of immune therapy for HGG, and to "dissect" different aspects of the immune therapy in order to find a putative ideal vaccination strategy. Cohorts have been built up on the most recent insights in vaccination strategy available at time of preparation of the cohort protocol (**Figure 2**).

- *Cohort A*. The DC vaccination schedule existed of five intradermal injections of autologous mature DC loaded with autologous tumor antigens. DC maturation was induced with the classical cytokine cocktail (IL-1b, TNF-a, PGE2). The latter cytokine cocktail was based primarily on the so-called Jonuleit cocktail (76). Already from the beginning, we omitted IL-6 out of the cocktail. IL-6 was known to play a major role in the induction of a Th17 phenotype of T cell response (77). Injections were administered at week 1, 3, 7, 11, 15.
- *Cohort B*. Based on the observations made in the patient group treated according to the vaccination schedule in cohort A, injections with autologous mature DC loaded with tumor-derived antigens were administered at week 1, 3, 5, 7, (9) and further each 4 weeks.
- *Cohort C*. Based on further observations made in the patient groups treated according to both prior vaccination schedules and based on recent insights in *in vivo* models upon priming with DC and boosting with lysate instead of DC (78), patients were treated with 4 weekly DC-HGG-L injections followed by monthly boosting with HGG-L.
- *Cohort D*. In this cohort, we omitted PGE2 out of the maturation cocktail. PGE2 was already long time ago linked to the induction of a DC2-type (79). Because of its importance for the induction mainly of the mobility of DC (80), it was kept in the classical maturation cocktail. However, PGE2 was later-on also shown to induce IDO activity in human DC, thereby creating a tolerizing DC phenotype (81). Moreover, PGE2 upregulated CD25 on DC, as such believed as a marker of strong DC maturation, but a marker, of which was shown that it was shed in the surrounding thereby consuming the IL-2 needed for autocrine T cell activation. Because not-fully maturated DC themselves play a role in tolerance induction (82), we wanted to apply a method to induce with imiquimod *in vivo* DC maturation after injection (83–86). Imiquimod binds to Toll-like receptor 7 and induces strong DC maturation and activation. Moreover, its role in generating immune responses in a preclinical *in vivo* model of HGG has been described (85). Based on this rationale, PGE2 *ex vivo* maturation was replaced by local application

of imiquimod to increase *in vivo* maturation and activation of loaded DC. Within this cohort, we switched at a certain time point from the open cell culture methodology toward a closed cell culture methodology. This group of patients was defined as cohort D(e). The monocytes were isolated with Elutra instead of plastic adherence. Elutriation allows for fast and easy enrichment of monocytes within a closed system, and is superior to other GMP-approved methods (87–89). DCs were cultured in VueLife tissue culture bags instead of Falcon culture flasks. The cytokines used for differentiation and maturation were GMP-certified. Finally, four batches of GMP-DCm-HGG-L were produced at the same time, of which the first was injected immediately as vaccine, while the three other batches were frozen until use. For each of the three remaining induction vaccinations, a batch was thawed and washed once before injection. Of note, the open cell culture methodology continued to include children with relapsed HGG, because the closed culture systems could not be applied to the leukapheresis product of children.

Updated Clinical Results

Patients suspected of a relapse of HGG, who could be taken into consideration for immunotherapy, were re-operated upon to maximally remove the tumor and in order to obtain tissue as a source of tumor proteins. Part of the tumor was provided for pathology diagnosis, part was placed immediately in a sterile vial, to be stored at −80°C. Because of the large amount of tumor tissue needed for vaccine production, in rare cases it was impossible for the pathologist to unequivocally prove the recurrent pathology: in these cases, radiological evolution and sometimes amino acid PET scan results were consulted to conclude a relapsing, progressive HGG.

Patients with relapsed HGG were entered into the trial. About 40% of the included patients combined or consecutively applied neurosurgery and immunotherapy with other types of treatment like re-irradiation or chemotherapy upon decision of the referring physician. We obtained clinical results from 366 patients (48 children younger than 18 years and 318 adults above the age of 18 years). These patients belong to the "as treated" group from whom also the RPA was estimated and who received new resection and only immunotherapy till the next event. Median PFS of these children and adults were 3.8 and 2.6 months, respectively; median OS was both 10.6 months. Most importantly, the 2-year OS for these patients with relapsed HGG was 20% (SEM = 6) for children and 22% (SEM = 2) for adults. When the subgroup of 33 children and 247 adults with relapsed GBM was taken separately, median PFS was 2.5 months for children and 2.6 months for adults, median OS was 8 and 9.9 months with a 2-year OS of 10% (SEM = 6) and 17% (SEM = 3), respectively. Thirteen percent (SEM = 8) of adults with relapsed GBM remained free of recurrence for more than 18 months, and 10% (SEM = 2) lived longer than 3 years. Although hard to compare with literature data, the tail of the OS curve seems beneficial to data published on repeated re-operations combined with drug-based adjuvant therapies (11). Our data are difficult to compare to published data on PFS and OS upon new chemotherapy (8) or radiochemotherapy (9, 10). To compare future clinical trials, data should be presented according to prognostic models as has been published after radio(chemo)therapy (90) or immunotherapy (25). Moreover, besides PFS at 6 months and median OS, we believe that long-term OS (2 years or more) should also be considered as further outcome of patients with relapsed HGG in the context of immunotherapy.

Having included a large series of patients with relapsed HGG and treated with neurosurgery and immunotherapy, it became indeed obvious that clinical risk factors were influencing the prognosis of the patients. This was considered as very important for counseling of the patients and for stratification while designing future RCTs for such patients. Therefore, a novel recursive partitioning analysis (RPA IMMUNO) classification was developed for adults above the age of 18 years with relapsed HGG, and survival data were analyzed on the 117 first included adult patients (25). The RPA classification was based on the age of the patient, the grading of the relapsed tumor (grade III or grade IV), the Karnofsky Self Performance Scale and the estimated mental status. We internally validated the RPA IMMUNO in an extended group of 251 adults with relapsed HGG treated in patient cohorts of the HGG-IMMUNO-2003 protocol and from whom we could retrieve the data for RPA classification. These patients were equally distributed into the four cohorts of patients. Patient characteristics are described in **Table 1**. As shown in **Figure 3**, the PFS and the OS of patients belonging to the different RPA risk classes were significantly different.

The immunotherapy was feasible without major treatment-related toxicities. Almost all patients were treated in an ambulatory setting.

	induction vaccines	boost vaccines	DC maturation	adjuvans
Cohort A	**DC week 1 and 3**	DC monthly	TNFa, IL-1b, PGE2	none
Cohort B	**DC week 1,3,5 and 7**	DC monthly	TNFa, IL-1b, PGE2	none
Cohort C	**DC week 1,2,3,4**	**Lysate monthly**	TNFa, IL-1b, PGE2	none
Cohort D	DC week 1,2,3,4	Lysate monthly	**TNFa, IL-1b**	**imiquimod**
Cohort D(Elutra)	**ElutraDC week 1,2,3,4**	Lysate monthly	TNFa, IL-1b	Imiquimod

FIGURE 2 | HGG-IMMUNO-2003 cohorts.

TABLE 1 | Patient characteristics.

	HGG-IMMUNO-2003	HGG-2006
Age (median, range)	49 (18–77)	57 (27–70)
Sex (M/F)	161/90	49/28
Grade III/IV/no grading tumors	43/205/3	0/77/0
Number of events (median, range)	2 (2–7)	1
Number of vaccines	6 (4–24)	8 (0–30)
Cohort A/B/C/D/D(e)	11/15/26/72/127	–

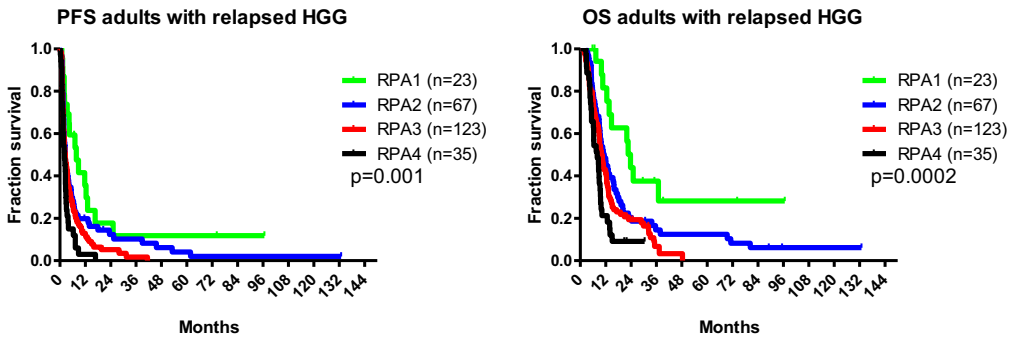

FIGURE 3 | PFS and OS of adults with relapsed HGG.

Immunotherapy for Patients with Newly Diagnosed GBM

HGG-2006 Phase I/II Trial

Rationale

As next step in our program, we wanted to integrate immunotherapy within the multimodal standard treatment for adults with newly diagnosed and histologically proven GBM (3, 4). A complex rationale was elaborated for the design. (1) Leukapheresis was scheduled after the surgical resection and before radiochemotherapy. After resection of GBM, a functional immune system is normally recovered within 1 week (91). Pro-inflammatory activity after irradiation might influence the activation state of monocytes and hence their differentiation capacity toward DC (92). Moreover, although grade III and IV hematologic toxic effects after radiochemotherapy were minimal (3), mild reduction of the monocyte count cannot be excluded. (2) The four induction vaccines were administered immediately after the radiochemotherapy. The immune suppression after 6 weeks concomitant TMZ was shown to be minimal but still might exist (3). The concept of tumor-specific immunization at time of immune reconstitution after chemotherapy has been demonstrated in several animal models (93, 94) and in clinical practice (95). Moreover, besides the induction of pro-inflammation (92), local radiotherapy might remove suppressor T cells, thus permitting a more effective T cell stimulation *in loco* (96). Another important reason to immunize prior to maintenance TMZ was the finding that the sensitivity of GBM to chemotherapeutics, among which TMZ, after prior vaccination was significantly increased (97, 98). (3) We further continued the boost vaccines during the TMZ maintenance therapy. Injection of lysate-loaded DCs for the priming, followed by boosts with tumor cell lysate alone generated the most effective antitumor effects in a preclinical model. The protocol allowed better CTL responses and also triggered an antitumor humoral response (78). The experiences in cohort C with induction vaccines with DCm-HGG-L and boost vaccines with HGG-L as immunotherapeutic strategy supported the concept for the HGG-2006 trial.

Updated Clinical Results

The first aim of this study was to assess the feasibility/toxicity to integrate tumor vaccination within the global treatment plan for an adult patient with newly diagnosed and GBM WHO grade IV, which could at least subtotally be removed. The major primary aim was the PFS at 6 months after diagnosis. To fulfill both the aims of (1) monitoring toxicity (phase I) of this treatment in the newly diagnosed patients and (2) detecting a potential benefit as a treatment strategy (phase II), we included a "STOP and GO" design.

The results of the pilot phase and the full trial phase have been published recently (27, 28). The trial was feasible without major immunotherapy-related toxicities. The integrated immunotherapy did not affect quality of life. We here present the last updated results (31 July 2014) of the PFS and OS of patients from the HGG-2006 study, divided into the EORTC RPA risk profiles three to five (**Figure 4**). Patient characteristics are described in **Table 1**. The data represent the intent-to-treat analysis. The 5-year OS for the EORTC RPA class III and class IV patients was 35.9% (asymmetrical CI95%: +25.4, −24.2) and 11.5% (asymmetrical CI95%: +10.2, −6.9), respectively. As compared to the historical control data of patients belonging to the same EORTC RPA risk profiles (4), patients from EORTC RPA class III had a better OS when immunotherapy was added to the standard treatment. These data were used to power the HGG-2010 trial.

HGG-2010 Prospective Placebo-Controlled Double Blind Randomized Clinical Trial

A prospective placebo-controlled double-blind phase IIb RCT was designed to explore the benefit of immunotherapy as fourth treatment modality to be included within the standard primary treatment strategy for patients with GBM (**Figure 5**). Supported by our experiences with patients included in HGG-2006, the design of the experimental arm (immunotherapy) is almost similar to HGG-2006. DCm-HGG-L is prepared and maturation is induced similar to Cohort D of the HGG-IMMUNO-2003 trial, using TNF-a, IL-1b, and Imiquimod skin preparation (aimed for TLR7-mediated DC activation). The design of the control arm is the current standard primary treatment: surgery, radiochemotherapy with TMZ, and maintenance chemotherapy with TMZ (3, 4). Randomization is performed with age as stratification variable (99). MGMT (*O*(6)-methylguanine DNA methyltransferase) methylation is not used for stratification. There is emerging evidence that other cytogenetic abnormalities outside MGMT methylation are of strong prognostic value as well (100–102). Primary endpoint of the trial is the PFS after six cycles of maintenance chemotherapy with TMZ. Secondary endpoints are quality of life assessments, OS, and induction of immune responses in both arms.

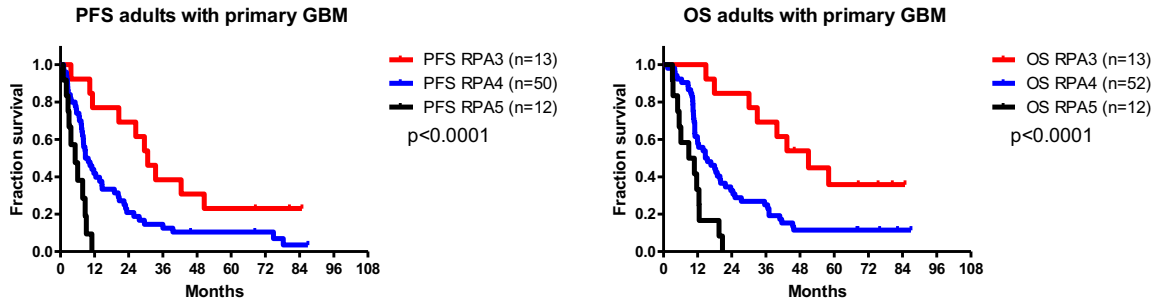

FIGURE 4 | PFS and OS of adults with primary diagnosis of GBM.

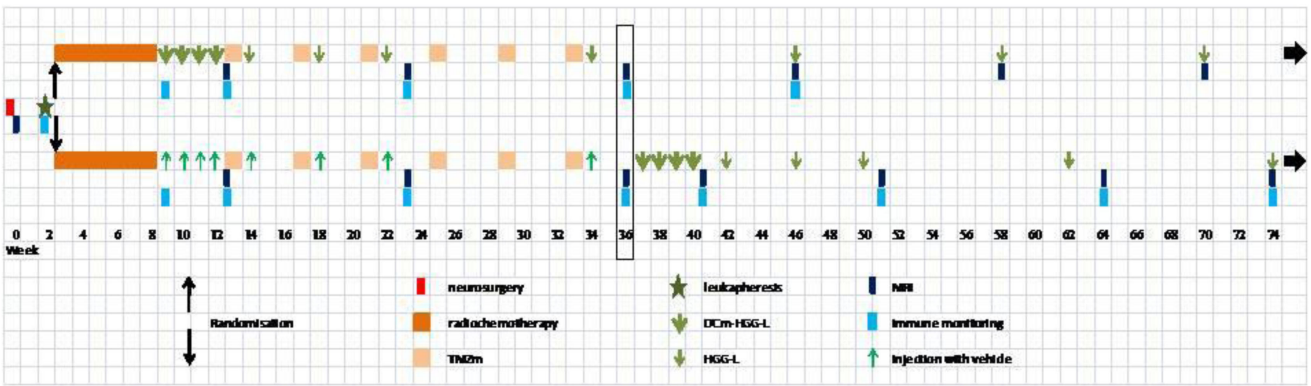

FIGURE 5 | Outline of the phase IIb randomized clinical trial HGG-2010.

Patients are unblinded after the assessment of disease status at time of MRI after the sixth cycle of TMZ or at time of progression if earlier progression occurred before the end of the sixth cycle of TMZ. Patients treated in the placebo arm and not yet relapsed (or with a compatible salvage treatment and no steroids after relapse) are treated with the immunotherapy regimen at this later stage, allowing to compare with immunomonitoring early vaccination efficacy during multimodal therapy with late vaccination after multimodal therapy.

The data of this RCT will be subject to the consortium Computational Horizons in Cancer (www.chic-vph.eu) to develop a hypermodel based on granular hypomodels in order to predict for which patient immunotherapy might be of added value. Clinical, radiological, immunological, and molecular data at diagnosis and at early evolution upon the radiochemotherapy will serve as incoming data into the different hypomodels.

New Preclinical Research Perspectives in 2014

Targeting Galectin-1 as Strategy for Immunomodulation

GL261 Orthotopic Mouse Model

Galectin-1 is a glycan-binding protein which is involved in the aggressive nature of GBM by stimulating angiogenesis, cell migration, and proliferation. In different cancer models, galectin-1 has been demonstrated to play a pivotal role in tumor-mediated immune evasion especially by modulating cells of the adaptive immune system. It was unknown, however, whether the absence or presence of galectin-1 within the glioma microenvironment also causes qualitative or quantitative differences in innate and/or adaptive antitumor immune responses. We explored the role of galectin-1 in the orthotopic GL261 mouse glioma model (19). Stable galectin-1 knockdown was achieved via transduction of parental GL261 tumor cells with a lentiviral vector encoding a galectin-1-targeting miRNA. We demonstrated that the absence of tumor-derived but not of host-derived galectin-1 significantly prolonged the survival of glioma-bearing mice as such and in combination with DC-based immunotherapy. Both flow cytometric and pathological analysis revealed that the silencing of glioma-derived galectin-1 significantly decreased the amount of brain-infiltrating macrophages and myeloid-derived suppressor cells (MDSCs) in tumor-bearing mice. Additionally, we demonstrated a pro-angiogenic role for galectin-1 within the glioma microenvironment. The data provided in this study point to a pivotal role for glioma-derived galectin-1 in the regulation of myeloid cell accumulation within the glioma microenvironment, the most abundant immune cell population in HGG. Furthermore, the prolonged survival observed in untreated and DC-vaccinated glioma-bearing mice upon the silencing of tumor-derived galectin-1 strongly suggests that the *in vivo* targeting of tumor-derived galectin-1 might offer a promising and realistic adjuvant treatment modality in patients diagnosed with GBM.

Galectin-1 in the Serum of Patients

In parallel to this preclinical work, we questioned whether increased galectin-1 expression levels were exclusively found at the tumor site or whether galectin-1 could also be detected in the serum of HGG patients. Galectin-1 serum levels were analyzed in a prospective dataset of 43 healthy controls and 125 patients with newly diagnosed or recurrent HGG (103). Samples were taken at the moment of surgical resection and/or 2–3 weeks after surgery. Galectin-1 serum levels were determined using an ELISA for galectin-1. Galectin-1 serum levels depended significantly on age and sex in the control group. Age- and sex-adjusted galectin-1 serum levels were significantly higher in all patient subgroups compared to healthy controls with a high discriminative ability that increased with age. We did not observe a significant decrease in the galectin-1 serum levels upon surgical resection of the tumor. Collectively, the data may represent a first step to establish galectin-1 as a serum biomarker in HGG disease monitoring.

Further longitudinal evaluation is required and ongoing to investigate the value of galectin-1 serum levels in HGG patients as an additional diagnostic marker, but more importantly as a predictor of treatment response and prognosis. Furthermore, galectin-1 serum levels can also provide an important tool for the identification of HGG patients that can benefit from galectin-1-directed therapies that are currently under development.

Oncolytic Virus Therapy

The oncolytic features of several naturally occurring oncolytic viruses have been shown on GBM cell lines and in (subcutaneous) xenotransplant models (104). However, orthotopic glioma studies in immunocompetent animals were lacking. We investigated Newcastle disease virus (NDV) in the orthotopic, syngeneic murine GL261 glioma model (105). Seven days after tumor induction, mice were treated intratumorally with NDV. Treatment significantly prolonged median survival of treated animals and 50% showed long-term survival *versus* none in the control group. We demonstrated immunogenic cell death (ICD) induction in GL261 cells after NDV infection, comprising of calreticulin surface exposure, release of HMGB1 and increased expression of PMEL17 cancer antigen. Uniquely, we found absence of secreted ATP. NDV-induced ICD in GL261 cells was shown to occur through programmed necrosis or necroptosis. *In vivo*, elevated infiltration of IFN-γ^+ T cells was observed in NDV-treated tumors, along with reduced accumulation of myeloid derived suppressor cells. The importance of a functional adaptive immune system in this paradigm was demonstrated in immunodeficient Rag2$^{-/-}$ mice, in which NDV induced a slight prolongation of survival, but failed to induce long-term survival. After secondary tumor induction in mice surviving long-term after NDV treatment, protection against glioma outgrowth was seen in 80% of animals, demonstrating induction of long-term antitumor immune memory after NDV therapy. We thus demonstrated for the first time that NDV has therapeutic activity against GL261 tumors, evidenced in an orthotopic mouse model. The therapeutic effect relies on the induction of a unique ICD route in the tumor cells, which primes adaptive antitumor immunity. The data change the paradigm that the use of oncolytic viruses for anti-cancer therapies should be performed in combination with suppression of potential antiviral immune responses. These insights are of high importance when using oncolytic viruses in combination with tumor vaccines within a multimodal treatment strategy.

Clinical Experiences on Immunotherapy Obtained in Other Centers

Active specific immunotherapy has been widely studied in many centers in phase I and/or phase II trials. Reviewing 37 reports on DC vaccines between 2000 and 2014, the patient number in each report was in median 15 ranging from 1 to 146. All these trials have been designed in different ways making read-outs hardly comparable. Moreover technologies for the vaccine production and administration routes were different as well. Characteristics of these trials are described in **Table 2**. Besides, the methodology to perform immune monitoring was variable: DTH tests, relative immune phenotypes of circulating lymphocytes, T cell proliferation and CTL assays, NK cell assays, IFN-γ production (serum, ELISPOT, mRNA expression, FACS), and recent thymic emigrant assay. In spite of all these differences, some general conclusions can be made. Immunotherapy for patients with (relapsed) HGG is feasible, and is safe. Only two immunotherapy-related serious adverse reactions have been reported: an overwhelming inflammatory reaction in a patient with large residual disease (21) and a cutaneous GBM growth after DTH testing of tumor

TABLE 2 | Overview of DC-based clinical trials.

Study phase	Case report	(20, 148)
	Phase I	(21, 27, 149–161)
	Phase I/II	(22–26, 28, 162–171)
	Phase II	(106, 172)
HGG grade	Grade III	(24, 148)
	Grade III and IV	(23, 25, 106, 149–151, 153, 154, 158, 160, 162, 164–169)
	Grade IV	(20–22, 26–28, 97, 152, 155–157, 159, 161, 163, 170, 172)
Disease status	Relapse (R)	(20–26, 148, 150, 151, 160–162, 165–167, 171)
	New diagnosis (ND)	(27, 28, 97, 149, 152, 155, 156, 159, 169, 170, 172)
	R and ND	(106, 153, 154, 157, 158, 163, 164, 168)
Tumor antigen	Lysate	(20–28, 97, 106, 153, 155, 158, 161–164, 166, 169)
	Peptides	(97, 148, 149, 152, 156, 160, 167, 171, 173)
	Tumor cell mRNA	(151)
	Cancer stem cell mRNA	(159)
	Tumor cell suspension	(154)
	IFN-g-treated tumor cells	(168)
	Apoptotic tumor cells	(170, 172)
	Fusions	(150, 165)
Route	ID	(20–28, 148, 150, 152–154, 156–161, 165)
	SC	(97, 106, 149, 164, 168, 170, 172)
	ID + intratumoral	(162, 166)
	ID + IV	(151)
	Intranodal	(167)

cells which were presumably radio-resistant (106). Induction of autoimmune reactions has not been observed at all, in spite of the fact that crude lysate of tumor tissue used in several trials contained also normal tissue antigens. In most of the trials, an effect is observed being long-term surviving patients and/or immune responses. Immune monitoring data were hardly correlated with clinical data. Most importantly for the further development, a first meta-analysis on the available data shows clear clinical benefit of DC-based immunotherapy for patients with HGG (44).

Modulation to Escape Immune Evasion Mechanisms

There are numerous factors that are responsible for HGG immune evasion (107). Intrinsic mechanisms include low expression of MHC class I and MHC class II molecules on the HGG tumor cells, microglia cells that produce IL-10 and IL-6, and an unbalance of the Th1/Th2 ratio in favor of Th2. Moreover Tenascin-C in the extracellular matrix in glioma prevents efficient immune cell to tumor cell contact. HGG cells produce a lot of immunosuppressive factors like TGF-b and PGE-2. Tumor cells lack costimulatory signals and might induce T cell anergy upon recognition. Moreover, stat-3 expression in the tumor cells promotes tumor immune evasion by inhibiting pro-inflammatory cytokine signaling and by amplifying Tregs. The PD-1L-1 expression on HGG is identified as a strong inhibitor of CD4+ and CD8+ T cell activation. The expression of HLA-E, HLA-G, and the presence of TGF-b and lectin-like transcript 1 are responsible for the absence of an NK attack to HGG. HGG cells express fas and fasL as well as CD70, and produce gangliosides and galectin-1. All these mechanisms are responsible for apoptosis of immune cells. Immune checkpoint blockade in combination with immunotherapy for glioma is therefore an emerging area of research (108). The most important immune evasion mechanisms are, however, the presence of myeloid-derived suppressor cells and especially Tregs.

The presence of Tregs in HGG tumors was found for the first time in 2006 (109). The number of Tregs infiltrating the brain was correlated with the WHO grade of the glioma (110). The suppressive activity of HGG-derived Tregs was demonstrated (109, 111–113). In preclinical research, we clearly showed the role of Tregs not only to block the antitumoral immune response (18) but also to change the inflammatory tumor microenvironment (114). Tregs have been shown to play a role on M2 macrophage differentiation (115) and MDSC functioning (116) in rodents. Tregs are particularly recruited into HGG by the production of CCL2 and CCL22 (117). Moreover, Tregs in HGG patients have a higher expression of the CCL2 receptor CCR4 as compared to controls. In the peripheral blood, a relative increase of the Treg fraction in the CD4 compartment as compared to controls was also described (118). Functional studies on Tregs from HGG patients became possible through isolation and characterization of this population as CD4 + CD127dim cells (119). These clinical data clearly show the presence and function of Tregs within the tumor microenvironment and even systemically.

Treg depletion and Treg inhibition are a widely discussed strategy in cancer (120). TLR ligands have been shown in preclinical models to inhibit Treg function and enhance *in vivo* tumor immunity (121, 122). Also TMZ (117, 123, 124) and gemcitabine (125) have been found to affect Treg infiltration in rodent models. Treatment with Sunitinib (126–128) or low dose paclitaxel (129) decreased the number of Tregs in cancer patients. Specific Treg depletion strategies have been performed in humans with anti-CD25 mAb daclizumab or with IL-2 diphtheria toxin conjugate denileukin diftitox (Ontak) (130–132). Treg depletion and immunological benefits could be obtained, especially with daclizumab. However, a trial had to be stopped because of availability of the product (130). The most important depleting strategy is the metronomic use of CPM (133–140). CPM suppresses *in vitro* induction of Tregs (141). The Treg depleting activity of CPM has been demonstrated in murine models in the context of vaccines (142). Some studies in humans have shown improvement of T cell effector function associated with a reduction in Treg numbers after low dose CPM (135). The timing and dose are critical for a robust CPM-based protocol able to induce significant ablation of Treg inhibitory functions in patients. Because the Treg depletion is aimed to be performed shortly after neurosurgery, potential interaction with used corticosteroids as described in mice should be taken into account (143).

Toward a New Health Care Model for Advanced Therapy Treatments

Autologous mature DCs loaded with autologous tumor lysate belong to the category of advanced therapy medicinal products (ATMP). According to EU Regulation 2007/1394/EC, ATMP for human use means (1) a gene therapy medicinal product as defined in Part IV of Annex I to Directive 2001/83/EC; (2) a somatic cell therapy medicinal product as defined in Part IV of Annex I to Directive 2001/83/EC; or (3) a tissue engineered product. In that context, DCs differentiated out of monocytes are defined as ATMPs. The boost vaccines consisting of HGG-L are regulated by the Directive 2004/23/EC. ATMPs in academic hospitals can be produced under the hospital exemption clausule. Hospital exemption means preparation of ATMPs on a non-routine basis according to specific quality standards, and used within the same Member State in a hospital under the exclusive professional responsibility of a medical practitioner in order to comply with an individual medical prescription for a custom-made product for an individual patient.

The production and administration of personalized ATMPs together with other anti-cancer therapies in a multimodal treatment approach for very diseased patients should be considered as Advanced Therapy Treatment for these patients, preferentially performed in centers of excellence by fully equipped specialty teams with particular multidisciplinary knowledge on basic, translational, and clinical science around the ATMP within the given clinical context. From the beginning of the translational research program, the working model was organized as a multicentre collaboration. The goal was to make this experimental

treatment strategy in clinical trials easily accessible for all potential patients in and outside the country. By doing this, a multiple "win" situation was created: the accessibility to immunotherapy program was easy for each patient, the referring specialist remained involved in the patient care (vaccination in ambulant setting) and in the scientific evolutions of the program, and the vaccination center obtained large series of patients so that experience could be maximized and scientific data generated within short periods. It might take time before patient-specific ATMPs that are used within a very complex clinical context, will reach industrialization for their production. In their report to the European Parliament and the Council in March 2014, the reporters from the European Commission pointed to creating a more favorable environment for ATMP developers working in an academic or non-for-profit setting, including by promoting early contacts with the authorities through the application of the fee reduction for scientific advice and by extending the existing certification scheme to these developers (144). Nevertheless, the DCVax®-L vaccine is developed by Northwest Biotherapeutics as an adjunct to the treatment of GBM, and is currently under evaluation in a phase III trial (145).

Obviously, the use of autologous *ex vivo* cultured mature loaded DCs is labor-intensive and expensive. This means a small-scale production for each individual patient as well as an adapted health care model to develop and provide such technologies. Meanwhile, strategies are searched for targeting DCs in the patient themselves. Appropriate pattern recognition receptors ligands are bound to tumor antigens to provide necessary adjuvant immune signals. Antigens are bound to antibodies which target particular receptors on DCs for internalization of the antigen and subsequent presentation (146). Besides antibody-based DC targeting, nanoparticles are rapidly emerging as new vehicles for delivering vaccines. Nanoparticles are a platform for co-encapsulating TLR ligands with the tumor antigen, and for targeting DCs through monoclonal antibodies or carbohydrate ligands (147).

Conclusion

Immunotherapy for HGG is feasible and has shown promising clinical results in a subgroup of patients without major adverse events. Decisive scientific results from large randomized trials are needed and awaited before the true position of DC vaccination in the therapy of HGG can be established. In parallel, patients who can benefit from this technology are characterized and defined. With current available basic science knowledge, further improvements of techniques and treatment strategies are reachable. However, administrative burdens to produce individualized vaccines remain a major threat, so that research focusses on as much as possible standardized off-the-shelf consumables for their production.

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Experiences and expectations for glioma immunotherapeutic approaches

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Malignant gliomas are the most prevalent type of primary central nervous system (CNS) tumor in adults. Despite progress in brain tumor therapy, the prognosis for malignant glioma patients remains dismal. Standard treatment with temozolomide and radiotherapy for patients with newly diagnosed glioblastoma has increased the median overall survival (OS) by 15–20 months (1), but tumor recurrence is inevitable. Salvage treatments upon recurrence are palliative at best and rarely provide significant survival benefit. Among the new treatments currently being investigated for malignant glioma, immunotherapy is theoretically attractive, because it offers the potential for high tumor-specific cytotoxicity (2). Although recent clinical trials of immunotherapy protocols for malignant gliomas focused on initiating and amplifying a host response with some clinical success, most of them failed to induce objective tumor shrinkage in patients (2). Antitumor activities of tumor cytotoxic T cells (CTL) and antibodies induced by these therapies are insufficient to overcome tumor growth because tumors have immune evasion mechanisms instigated by myeloid derived suppressor cells (MDSCs) and regulatory T cells (T_{reg}) (3). In this paper, we will review past experiences and discuss the promising future of immunotherapeutic approach for glioma treatment.

WHAT HAVE WE LEARNED FROM PREVIOUS CLINICAL TRIALS?

Preliminary results from recent immunotherapeutic clinical trials (2, 4–6) with dendritic cells or peptide vaccines for malignant glioma patients are encouraging. However, these trials have some limitations, and we will have to await the results of several phase III trials to make definitive conclusions. There are several concerns from past experiences.

1. The immune responses such as CTL and antibody production were not sufficient to overcome glioma progression, and were not correlated to clinical outcomes.
2. New issues have emerged regarding the evaluation of disease response, and with the identification of patterns such as pseudoprogression (7) that is frequently indistinguishable from disease progression. Additionally, there are delayed radiation responses after radiotherapy. In short, there are pitfalls in distinguishing the response of radiotherapy to that of immunotherapy.
3. There are prognostic variations and long term survivors among glioblastoma patients (8). We therefore have to develop molecular markers to predict the prognosis of the patient more precisely to conduct clinical trials with less bias.
4. We have to develop biomarkers that predict patients' responses to individualized immunotherapy. To do so, we have to conduct clinical trials that exclude patients with pseudoprogression, a delayed radiation response and a biologically good prognostic group.
5. Most immunotherapy clinical trials state that the therapy is safe. This is a concern because the adverse events of immunotherapy are usually interpreted as those of the clinical course of glioma. We have to continue to carefully monitor patients, because acute disseminated encephalomyelitis and neuropathic syndrome following vaccination against human papillomavirus for cervical cancer are now serious problems (9).
6. In recent years, there has been a significant increase in OS and progression free survival (PFS) owing to improvements in standard of care (10). In phase II clinical trials, survival data are usually compared to that of a decade ago, so emerging therapeutics are easily misconstrued as effective therapies.
7. In Japan, bevacizumab was approved for glioblastoma in June 2013. Therefore, we should reconsider whether an immunotherapeutic approach for glioma could be a new standard of care.
8. In Japan, medical oncologists are expected to participate in the development of global immunotherapeutic protocols for glioblastoma.

PROGNOSTIC MARKERS FOR GLIOBLASTOMA

The World Health Organization (WHO) currently has the most widely used system for prognostic markers; a high WHO grade correlates with clinical progression and decreased survival rate (11). However, individual fates vary within diagnostic categories. There are several prognostic factors that are associated with longer survival of glioblastoma patients, including age, performance status (PS), *MGMT* status, and *IDH1* mutation. The inadequacy of histopathological grading is shown, in part, by the inability to recognize patients prospectively. We and other researchers have developed a predictive method for patient outcome that enables clinicians to make optimal clinical decisions using microarray technology (8, 12–14). Our work described an expression profiling study of glioblastoma patients

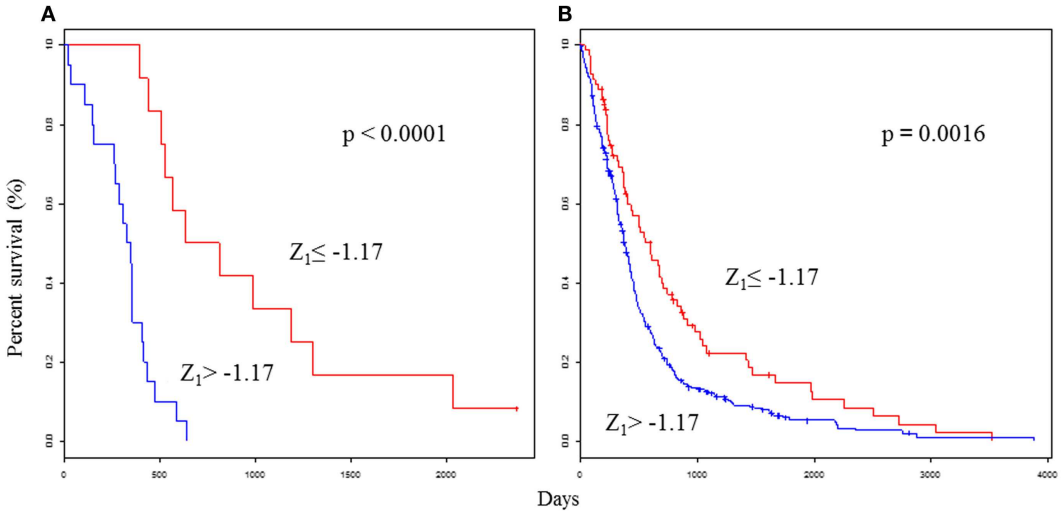

FIGURE 1 | Survival analyses using the selected 25 gene classifiers show prognostic value for glioblastoma. Kaplan–Meier curves that compare groups classified by the Z_1 PPS with the 25 gene model in the test set **(A)** and validation set **(B)**. Permission for reuse was obtained from John Wiley & Sons Ltd. and ©2013 Japanese Cancer Association.

for the identification of genes that predict OS using random survival forests models (8). The gene expression predictor, which we named the Prognosis Prediction Score (PPS), was computed from a linear combination of 25 selected genes and was calculated for each tumor as follows:

$$\begin{aligned} Z_1 = {} & 0.27 \times GPNMB + 0.09 \times EFNB2 \\ & - 0.22 \times ASF1A + 0.02 \\ & \times LOC283027 + 0.15 \times AMIGO2 \\ & + 0.22 \times IL13RA2 + 0.25 \times ITGA7 \\ & + 0.15 \times LDHA - 0.01 \times C11orf71 \\ & + 0.15 \times AFTPH + 0.15 \\ & \times TBC1D19 - 0.21 \times MED29 \\ & + 0.02 \times ACN9 + 0.29 \\ & \times SLC25A19 + 0.16 \times RPL12 \\ & - 0.09 \times ALS2CR4 - 0.14 \\ & \times C10orf88 - 0.11 \times ARHGAP39 \\ & + 0.18 \times LMAN2L + 0.29 \times CASP8 \\ & - 0.28 \times ST6GAL2 + 0.33 \times LOXL3 \\ & + 0.08 \times ANGPTL1 + 0.22 \times MRRF \\ & - 0.33 \times ARHGAP32. \end{aligned}$$

As expected, the predictor performed well in terms of patient prognosis: the improved prognosis group ($Z_1 \leq -1.17$) had a median survival time of 721 days, while the poor prognosis group ($Z_1 > -1.17$) had a significantly lower median survival time of 335 days ($P < 0.0001$; **Figure 1A**). For more practical purposes, the PPS could also be computed from a linear combination of three genes and was calculated for each tumor as follows:

$$\begin{aligned} Z_2 = {} & -0.63 \times ASF1A + 0.62 \times ITGA7 \\ & + 0.47 \times AFTPH. \end{aligned}$$

As expected, the predictor performed well in terms of patient prognosis: the improved prognosis group ($Z_2 \leq -0.76$) and the poor prognosis group ($Z_2 > -0.76$) had identical median survival times and significance scores as Z_1. The Z PPS results were compared with traditional individual indicators. Z_1, Z_2, age, PS, and subtype were significantly associated with OS in univariate analyses. Z_1 was significantly associated with OS by multivariate analyses. The PPS was the most significant feature of these clinical parameters. The PPS formula was validated in the validation set ($n = 488$), which was derived from glioblastoma patients in four external data sets (12–15). As expected, the OS was significantly higher in the improved prognosis group ($Z_1 \leq -1.17$) than in the poor prognosis group ($Z_1 > -1.17$) ($P = 0.0016$; **Figure 1B**). Two-year survival rates were 36.3 and 30.8% in the improved prognosis group, and 4.7 and 11.8% in the poor prognosis group, using the test and validation data sets, respectively. Even among glioblastomas in both test ($n = 32$) and validation sets ($n = 488$), the OS ranged between 0 and 3,880 days. Fifty-two patients (10%) survived for longer than 1,000 days. Class prediction models based on defined molecular profiles allow the classification of malignant gliomas in a manner that will better correlate with clinical outcomes than with standard pathology. Glioblastomas have a wide-ranging survival time, which requires a more precise prognostic scoring system to study novel therapeutic approaches. Therefore, the identification of molecular subclasses could greatly facilitate our ability to develop effective treatment protocols.

FUTURE PERSPECTIVES

The genetic landscape of gliomas has been revealed by the advancements of genome sequencing technology (15, 16). Researchers are now trying to develop novel therapeutic strategies based on these exciting discoveries. New therapeutic strategies, such as targeted therapies and anti-angiogenic treatments that appear promising with regard to improving the results have been reported (17). Immunotherapies have also shown promise for treating advanced solid tumors. In particular, monoclonal antibodies that block inhibitory immune checkpoint molecules and enhance the immune response to tumors such as

cytotoxic T-lymphocyte-associated antigen 4 (CTLA4) and programed cell death protein 1 (PD-1) (18, 19). Another forefront of immunotherapy research is genetically engineering T cells to target tumor cells (20). Future efforts will need to focus on development of novel therapies that appear active as monotherapies or in combinatorial regimens that modulate the host immune system. Although it is still unknown whether these novel discoveries will be suited for use in the CNS microenvironment, we are awaiting the next generation of progress for glioma immunotherapy based on the fundamental pathophysiology of this challenging disease.

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