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Cellular Immunotherapy for Malignant Brain Tumors

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1. Introduction

Glioblastoma multiforme (GBM) is the most common and most aggressive adult brain tumor with a patient median survival of 15 months from the time of diagnosis, and less than 20 weeks for patients with recurrent tumors. Current standard of care consists of multi-modality therapy including image-guided tumor resection, fractionated radiotherapy, and chemotherapy. This aggressive therapy is non-specific and highly toxic, leaving collateral damage to surrounding normal brain and systemic tissue, and is often debilitating to patients. Thus, there is a dire need for a more effective therapy that more specifically targets tumor cells while minimizing damage to surrounding eloquent cerebral cortex. Immunotherapy is based on the premise that the inherent sensitivity and specificity of immunologic reactivity could deliver tumor cell-specific therapy. Cellular immunotherapy aims to utilize the patient's own immune cells that are harvested, expanded *ex vivo*, primed against tumor antigens, and returned to the host, in order to direct an anti-tumor immune response with specificity and efficiency.

During early efforts in immunotherapy, tumor specific antigens were unknown and it was unclear whether tumor antigens could be recognized and targeted by the immune system. The identification of tumor antigens began with those expressed in malignant melanoma, and soon there was an explosion in the development of antigen specific immunologic treatments against solid tumors. In the past several years, pre-clinical models of cancer have reliably demonstrated that the immune system is capable of targeting tumor antigens and eradicating malignancies. It has also been demonstrated clinically that the human immune system is capable of recognizing antigens within malignant tumor cells with precision, and current immunotherapy research aims to induce potent antitumor immune responses to prolong patient survival. It was initially unclear if a potent immune response was inducible against brain tumors because of the immunoprivileged nature of the nervous system, but studies have demonstrated that immune effector cells can infiltrate the central nervous system (CNS) and induce efficient immune responses against intracranial tumors.

Current research in cellular immunotherapy against cancer is directed at eliciting a specific immune response against tumor antigens using active immunization with cellular vaccines or adoptive transfer of *ex vivo* activated lymphocytes. Clinical studies testing the safety and efficacy of cellular vaccines in patients with grade III or grade IV gliomas include the

administration of dendritic cell (DC) vaccines, autologous tumor cell vaccines, and tumor cell-antigen presenting cell fusions. Clinical studies using adoptive cell transfer employ a variety of techniques to expand tumor-specific lymphocytes *in vitro* prior to adoptive transfer to recipients with invasive brain tumors. This chapter will discuss both pre-clinical and clinical research in cellular immunotherapy targeting malignant gliomas.

2. Immune privilege

Cellular immune responses must afford protection without causing collateral damage to normal tissue. This is particularly important in the brain where passive and active mechanisms maintain a state of immunological privilege that limits the magnitude of the immune response. It has been demonstrated that immune responses in the CNS can be induced, the magnitude of this response is strictly regulated by the presence of the blood-brain barrier. Cerebral interstitial fluid (CIF) is secreted at the blood-brain barrier and flows within the spaces of the brain parenchyma. Cerebrospinal fluid (CSF) is formed by the choroid plexus within the ventricles and subarachnoid membrane, then flows through the ventricles to the basal cisterns, then through the subarachnoid space [1-3]. Antigens within the CNS enter the lymph nodes via the CSF which drains into the Virchow-Robbin spaces to the deep cervical lymphatic's via perivascular sheaths and through the subnasal mucosa [2, 4, 5]. The flow of CSF exits the subarachnoid space through the arachnoid granulations and through drainage along the olfactory nerve across the cribriform plate into blood circulation and cervical lymph nodes [4, 6, 7]. Antigens draining to cervical lymph nodes encounter cognate B cells and can also be processed and presented to T cells [4, 6, 7].

Immune activation occurs with a distinct hierarchy in terms of the types of responses induced [6]. Antigens that drain into the periphery via the cervical lymph nodes induce a response characteristic of a strong antibody response and the priming of cytotoxic T cell responses, but an absence of delayed-type hypersensitivity (DTH) responses with a skewing towards a Th2 phenotype [1, 2, 6, 8]. Strong humoral responses are induced in response to antigenic challenge. T cells are not endogenously found in the brain, but T cells and antibodies [9] have access to antigens in the brain, indicating that the blood-brain barrier does not entirely prohibit immune responses. Activated T cells "patrol" the CNS and return to systemic circulation, exiting through the cribriform plate, through the nasal mucosa, and then the cervical lymph nodes [1, 10]. Some studies suggest that T cells that encounter their cognate antigen are retained within the CNS [11], but do not proliferate and undergo apoptosis [12]. Alternatively, other studies have demonstrated that T cells encountering cognate antigen proliferate and differentiate into tumor-specific T cells, with enhanced effector function [1].

Professional antigen presenting cells (APC) such as DCs have not been described in the CNS. Microglia are the resident antigen presenting cells in the CNS, but DCs are present in the choroid plexus and meninges [10, 11, 13, 14]. Immunologic responses in the CNS require complex interactions between resident immune cells such as microglia and astrocytes, and peripheral macrophages, lymphocytes, and DCs [14-18]. Microglia constitutively express MHC class II antigens and T cell co-stimulatory molecules. Microglia are bone marrow derived cells that are capable of presenting antigen to T helper cells *in vivo* [19].

3. Glioma Immunology

In the past decade, tumor-associated antigens that are recognizable by cytotoxic T lymphocytes (CTL) have been identified and have the been the basis of cancer immunotherapy. In cancer patients, tumor-specific endogenous immunity can be elicited when tumor antigens are overexpressed, however the immune response is incapable of preventing tumor growth. The immunosuppressive tumor microenvironment, the low avidity of the T cells for tumors, and the low grade immune response are all contributing factors to the inhibition of the endogenous antitumor response. Glioma cells secrete immunosuppressive cytokines including transforming growth factor beta (TGF-β) and vascular endothelial growth factor (VEGF) [20-22] that contribute to tumor immune evasion. In addition, the increased frequency of T regulatory cells in tumor bearing patients plays a critical role in tumor tolerance [23-25].

Cancer vaccines are designed to augment patient immunity by boosting low-level immunity and stimulating the proliferation of higher-avidity T cells. Clinical studies have reported that immunotherapy by systemic administration of antigen-specific DCs and peptide antigens is capable of inducing an antitumor response against malignancies, including CNS malignancies [26-30].

In 1991, van der Bruggen *et al.* [31] identified a gene encoding a tumor-associated antigen recognizable by cytotoxic T lymphocytes in melanoma. Tumor associated genes and peptides were subsequently identified with potential use for cancer vaccines [32]. Peptide based vaccines consist of amino acids capable of binding to a major histocompatibility complex (MHC) class I antigen with the ability to activate tumor reactive T lymphocytes [20]. The immune response targets specific antigenic proteins generally classified as tumor specific antigens (TSA) or tumor associated antigens (TAA). TSAs are antigenic proteins uniquely expressed by tumor tissue while TAAs have a relatively much higher degree of antigen expression relative to normal tissue. Tumor antigens expressed by malignant neoplasms are broadly classified as (i) differentiation antigens, (ii) the products of viral, mutated, differentially spliced, or over-expressed genes, or (iii) metabolic pathway antigens[20]. There have been a few glioma associated antigens identified that are over-expressed in GBM, a few examples include interleukin 13 receptor alpha 2 (IL13Rα2) [33]which is a member of a group of antigens called cancer-testes antigens, and is thought to activate downstream transforming growth factor beta-1 (TGFβ-1) [34]. EphA2 is a tyrosine kinase receptor thought to play a role in mediating developmental processes, and is an antigen also over-expressed on the plasma membrane of GBM tumor cells and tumor-associated vasculature [35]. Survivin expression, which is documented in both gliomas and medulloblatomas [36, 37], inhibits caspase activation, leading to the negative regulation of apoptosis in tumor cells [38]. Telomerase is a ribonucleoprotein that maintains the length of telomeres and thus controls cell proliferation [39], and high telomerase activity has been documented in brain tumor cells [40, 41], particularly brain tumor stem cells [42]. The expression of cytomegalovirus (CMV) antigens IE1 and pp65 have been identified in glioma tissue, and in very low to undetectable levels in non-tumor tissue in the brain [43]. EGFRvIII is an exquisitely tumor-specific antigen and has the most potential for specific immunotherapy.

4. Immunosuppression in GBM

Patients with brain malignancies have impaired B and T cell immune function in part due to tumor secreted factors, but greatly due to depressed cellular immunity and increased levels

of T regulatory cells [25, 44]. T regulatory cell frequency is increased CD4+ T cell subset in lymphopenic patients bearing malignant gliomas [25, 45]. Peripheral blood lymphocytes from glioma patients proliferate poorly in response to T cell mitogens, anti CD3, and T and B cell dependent mitogens [46-48]. The total T cell compartment has limited capabilities to respond to mitogen stimulation [46, 47, 49, 50].

4.1 Immunosuppressive cytokines

Two immunosuppressive cytokines secreted by gliomas are TGF-β and VEGF. TGF-β has been isolated from malignant glioma cell supernatants, and the gene encoding for TGF-β2 was cloned from a glioma cell line [51]. TGF-β suppresses the generation of cytotoxic T lymphocytes from PBLs and tumor-infiltrating lymphocytes by inhibiting IL-2 receptor expression on T cells, reducing IL-1 and IL-2, and depressing natural killer cell activation. TGF-β also inhibits the differentiation of cytotoxic T lymphocytes, reduces IFNγ production, and downregulates MHC class II-dependent antigen expression [52, 53]. In an *in vivo* experiment using a highly immunogenic fibrosarcoma cell line, tumor cells were transfected with TGF-β cDNA and stable clones were used *in vitro* and *in vivo* to determine the effects of TGF-β on the induction of immune responses [54]. Tumor cells producing TGF-β failed to stimulate cytotoxic T lymphocyte responses, and TGF-β expressing tumors grew progressively *in vivo*, promoting a means for a immune escape [54], subsequently negatively impacting any potential antitumor efficacy of immunotherapies.

VEGF is produced by most solid tumor cells and plays an important role in tumor immunosuppression by inhibiting the maturation of bone marrow derived DCs [55, 56] by inhibiting NF-KB signaling in hematopoietic progenitor cells. In the context of DC vaccination in tumor bearing mice, inhibition of VEGF production with a blocking anti-VEGF monoclonal antibody enhanced antitumor efficacy [57], demonstrating that attenuating VEGF-mediated immunosuppression is vital to proper function of immunotherapy. VEGF and TGF-β production by tumors contribute to tumor vascularization and immune evasion, contributing to the systemic immunosuppression found in glioma patients. Monoclonal antibodies against VEGF are used therapeutically (bevacizumab) and have been shown to be efficacious against malignant gliomas [58-60]. Preclinical studies conducted in xenogeneic systems with human brain tumor bearing immunodeficient mice have demonstrated that inhibition of VEGF is efficient in prohibiting angiogenesis, leading to subsequent growth suppression of tumors [61].

4.2 T regs

The $CD4^+FOXP3^+CD25^+$ T regulatory cell subset normally comprises of 5-10% of the total $CD4^+$ compartment [62-64]. T regulatory cells inhibit T cell cytokine secretion while inhibiting endogenous or induced immune responses [65, 66]. T regulatory cells play a significant role in hindering immunity to normal and tumor antigens [67, 68], and represent an increased frequency of CD4+ cells in the peripheral blood of GBM patients [44]. Targeting T regulatory cell activity to counter their immunosuppressive effects enhances antitumor immunity in murine and human hosts. Fecci *et al.* [44] demonstrated that in a murine model of a spontaneously arising GBM, administration of anti-CD25 antibody eliminated T regulatory cell immunosuppressive function. Though T regulatory cell numbers were only partially reduced, anti-CD25 administration inhibited their function, and anti-CD25 monoclonal antibodies enabled T lymphocyte proliferation and IFNγ responses and increased tumor-specific lysis *in vitro*. In tumor challenged mice,

administration of anti-CD25 in combination with DC vaccination provided 100% tumor protection without inducing autoimmunity. Further developing strategies to deplete and inhibit T regulatory cells using monoclonal antibodies, CD25-binding immunotoxins, or pharmacologic inhibition of T regulatory cell activity is important in augmenting immunosuppression in brain tumor patients [25, 67, 68].

5. Immunotherapy

5.1 Antibody-based immunotherapy

Therapeutic use of antibodies aims to alter patient immunity by delivering monoclonal antibodies (mAb) that are targeted against TSAs or TAAs. Antitumor antibodies have been used as either naked antibodies or as vehicles to deliver radioisotopes or toxins to tumors. It is imperative that the mAb can recognize and bind to tumor tissue with high specificity and affinity, without accumulation in normal tissue. Antibody based immunotherapy has been successful for lymphomas (rituximab) and breast cancer (trastuzumab). Bevacizumab, a monoclonal antibody against the angiogenic regulator, vascular endothelial growth factor (VEGF), was approved by the FDA for the treatment of recurrent glioblastoma in 2009 [69]. Blocking VEGF is effective in normalizing abnormal tumor vasculature and increasing tumor response to radiation and chemotherapy [70].

EGFRvIII is currently the only TSA found on malignant glioma cells, but is absent from normal brain tissue. EGFRvIII consists of an in-frame deletion of exons 2-7 from the extracellular domain of the EGFR that splits a codon and produces a novel glycine at the fusion junction [71, 72]. The new glycine inserted at the fusion junction of normally distant parts of the extracellular domain results in a tumor-specific epitope not found in any normal tissue. This tumor-specific mutation encodes a constitutively active tyrosine kinase that enhances tumorigenicity [73-75] and migration of tumor cells that confers radiation and chemotherapy resistance [76-78]. The EGFRvIII mutation is expressed on the plasma membrane of up to 100% of glioma cells and is frequently found in GBM patients [79, 80]. Through the use of reverse transcriptase-polymerase chain reaction (RT-PCR) and fluorescent in situ hybridization (FISH) studies have detected the EGFRvIII mutation on 6-21% of grade III/IV gliomas that have amplified EGFR [80-82]. In addition, analysis using FACS found EGFRvIII expression in 50% of GBM samples [83]. The expression of this mutation confers a negative prognosis for GBM patients. The tumor-specific clonal expression of EGFRvIII on GBMs and its absence from normal tissues make EGFRvIII an ideal target for anti-tumor immunotherapy.

In pre-clinical systems, EGFRvIII expressing cell lines or PEPvIII, an EGFRvIII-specific 14-amino acid peptide, has been used for the generation of EGFRvIII-specific antibodies [79, 84-86], induction of cellular immune responses, or derivation of targeted toxins [87, 88]. Both murine and human chimeric EGFRvIII antibodies have been cloned for use in diagnostic immunohistochemistry and FACS [79]. Monoclonal antibodies binding EGFRvIII are rapidly internalized and have been successfully used *in vivo* in models for therapeutic radioimmunotherapy [86, 89-91]. Unarmed antibodies against EGFRvIII have demonstrated significant antitumor efficacy *in vitro* and *in vivo* in murine models. With a single intratumoral injection of Y10, an unarmed IgG_{2a} anti-EGFRvIII antibody, median survival significantly increased in mice bearing an EGFRvIII expressing intracranial tumor by an average of 286% [85] and produced 26% long-term survivors (n=117). *In vitro* experiments

demonstrated that Y10 inhibits DNA synthesis and cell proliferation in tumor cells expressing EGFRvIII by inducing complement mediated, and antibody dependent cell-mediated cytotoxicity [85, 92]. The mechanism identified for Y10 antitumor activity was shown to be Fc receptor dependent. A human chimeric antibody based on Y10 has been developed for clinical use and has been shown to induce lysis of human EGFRvIII positive malignant glioma cell lines. These data on the specificity of anti-EGFRvIII antibody mediated responses support the logic for further investigation into using tumor-specific antibodies as biologic response modifiers.

It has long been established that EGFR and its downstream signaling pathway plays a role in oncogenesis and tumor progression in malignant brain tumors. Thus arose efforts to block the EGFR pathway with the aim of inhibiting tumor cell proliferation with anti-EGFR monoclonal antibodies developed for clinical use. Faillot *et al*. [92], demonstrated the ability of anti-EGFR antibody EMD55900 to bind specifically to malignant gliomas in human patients when administered in a single dose [92]. A phase I/II clinical trial involving multiple intravenous administration of EMD55900 in 16 patients, however, did not observe measurable tumor regression [93], despite evidence of antibody accumulation at the tumor site. Imaging studies have demonstrated that systemically administered anti-EGFR antibodies are capable of reaching intracranial tumors.

EGFRvIII has also been shown to be immunogenic in humans [94]. While anti-EGFRvIII antibodies have not been identified in normal volunteers, patients with malignant gliomas develop EGFRvIII specific antibodies. Weak CTL epitopes restricted by MHC class I and class II have been identified and are sufficient to induce EGFRvIII-specific lymphocyte proliferation and cytokine production. Phase I/II clinical trials targeting this mutation demonstrated that vaccines targeting EGFRvIII are capable of inducing antitumor immunity. In a phase II multicenter trial between Duke University Medical Center and M.D. Anderson Cancer Center (FDA BB-IND-9944), 18 patients with EGFRvIII expressing primary GBMs were treated with an EGFRvIII peptide vaccine called PEPvIII, which is a 13- amino-acid peptide with an additional terminal cysteine that spans the entire *EGFRvIII* mutation [95]. The progression free survival from time of histologic diagnosis was 14.2 months. Six months after histologic diagnosis, 94% of patients were alive without evidence of progression. Six months after PEPvIII vaccination, 67% of patients were alive and progression free. Six patients developed EGFRvIII-specific antibody responses, and their median overall survival from histologic diagnosis was 47.7 months. However, those who did not develop antibody responses had an overall survival time of 22.8 months [95]. In another multicenter phase II trial at Duke University and M.D. Anderson Cancer Center, PEPvIII vaccinations were administered in 22 patients undergoing either standard-doses of temozolomide (TMZ) (200mg/m^2 per 5 days) or dose-intensified (DI) TMZ (100mg/m^2 per 21 days) [96]. This study assessed the immunogenicity of the EGFRvIII peptide vaccine under different degrees of lymphopenia in patients. At 6 months after vaccination, 75% of patients who received standard TMZ were alive and lacked evidence of radiographic progression, while 90% of patients who received DI TMZ were alive and lacked evidence of progression. According to Curran's recursive partitioning analysis, 17 of 22 vaccinated patients had better outcomes than expected when compared to historical controls (p=0.008) [96]. Anti EGFRvIII vaccines have demonstrated the capacity to induce antitumor immunity in the clinical setting, thus warrants investigation in a phase III trial.

5.2 Radiolabeled antibodies

Unlabelled antibodies can be used as delivery vehicles to administer effector molecules such as toxins or radiation directly to tumors. The specificity of tumor associated antigens guide molecules to targets using the specificity of antibodies. The most common effectors conjugated to antibodies are radionucleotides. Despite the expression of EGFRvIII, tenascin has been the most widely evaluated antigenic target. Tenascin is an extracellular matrix protein that is highly expressed in gliomas [97] and its expression increases with tumor progression and is a logical target of trials using radioimmunotherapy. Conjugating antibodies with radioisotopes has been a focus in clinical studies.

The antibody 81C6 is a radiolabeled antibody used in a number of clinical studies [98-102]. 81C6 reacts with an alternatively spliced segment of tenascin at the fibronectin type III domain. Its tumor reactivity and specificity to gliomas is superior to other anti-glioma mAbs and has been proven to be clinically safe. In a safety study at Duke University, antitenascin 81C6 labeled with 131-I was administered into the surgical resection cavity of 21 newly diagnosed GBM patients to achieve a 44-Gy boost specifically to the 2-cm margin of the resection cavity [103]. In 17 patients, 131-I was administered prior to external beam radiotherapy (XRT), and 3 patients 131-I was administered after XRT. Conventional XRT and chemotherapy was then administered. One patient opted not to receive XRT or chemotherapy. Twenty out of twenty-one total patients enrolled received the targeted 44-Gy boost and at a median follow-up of 151 weeks, medial overall survival times for all patients was 96.6 weeks [103]. This study demonstrated that this radioimmunotherapy was well tolerated with encouraging survival in patients with malignant gliomas. Other studies have demonstrated that 81C6 increased survival in patients with leptomeningeal neoplasm as well as recurrent and newly diagnosed gliomas [98, 99, 101, 102]. In a study conducted conducted at Duke University [102], 33 patients with previously untreated malignant glioma (GBM, n=27; anaplastic astrocytoma, n=4; anaplastic oligodendroglioma, n=2) were given 81C6 into the surgical resection cavity followed by conventional XRT and chemotherapy. The observed median survival for all patients was 86.7 weeks, and 79.4 weeks for GBM patients. The median survival of patients treated with 131-I in this study exceeded that of historical controls treated with conventional therapy.

211At is an alpha-emitting radionucleotide, and also emits K X-ray of sufficient energy to allow both γ-counting of tissue samples and external imaging [104]. This α-emiting nucleotide is more advantageous to gliomas than other isotopes. For example, since damage to normal tissue in the brain is most detrimental to the patient's cognitive function, specificity of isotope delivery is essential. The range of 211At particles is only up to 2 mm, thus toxicity is confined to the peritumoral area, minimizing collateral damage to normal tissue. 211At α-particles have a linear energy transfer that is ideal for maximizing biologic efficacy. The distance between ionizing events is approximately the distance between DNA strands, thus increasing the likelihood of inducing irreparable DNA breaks, thereby increasing cytotoxicity [104]. In a phase I safety study, 18 patients with histologic diagnosis of recurrent supratentorial primary malignant brain tumors were treated with 211At-labeled anti-tenascin mAb administered into the surgical resection cavity and treated with salvage chemotherapy [105]. No toxicities of grade 3 or higher were observed. The median survival in patients with recurrent GBM was 54 weeks, patients with anaplastic astrocytoma or oligodendroglioma had a median survival of 52 and 116 weeks respectively. Local administration of 211At-81C6 is safe, feasible, and may potentially provide a survival benefit in recurrent malignant brain tumor patients.

5.3 Dendritic cells and tumor immunotherapy

DCs induce, regulate, and maintain T cell immunity and are essential for the foundation of immunotherapy [106, 107]. DCs take-up and process antigens, thus playing a critical role in T cell priming and regulation of the immune response. DCs are equipped with antigen-processing machinery (APM) essential for uptake and processing of tumor-derived antigens so that tumor-derived epitopes can be cross-presented to T cells [20]. Immature (non-activated) DCs present self-antigens to T cells, inducing a tolerizing immune response by activating T regulatory cells [108]. Immature DCs do not have the ability to stimulate naïve or antigen-specific T memory cells [109, 110]. Immature DCs can take-up antigens via receptor- or nonreceptor-mediated mechanisms. Upon internalization, tumor antigens are processed and split into peptides in the cytosol or endocytic vesicles, then expressed on the cell surface in association with MHC molecules [20, 111].

Activated mature antigen-loaded DCs are responsible for antigen-specific immune responses that lead to T cell activation and proliferation into T helper and effector cells [111]. The two major DC subsets are the classical DCs (myeloid DCs) and plasmacytiod DCs. Plasmacytoid DCs are responsible for the antiviral immune response, producing high amounts of type I IFNα/β in response to viruses [112]. Classical DCs are further categorized in subsets displaying different phenotypes and functions. The skin contains Langerhans cell (LC) found in human epidermis, and the dermal layer contains two subsets, $CD1a^+$ DCs and $CD14^+$ DCs [113, 114]. $CD14^+$ DCs are geared toward mounting humoral immunity. LCs prime high avidity antigen-specific $CD8^+$ T lymphocytes [115].

Ex vivo generation of DCs has been used as a therapeutic vaccine in patients with metastatic disease for over a decade [107, 116]. DCs have the ability to activate and expand T cells that are specific for self-proteins overexpressed in tumors. To generate *ex vivo* derived DC-based vaccines from patient leukapheresed peripheral blood, the combination of cytokines used to differentiate monocytes into DCs may play a role in determining the quality of the elicited T cell response [111, 116]. DCs generated with GM-CSF and IFNα are highly potent in priming T cells [117]. DCs generated in GM-CSF and IL-15 are phenotypically Langerhans cells and are more efficient in priming melanoma antigen-specific $CD8^+$ T cells *in vitro* than DCs generated in GM-CSF and IL-4 [118]. Not all DC maturation signals are equal, thus the selection of methods for activating DCs *in vitro* also represents a critical factor in designing DC vaccines [111]. The capacity to generate large numbers of DCs *in vitro* has led to the emergence of *ex vivo* loading of DCs with tumor antigens, thus cellular DC vaccination for the induction of antitumor immunity.

A number of phase I safety and feasibility clinical studies have evaluated the use of antigen-loaded DC vaccination for the treatment of malignant glioma [26, 27, 119-121]. Yu *et al.* [122] was the first study to demonstrate that tumor-specific cytotoxicity was developed in four out of seven patients who received autologous glioma peptide-pulsed DCs. Two of the four that underwent a second surgical resection demonstrated a robust CD8+ and CD45RO+ memory T cell infiltration into the tumor [122].

EGFRvIII is an evident target for tumor-targeted immunotherapy since it is the only tumor-specific antigen in gliomas. Duke University Medical Center conducted a phase I clinical trial whereby 16 glioma patients received intradermal immunizations with autologous DCs pulsed with PEPvIII, a keyhole limpet hemocyanin (KLH) conjugate of a peptide spanning the mutated region of EGFRvIII. The logic follows that DCs injected intradermally will migrate to lymph nodes, subsequently presenting antigen to T lymphocytes [123, 124]. The

patients in this study were adults with malignant gliomas who underwent resection and radiotherapy. Patients underwent leukapheresis to collect autologous peripheral blood mononuclear cells from which to generate DCs *in vitro* using GM-CSF and IL-4. DCs were then pulsed with PEPvIII and matured in a combination of TNF-α, IL-1β, and IL-6 before administered to the patient in three bi-weekly intradermal injections [125]. No adverse events occurred upon completion of the vaccinations. Prior to vaccination, none of the patients had positive DTH reaction to neither KLH nor PEPvIII; however, after vaccination 13 of 13 evaluable patients reacted to KLH, and 5 of 13 responded to PEPvIII. *In vitro* culture of patients' cells demonstrated *in vitro* proliferation of lymphocytes in response to PEPvIII in 10 of 13 patients, and to KLH in 12 of 13 patients. Two patients in the study had a nearly complete response and remained stable for 66.7 and 56.9 months. Of the 14 patients without radiographically evident disease, the median time to progression was 13.2 months. For the patients with GBM in this study the median survival time was 110.8 weeks, significantly prolonged over the 60 week median survival of patients who undergo the standard of care. This study suggests that autologous tumor specific PEPvIII-pulsed DCs are safe and might potentially induce a potent antitumor response in glioma patients.

In a phase I trial, 12 GBM patients were given DCs pulsed with peptides eluted from the surface of resected autologous tumor in three bi-weekly intradermal injections [119]. In addition to demonstrating no adverse events occurring after DC vaccinations, the study demonstrated increased systemic and intracranial immunologic responses against autologous tumor in 50% of treated patients with a median survival of 23.4 months[119].

De Vleeschouwer *et al.* [126] reported the results of 56 patients with recurrent GBM given at least three vaccinations with autologous tumor lysate-pusled autologous mature DCs. Only one serious adverse event occurred of vaccine-related edema in a patient with gross residual disease. The total population median progression free survival was 3 months, while overall survival was 9.6 months. Fourteen percent of patients had an overall survival of 2 years. Patients were divided into three cohorts, each with shorter vaccination intervals per cohort. The authors observed an improved progression free survival in patients with the shorter vaccination intervals of four vaccinations a week apart, plus a boost with an intradermal injection of tumor lysate [126]. Although there was a limited clinical response, an observed two-year overall survival in some patients is encouraging.

Wheeler *et al*. [127] demonstrated a correlation between vaccination and immune response in GBM patients. Patients who received tumor lysate-pulsed DCs demonstrated a statistically significant correlation between vaccine-induced immunity and time to tumor progression and time to survival. Patients who received tumor lysate-pulsed DCs had a greater than a 1.5 fold increase of IFNγ production relative to pre-vaccination levels. Time to survival was significantly longer ($p=0.041$) in responders, 642 ± 61 days, than in non-responders, 430 ± 50 days when both recurrent and newly diagnosed GBM patients were analyzed.

Prins *et al*. [128] conducted a safety and feasibility trial using autologous tumor lysate-pulsed DC vaccination coupled with toll-like receptor (TLR) agonists in GBM patients. Patients received either imiquimod, a TLR-7 agonist, or poly-ICLC, a TLR-3 agonist. Previous preclinical studies by this group demonstrated that TLR agonists are capable of enhancing DC activation and migration, and T cell antitumor immunity in glioma models [128, 129]. In this clinical study, 23 GBM patients were enrolled and received three biweekly injections of glioma lysate-pulsed DCs followed by either imiquimod or poly-ICLC adjuvant

until tumor progression. The median overall survival was 31 months with a 47% three year survival rate.

5.4 RNA-pulsed DCs

Vaccine treatments dependent on large amounts of autologous tumor tissue can be limited in patients with brain tumors because of small amounts of material available after resection. Small amounts of tumor tissue is also a limitation to tumor-lysate based DC therapy because It has been argued that continuous boosting is required to maintain antitumor protection [130, 131]. The use of tumor antigen RNA-pulsed DCs demonstrably stimulates potent antitumor immunity in both murine and human cells [132, 133]. Both murine and human tumor-derived RNA can be isolated and amplified without loss of function, thus an RNA based platform will not be limited by the availability of tumor tissue [132]. RNA transfection has also been demonstrated to be a superior method for antigen-loading of DCs [134-136], in addition, RNA-loaded DCs have been found to be better stimulators of antigen-specific T cells than other methods of loading DCs [135]. In an *in vitro* comparison, electroporation is a superior method of loading RNA into DCs than lipofection and passive pulsing of RNA [134].

In early studies with prostate cancer, DCs transfected with prostate-specific antigen RNA and were capable of inducing cytotoxic T lymphocyte responses specifically against prostate-specific antigens, but not kallikrein antigens, a protein that shares homology with prostate-specific antigens. This demonstrates the specificity of the elicited immune response [133]. RNA-pulsed DC responses are not restricted to single MHC haplotype, nor a specific T cell subtype, enabling activation of both cytotoxic T lymphocytes and T helper cells [137-139].

In a phase I clinical study by Caruso *et al.* [140], tumor-RNA-loaded DCs were used to vaccinate 7 children with recurrent brain malignancies: anaplastic astrocytoma (n=1), GBM (n=2), ependymoma (n=2), pleomorphic xanthoastrocytoma (n=1), ependymoma (n=1) [141]. Two patients mounted tumor-specific immunity, and clinical responses were observed by magnetic resonance (MR) imaging in three patients (2 with stable disease, and 1 partial response). Because of the low number of patients in the study, the authors cannot demonstrate a clinical benefit, but have demonstrated the potential of this platform to elicit antitumor immunity.

Preclinical murine models of tumor challenge have demonstrated that DCs pulsed with unselected tumor-derived antigens induce potent protective immune responses without toxicity due to autoimmunity [142-145]; however in studies modeling large solid tumors, much stronger immune responses were required for protection [146, 147]. When such responses were generated against tumor-associated antigens not exclusive to tumors, severe autoimmunity was observed in some but not all mice [147]. This platform is capable of engendering a range of immune responses, and further studies are essential to find the balance between antitumor efficacy and prevention of toxicity.

Given the immense potential for the clinical use of DC-based tumor-specific immunotherapy, studies to examine strategies of maximizing DC potential are necessary. In the past decade, the ability of DC-based strategies to induce effective T-cell responses against malignant astrocytomas has been demonstrated using human DCs. DCs generated from tumor-bearing patients were fused with autologous tumor cells or pulsed with total tumor RNA or tumor lysate. Their respective abilities to generate a tumor-specific T cell

proliferation and cytotoxic response *in vitro* were examined and no significant differences were found between the various DC treatments in their capacities to stimulate T cell proliferation and induce cytotoxicity. The preclinical development of DC-based immunotherapy for gliomas warrants further investigation in the clinical setting.

5.5 Adoptive cell transfer

Adoptive transfer involves the transfusion of cells that were manipulated *ex vivo* into the patient. In the past decade, different cell types have been studied to best induce antitumor immunity in tumor bearing hosts. Different cell types that have been used include (i) peripheral blood mononuclear cells (PBMCs) or peripheral blood lymphocytes (PBL)[148, 149], (ii) lymphokine-activated killer cells (LAKS)[150-152], (iii) mitogen-activated killer cells (MAKs)[153, 154], (iv) tumor infiltrating lymphocytes (TILs)[155], and (v) antigen specific cytotoxic lymphocytes [156, 157].

In 1992, Riddell *et al*. [158] reported that the adoptive transfer of T cell clones restored viral immunity in patients undergoing hematopoietic stem cell transplant. Adoptive transfer of T cells was a way of preventing cytomegalovirus (CMV) reactivation post-transplant. Allogeneic donor peripheral blood lymphocytes (PBL) were cultured *in vitro* with CMV infected autologous fibroblasts, subsequently expanding clonogenic CMV specific CD8+ T cells, and were then transferred back into the patients. Additionally, transplants can cause reactivation of latent Epstein-Barr virus (EBV) infections that can subsequently lead to post-transplant lymphoproliferative disease (PTLD), and occurs in up to 20% of solid organ transplants. In 1994, Papadopoulos *et al.* [159] demonstrated that adoptive transfer of *ex vivo* expanded allogeneic cytotoxic T lymphocytes is capable of effectively treating EBV-associated PTLD. This was the basis of adoptive cell transfer and approaches have been expanded to target viral-associated malignancies. The development of adoptive transfer for the treatment of non-viral malignancies primarily occurred in the context of allogeneic hematopoietic stem cell transplants for treatment of hematologic malignancies and melanoma. Adoptive cell transfer was first studied in hematopoietic stem cell transplant in a non-myeloablative setting used for the treatment of chronic myeloid leukemia [160] and was further developed for solid tumors.

In 1984, Steinbok *et al.* [148] was the first to demonstrate the safety and feasibility if adoptive immunotherapy for brain malignancies, but saw no measurable benefit to patient outcome. This landmark study was based on previous observations that GBM patients had observed lymphocytic infiltrates at tumor sites [148], suggesting that there was an attempt to mount an immune response by endogenous immune cells [161, 162]. The logic follows that perhaps other systemic factors were preventing these lymphocyte infiltrates from properly reaching the tumor site, or preventing lymphocyte activation. To circumvent this and the known immune deficits of glioma patients, Steinbok and colleagues[148] collected PBMCs from patients and re-infused the cells into their post-surgical cavities. Though no beneficial clinical outcomes were observed, this study established the feasibility and beginnings of adoptive immunotherapy in CNS malignancies.

5.6 LAK cells

Lymphokine-activated killer cells (LAK) are *in vitro* activated PBMCs cultured in IL-2 that have cytotoxic capabilities. These cells demonstrably lyse autologous and allogeneic tumors, but not healthy tissue, as demonstrated in human melanoma [163]. Early human trials to treat solid tumors with LAK cells are limited however, because of dose-dependent toxicity

observed from the infusion of IL-2 into patients in attempts to expand LAK cells *in vivo*. To avoid the systemic toxicity by IL-2, Jacobs *et al.* [164] infused LAKS cells that were *ex vivo* expanded with IL-2 directly into the brain. Although this trial demonstrated a minimal benefit to patients, it did not show overall safety [165-167]. Hayes *et al.* [150] was able to demonstrate that autologous LAK cells delivered into the surgical resection cavity plus IL-2 therapy increased median survival in patients with recurrent GBM from 26 weeks in historical control patients receiving standard therapy, to 53 weeks in patients who received LAK cell therapy.

In another clinical trial, 40 GBM patients received $2.0 \pm 1.0 \times 10^9$ autologous LAK cells into their post-surgical cavity. The median interval from time of diagnosis to receiving LAK cell treatment was 10.9 months.The median survival from initial diagnosis for 31 GBM patients was 17.5 months [168]. Although this trial did not have clear survival benefits, it demonstrated the safety and feasibility of adoptive transfer of *ex vivo* manipulated cells into the CNS. The mechanisms of tumor recognition and cytotoxicity by LAK cells are unknown. Although the cells seem promising, there was limited specificity of LAK cells to tumors *in vivo*.

5.7 TILS and tumor-draining-lymph node T cells

In attempts to increase T cell specificity of adoptively transferred cells, Kitahara *et al.* [157] generated CTLs by isolating PBLs from cancer patients and cultured them *in vitro* with autologous tumor cells and IL-2. These *ex vivo* expanded cells were then re-administered back into the patient intracranially. Although this strategy generated activated tumor-specific cells, it was technically more cumbersome since it required the isolation of limited numbers of human tumor cells.

Another means of isolating tumor-specific lymphocytes is to isolate lymphocytes directly from the tumor. Autologous tumor infiltrating lymphocytes (TILS) were first demonstrated to mediate tumor regression in melanoma in 1988 [163]. In this early study, the response rate was 33%. Further studies in host preconditioning substantially increased the antitumor efficacy of TILS in melanoma [169], with clinical responses in up to 50% of patients.

The recovered TILS are found in the tumor by the time surgical resection occurs. These cells are already 'primed' against the tumor and thus have tumor-specific activation. In clinical studies, TILS were recovered from tumors and re-administered into the tumor post-surgical cavity in addition to IL-2 to enhance T cell proliferation. This was most studied in melanoma patients, but in a study by Quattrocchi *et al.* [155], six recurrent malignant glioma patients received TILS in a safety trial. Autologous TILs were isolated, *ex vivo* expanded in the presence of IL-2, then administered on treatment days 1 and 14 concurrently with IL-2. Patients also received standard chemotherapy. The study demonstrated that TILs had a dose-dependent cytotoxicity against autologous tumor, allogeneic tumor, and tumor cell lines. No significant therapy associated complications occurred above Grade 2 (by the NCI Common Toxicity Scale criteria). At the three and six month follow-up, three patients had a partial response, two had stable disease, and one patient progressed. At a 45 month follow-up, one patient had a complete response, 2 had partial responses at 48 and 47 month follow-up, and three patients expired (at 12, 12, and 18 months post-TIL administration). This pilot study demonstrated that immunotherapy with TIL intracranial administration is both safe and feasible without toxicity, but due to the small patient number of this trial, the authors cannot deduce a definitive clinical benefit [155].

In another trial, Kruse *et al.* [170]hypothesized that alloreactive cytoloxic T lymphocytes (CTL) that were sensitized to the MHC protein of the patients would provide tumor-selective targeted killing of glioma cells that express MHC. The authors collected CTLs from normal donors and cultured them with irradiated patient lymphocytes, sensitizing the normal CTLs to the patients' MHC over a 2 to 3 week period. *In vitro* assays demonstrated that the CTLs lysed targets expressing the patient MHC. CTLS were initially implanted into the tumor cavity, then patients received one to five treatment cycles every other month. Authors observed a transient toxicity at Grade 1-3. One patient showed no evidence of progression for 30 months from the start of adoptive immunotherapy. Two patients with oligodendroglioma had no evidence of disease after 80 months.

The adoptive transfer of *ex vivo* manipulated T cells that are targeted against tumor-specific antigens is an ideal platform for cellular immunotherapy. The fact that there are no known tumor-specific antigens that have been identified specifically in all glioma cells proves to be a limiting factor. Studies have successfully targeted EGFRvIII with precision using vaccination strategies, but no records of using T-cell mediated adoptive immunotherapy to target EGFRvIII have been demonstrated. Other potential glioma target antigens include IL-13R2a, survivin [171], and telomerase [172]. Interestingly, several groups have found viral antigens from human cytomegalovirus (CMV) to be expressed in nearly all GBMs, but not in surrounding healthy tissue [173]. CMV antigens could thus be an ideal target for immunotherapy. All these mentioned antigens lend themselves to generating highly tumor-specific T cell populations for the use in adoptive cell transfer.

Incredible advances in adoptive immunotherapy have been made in metastatic melanoma to maximize the clinical benefits of adoptive transfer methods by optimizing host conditioning, genetic manipulation of T cells, and optimizing *in vitro* T cell expansion conditions. Adoptive cell therapy in the context of lymphodepletion is the currently the most effective treatment for advanced refractory melanoma with objective responses greater than 50% [174].

6. Host conditioning and homeostatic proliferation

Lymphodepletion is well known to significantly enhance the antitumor efficacy of adoptive cell transfer and DC vaccination strategies in tumor bearing hosts. Lymphodepletion removes inhibitory T regulatory cells, decreases competition for homeostatic cytokines between host and transferred cells, and induces homeostatic proliferation of the few remaining host T lymphocytes. Homeostatic proliferation is a rapid expansion of T cells with the purpose of recovering normal lymphocyte counts [175]. An increase in serum levels of IL-7 and IL-15 help induce rapid proliferation of T cells with a lower activation threshold [175, 176] and differentiate into effector memory T cells that respond to antigen [45]. Lymphocytes must encounter cognate antigens and compete for these cytokines. Following this logic, B and T cells that are antigen-specific such as those provided as vaccines or as adoptively transferred antigen-specific T lymphocytes, have a competitive advantage over depleted host lymphocytes [177, 178]. Antigen-specific lymphocytes disproportionately expand to become over-represented in the host circulation both in murine models and human patients [177-179], therefore enhancing antitumor immunity [177, 178, 180].

In preclinical and clinical studies of adoptive immunotherapy in metastatic melanoma, lymphodepletion enhanced the expansion of adoptively transferred tumor-specific T cells and resulted in increased clinical responses with a greater than 50% objective clinical

response [174, 181-185]. Adoptively transferred cells undergo dramatic expansion and can constitute up to 90% of host T cell repertoire and persist for months [174]. These studies by Dudley and Rosenberg demonstrate a correlation between clinical regression of systemic disease, the frequency of tumor-specific T cells in peripheral blood, and the persistence of transferred cells *in vivo* [186]. In further studies, increased lymphodepletion to myeloablative levels that required bone marrow stem cell rescue further enhanced antigen-specific T cell proliferation as well as an increased antitumor efficacy [187]. Clinical trials conducted at the National Cancer Institute using tumor-reactive TILS and IL-2 infusion demonstrated that increasing intensity of lymphodepletion enhanced clinical responses. With maximum doses of lymphodepletion, 72% of patients demonstrated an objective response and 32% of patients had complete tumor regression [188]. Only 1 of 16 patients who achieved complete response recurred after 84 months.

7. Conclusion

Cellular immunotherapy is a highly specific therapy that is directed at eliciting an immune response against tumor antigens using passive or active immunization with cellular vaccines or adoptive transfer of ex vivo activated lymphocytes. Preclinical studies have demonstrated the clear antitumor efficacy of these therapeutic modalities. The breadth of clinical studies conducted demonstrates a lack of adverse toxicity related to immunotherapies. The curative potential of cellular immunotherapy has been successful in other solid and hematological malignancies and is currently in the early stages of use in CNS malignancies.

8. References

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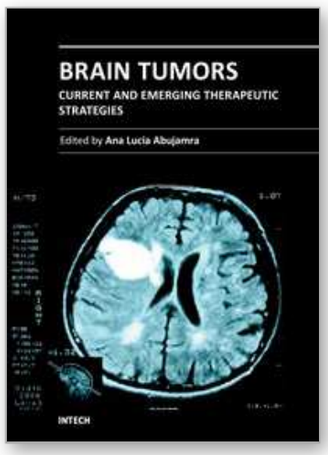

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