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The PEP-3-KLH (CDX-110) vaccine in glioblastoma multiforme patients

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Abstract

Conventional therapies for glioblastoma multiforme (GBM) fail to target tumor cells exclusively resulting in non-specific toxicity. Immune targeting of tumor-specific mutations may allow for more precise eradication of neoplastic cells. The epidermal growth factor receptor variant III (EGFRvIII) is a tumor-specific mutation that is widely expressed on GBM and other neoplasms and its expression enhances tumorigenicity. This in-frame deletion mutation splits a codon resulting in a novel glycine at the fusion junction producing a tumor-specific epitope target for cellular or humoral immunotherapy. We have previously shown that vaccination with a peptide that spans the EGFRvIII fusion junction (PEPvIII-KLH/CDX-110) is an efficacious immunotherapy in syngeneic murine models. In this review, we summarize our results in GBM patients targeting this mutation in multiple, multi-institutional Phase II immunotherapy trials. These trials demonstrated that a selected population of GBM patients who received the vaccines targeting EGFRvIII had an unexpectedly long survival time. Further therapeutic strategies and potential pitfalls using this approach are discussed.

Keywords

antigens; CDX-110; central nervous system neoplasms; epidermal growth factor receptor; immunotherapy; PEPvIII-KLH

1 Introduction: Glioblastoma multiforme & Conventional Therapy

The brain is the most frequent site of crippling and incurable human disease that account for more than 100,000 deaths each year in the United States [1]. The most common malignant primary tumor, glioblastoma multiforme (GBM), will arise in more than 15,000 Americans this year [2] and is uniformly fatal. Malignant primary brain tumors alone are more common than Hodgkin's disease and cause more deaths than melanoma or cancers of the bladder or kidney. Despite aggressive, computer-guided tumor resection [3], high doses of external beam radiation therapy [4] and multi-mechanistic chemotherapy delivered at toxic doses, the average lifespan of patients with GBMs is a little more than one year from the time of diagnosis [5,6] and patients with recurrent tumors have an even more dismal prognosis [4,7-11].

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The estimated cost of treatment for each patient with a malignant brain tumor is between $30,000 and several hundred thousand dollars annually. Thus, the annual treatment cost alone for these patients, not mentioning the lost earning potential of afflicted individuals, is greater than the entire annual budget of the National Institute of Neurological Diseases and Stroke. In fact, as a result of its ineffectiveness, conventional therapy for a malignant brain tumor represents the most expensive medical therapy per quality-adjusted life-year saved currently provided in the United States [12]. Moreover, the non-specific nature of conventional therapy for brain tumors often results in incapacitating damage to surrounding normal brain [13,14]. Thus, therapeutic strategies that are more likely to be effective against these tumors will have to precisely target tumor cells while minimizing collateral damage to neighboring eloquent cerebral cortex. The rationale for employing the immune system to target brain tumors is based on the premise that the inherent biologic specificity of immunologic reactivity could meet the clear need for more specific and precise therapy.

2 Central Nervous System Immunity

Immune responses are initiated by uptake of a protein within antigen presenting cells (APC), processing and subsequent presentation on major histocompatibility complex (MHC) class I or II [Adaptive immunity]. T cells recognize the antigen within the context of these MHC-antigen complexes via cell surface receptors – T cell receptor (TCR). More specifically, CD4 + (helper) T cells recognize peptide-class II complexes, whereas the CD8+ (cytotoxic) T cells recognize peptide-class I complexes. Full activation of a T cell requires co-stimulation resulting in the clonal expansion of the naïve T cell. Absence of co-stimulation or the presence of co-stimulatory inhibition markers can lead to a state of unresponsiveness or anergy in the effector T cell. The stimulated cytotoxic immune effector cells (CD8+) can destroy tumor cells using either perforin-induced cell lysis or Fas/APO-l receptor-mediated apoptosis.

The brain has been characterized as being “immunologically privileged”, based on the protective nature of the brain’s environment provided to allografts and xenografts. A number of vaccination strategies in experimental animals have been shown to be highly effective against tumors outside the central nervous system (CNS) but completely failed to have impact on tumors within the CNS. Patients successfully treated with biomodulators had tumor relapses within the brain despite remissions extracranially. Conventional dogma to explain the “immunological privilege” of the brain have included the absence of conventional lymphatics; however protein, lymphocytes, and macrophages can drain from the cranial subarachonoid space into the cervical lymph nodes [15]; the presence of the blood-brain barrier (BBB), but this is broken down during inflammatory processes; and the presumed paucity of APC within the CNS, however draining antigens to the cervical lymph nodes will be presented there.

In fact, CNS tumors are recognized by the immune system [16-18]; however these responses are insufficient for immunological clearance. Primed CD8+ cytotoxic T cells gain CNS access [19,20]; the lack of tumor eradication indicates that the T cells are functionally impaired within tumors. Lymphocytes in the CNS of healthy humans are a rare finding but during inflammatory responses lymphocytes are abundant within the CNS. Lymphocytes generally require activation prior to entry into the CNS [20] but antigen specificity is not necessary for entry. Further evidence supporting the fact that immunological responses can occur in the CNS is supported by the presence of antibody-secreting plasma cells in the CNS [21,22] and the therapeutic efficacy of systemically administered antibodies for the treatment of multiple sclerosis [23] and Alzheimer disease [24]. Both peptide vaccination approaches and direct instillation of glioma-specific antibodies intratumorally have been shown to mediate their in vivo efficacy via antibody dependent cellular cytotoxicity (ADCC) [25,26]. The potential limitation of immune clearance of tumors via antibodies is that large, bulky tumors may prevent sufficient antibody penetration. However, antibodies directed to the vascular component of the

tumor have demonstrated clinical success [11] as well as delivery via surgically created cavities [27]. Humoral immune responses have been underappreciated and under assessed in immune therapeutic clinical trials.

3 Immune suppression

A potential barrier to immunotherapy for patients with GBM is the well-documented impairment of T and B cell immunity in these patients. Specifically, cutaneous anergy, lymphopenia, impaired antibody production, reduced lymphocyte protein synthesis, and diminished lymphocyte responsiveness have been documented [16,17,28-40]. In addition, human GBM cell culture supernatants have been shown to suppress immune responsiveness *in vitro* [41-44], and lymphocytes recovered from such tumors and tested *in vitro* show a marked reduction in functional ability [18,42,44]. The presence of immunosuppressive activity in tumor cyst fluid [45], and the significant improvement in the immunological parameters after surgical removal of the tumor [16,28] indicate that factors secreted by these tumors mediate the immunosuppression. The impairment of cell-mediated immunity suffered by GBM patients is, in part, due to the tumor secretion of transforming growth factor-β (TGF-β) isoforms and other immunosuppressive molecules such as IL-10 and PGE_2 [41,42,46,47]. Human TGF-β1 and 2 have been isolated from GBM supernatants [42,46,47] and these cytokines have been shown to suppress the generation of cytotoxic T lymphocytes (CTLs) from peripheral blood lymphocytes and tumor-infiltrating lymphocytes by interleukin (IL)-2, to inhibit IL-2 receptor expression on T cells, to reduce IL-1- and IL-2-dependent proliferation of T and B lymphocytes, to depress the cytotoxicity of natural killer (NK) cells and their activation by γ-interferon (γ-IFN), to down regulate major histocompatibility (MHC) class II dependent antigen expression, to suppress Th1 cytokine synthesis, to inhibit the function of APCs, and to suppress the production of numerous pro-inflammatory cytokines such as tumor necrosis factor (TNF)-α, γ-IFN, IL-1, IL-6, and IL-8 [42,48-56]. The potential of immunosuppressive factors, such as TGF-β, to abolish a cell-mediated antitumor immune response has been confirmed experimentally [57]. Thus, immunosuppressive factors commonly secreted by primary brain tumors have a significant impact on the efficacy of active immunotherapies if not monitored and addressed.

Another contributor of this immune suppression is the presence of CD4+/CD25+ T_{regs} within the tumor or the T cell compartment that directly curtail immune responses. T_{regs} co-express CD4 and high levels of the high-affinity IL-2 receptor α (IL-2Rα)(CD25)[58], and can be identified by expression of the intracellular transcription factor, FoxP3. T_{regs} constitute 5-10% of peripheral CD4+ T cells in both mice and humans [58-62] and are a normal T cell subset set with the task of suppressing auto-reactive T cells. *In vitro* studies have revealed that T_{regs} potently inhibit T cell cytokine secretion and proliferation [60,63,64]. Antigen-specific stimulation through the T cell receptor (TCR) is required for activation of T_{regs}, but once activated, T_{reg}-mediated immune suppression is not antigen specific [65]. This immune suppression is mediated, at least *in vitro*, by cell-contact dependent mechanisms that are capable of inducing anergy in target cells [63,65]. T_{regs} also express cell-surface TGF-β [66] and induce other cells to secrete IL-10 and TGF-β [67] that mediate and amplify cell-contact independent inhibition of effector T cells. In a murine model, depletion of CD25+ cells from splenocytes and subsequent adoptive transfer of the remaining CD25- T cells into immune compromised mice resulted in a reproducible spectrum of autoimmune disease phenotypes [68-72], which were preventable by reconstitution with CD4/+CD25+ T_{reg} cells [58,73]. In mice, removal of CD25+ cells has not proven sufficient for eliciting experimental allergic encephalomyelitis (EAE). However, adoptive transfer of T_{regs} into EAE-susceptible mice proved capable of preventing or alleviating the autoimmunity [74-76], asserting an EAE-protective role for these cells. These combined inhibitory effects that T_{regs} exert on the responses of CD4+ and CD8+ T cells to both self and non-self antigens identify T_{regs} as a

unique and possibly potent barrier to antitumor immunotherapy. In support of this, T_{regs} are found in increased numbers in PBMCs and tumor infiltrating lymphocytes (TILs) of patients with several types of cancer [77-79], and the inhibition of tumor-specific, autologous CTL by T_{regs} have been demonstrated in a patient with colorectal cancer [80]. Furthermore, in preclinical murine studies, in vivo depletion of CD25+ cells in mice resulted in prolonged survival without concomitant autoimmunity in both tumor challenge and stringent therapeutic vaccination models [81-83].

Within the glioma microenvironment, the effector T cells can be critically suppressed/overwhelmed by the T_{regs}. Tissues from glioma patients obtained post-surgical resection have been dissociated and stained for the CD8+ and CD4+ subsets. The tumor-infiltrating CD8+ T cells were phenotypically CD8+CD25-, indicating that these cells were not activated or proliferating. CD4+ T cells were more numerous than $CD8^+$ T cells within glioma tissue and the majority of CD4+ T cells were T_{regs} [84]. The expansion of T_{regs} was significantly higher in patients within GBM than from control brain specimens [85]. Gliomas also elaborate the chemokine CCL2 that preferentially attracts T_{regs} into the tumor microenvironment especially in high-grade astrocytic tumors [86]. In murine models of syngeneic murine glioma, a time-dependent accumulation of T_{regs} was observed in the brain tumors [87]. Furthermore, Fecci et al showed that although individuals with malignant gliomas are lymphopenic with decreased CD4 counts; however, the fraction of T_{regs} is increased [88]. This increase in the T_{reg} fraction corresponded with a decrease in T cell effector functions. Moreover, *in vitro* removal of T_{regs} was shown to restore T cell function from malignant glioma patients. This data indicate that T_{regs} can not only inhibit initial systemic immune activation but also prevent the effector responses in the glioma microenvironment and as such are a potential therapeutic target.

Other mechanisms that contribute to tumor-mediated immune suppression include CNS microglia/macrophages that trigger T cell anergy by failing to provide co-stimulation [89], a paucity of professional APCs such as dendritic cells (DCs) within the CNS that can provide T cell re-stimulation necessary for optimal effector function [84], and the presence of glioma-associated mesenchymal cells and cancer stem-like cells that elaborate immune suppressive cytokines and induce T_{regs}, respectively [90]. Attempts at blocking a single immunosuppressive factor in glioma patients will likely be disappointing given the redundancy of pathways and mechanisms. Thus, combinational approaches that address the local tumor microenvironment in combination with immune activation are most likely to result in therapeutic success. Increasingly recognized by those that conduct immunotherapeutic clinical trials, is the need to negate the immune suppressive tumor microenvironment by either selecting for patients with minimal residual disease or patients early in their disease course. In the case of active immunotherapy with a vaccination approach, the balance may be tipped toward a preponderance of anti-tumor effector responses that could possibly overwhelm the immune suppressive mechanisms. However, in the case of the clinical trials with PEPvIII-KLH/CDX-110, the localized tumor microenvironment immunosuppressive effects were potentially negated by the selection of patients that had gross total resections. Despite patients with GBM having no endogenous immune responses detectable to epidermal growth factor receptor variant III (EGFRvIII), active immunization in this context is able to overcome immunosuppression sufficient to produce EGFRvIII-specific immune responses in almost all patients.

4 The prognostic impact of EGFRvIII expression

The most frequent genetic alteration associated with GBM is amplification of the EGFR gene, which results in over expression of the EGFR, a transmembrane tyrosine kinase receptor [91]. The majority of GBMs with EGFR amplification also contain the mutant EGFR gene, EGFRvIII [92], which is typically expressed in about 30% of newly diagnosed GBM patients.

The EGFRvIII is characterized by the deletion of exons 2-7, resulting in a sense mutation that has a truncated extracellular domain with ligand-independent constitutive activity [93]. Given the crucial function of EGFRvIII in mediating tumorgenesis [93], it would be anticipated that its expression would confer a poor prognosis in GBM patients. EGFRvIII is not an independent predictor of median survival in either gross total (1.1 years in EGFRvIII positive tumors versus 1.0 years in EGFRvIII negative tumors) [94] or sub-totally-resected patients [95]. The adjusted rate ratio for EGFRvIII expression is 1.07 (95% CI, 0.72-1.60) in a multivariate Cox model analysis. However, EGFRvIII is a negative prognosticator for long-term survival. In patients surviving for one year or longer, the expression of EGFRvIII is a negative prognostic indicator, with a median survival in GBM patients with EGFRvIII-positive tumors of 1.2 years versus 2.0 years in non-EGFRvIII expressing tumor patients ($p<0.0001$) [94]. This was confirmed in follow-up studies indicating that regardless of intervention (i.e. surgery, chemotherapy, radiation), that long-term GBM survivors do not usually express EGFRvIII [96].

5 Pre-clinical results of EGFRvIII peptide (CDX-110) vaccination

The EGFRvIII-specific 14-amino acid peptide, PEP-3 (H-Leu-Glu-Glu-Lys-Lys-Gln-Asn-Tyr-Val-Val-Thr-Asp-His-Cys-OH), is chemically conjugated to keyhole limpet hemocyanin (KLH) (PEPvIII-KLH/CDX-110) and has been used for the generation of EGFRvIII-specific antibodies [26,97-105], induction of cellular immune responses [25,106,107], and as a derivation of targeted toxins [108-111]. Unarmed murine antibodies targeting EGFRvIII have been shown to exert potent antitumor activity *in vitro* and *in vivo* [26]. Specifically, an Ig_{2a} antibody (Y10) was found to inhibit DNA synthesis and cellular proliferation in tumor cells expressing EGFRvIII and was capable of inducing autonomous, complement-mediated, and antibody-dependent cell-mediated cytotoxicity. While systemic therapy failed to increase median survival of mice with established intracerebral tumors, treatment with a single intratumoral injection of Y10 increased median survival by an average 286% and produced 26% long-term survivors. Another murine monoclonal antibody (IgG_{2b}) that targets EGFRvIII (mAb 806), but with reactivity against the wild-type EGFR especially when over-expressed, has also been shown to reduce tumor growth and angiogenesis, reduce EGFRvIII phosphorylation, increase tumor cell apoptosis, and down-regulate expression of the apoptotic protector Bcl-XL [103-105].

Active immunotherapy targeting EGFRvIII has also been effective [25,106]. Intraperitoneal vaccination with DCs mixed with PEP-3-KLH increased median survival by >552% (>300 days, $P<0.001$) in C3H mice challenged with intracerebral tumors [106]. The majority of mice vaccinated using this approach survived long-term without evidence of tumor, and all survived rechallenge with tumor suggesting the development of long-lasting immunological memory. More significantly, C3H mice with well-established intracerebral tumors that received a single vaccination of PEP-3-KLH in complete Freund's adjuvant without DCs showed a 26% increase in median survival time with 40% of the mice surviving long-term ($P=0.007$)[25]. To determine the EGFRvIII status of mice that failed to respond to the PEPvIII-KLH vaccination, relapsing subcutaneous tumors were evaluated by immunohistochemistry. EGFRvIII expression by immunohistochemistry was lost in 80% of relapsing tumors (n=5) after PEPvIII-KLH vaccination indicating that although there was a growth delay, EGFRvIII negative escape variants were a potential mechanism of treatment failure in active immunotherapy.

Mice vaccinated with PEP-3-KLH in the presence of an appropriate adjuvant have produced EGFRvIII-specific IgG_1 and IgG_{2a} antibodies. The induction of the IgG_{2a} antibodies appeared to correlate with an antitumor immune response [106]. Consistent with this finding, sera from successfully vaccinated mice mediated potent antibody-dependant macrophage-mediated cytotoxicity (ADMC) and passive transfer of immune sera protected against tumor challenge [25]. In C57BL/6 ($H\text{-}2^b$) mice, antitumor immune responses were shown to be dependent on

both NK and $CD8^+$ T cells. Despite this, EGFRvIII-specific cellular immune responses were not detectable in tumor-naïve vaccinated C57BL/6 mice [25] although they have not been examined in C3H ($H\text{-}2^k$) mice where the MHC Class I peptide binding site affinity is more favorable [25,106]. This suggests to us the possibility that a secondary $CD8^+$ T cell response may be induced which is critical to the antitumor effect, but which possibly may not be EGFRvIII-specific. While clinical evident autoimmune responses were not identified in these mice, the generation of secondary immune responses that may not be tumor-specific remains a possibility.

The PEPvIII-KLH vaccination demonstrated suboptimal efficacy in the C57BL/6J background (H-2B) likely secondary to the suboptimal binding of peptides spanning the EGFRvIII mutation to class I MHC as predicted at http://bimas.dcrt.nih.gov/molbio/hla_bind/. However, in the C3H background (H-2K), PEPvIII was predicted to have excellent binding to class I MHC. A similar problem may arise in certain patient haplotypes. Based on the range of binding affinities (http://bimas.dcrt.nih.gov/molbio/hla_bind/) of other tumor-specific peptides antigens capable of eliciting lymphocyte responses (http://www.cancerimmunity.org/peptidedatabase/tumorspecific.htm) patients with a haplotype background of HLA-B4403, -B2705, -B5201, -B60 or -B61 would be predicted to have homologous binding affinities to PEPvIII-KLH. One of these haplotypes, HLA-B27, is more common in glioma patients at a frequency of 19% compared to 7.5% within the general population [112]. Therefore, the overall chance that any given glioma patient would have one of the aforementioned class I haplotypes predicted to have binding to PEPvIII-KLH is 64%. It is conceivable that many patients will have efficacious vaccine responses to uncommon HLA Class I haplotypes. Although an HLA-2 restricted epitope of PEPvIII has been determined, full epitope mapping has not been performed and it is unclear whether any efficacious immune responses are dependent on HLA class for this antigen. Furthermore, responses in the context of HLA Class II and antibody responses might also be important in the immune response. Specifically, in a phase I clinical trial of glioma patients vaccinated with dendritic cells pulsed with PEPvIII-KLH and GM-CSF, PEPvIII-KLH delayed type hypersensitivity reactions were observed, which would indicate that the PEPvIII-KLH/CDX-110 is capable of elaborating class II responses as well. Given the paucity and potential unreliability of epitope mapping using currently available methods, we do not believe that screening patients on HLA type for inclusion in the ACTIVATE and ACTII study were appropriate.

6 Clinical Results

Once PEPvIII-KLH/CDX-110 was demonstrated to be efficacious in mice, clinical trials under BB-IND-9944, approved by each participating institutions IRB, were initiated. For the clinical trials with PEPvIII-KLH/CDX-110, all patients had a newly diagnosed GBM that underwent at least a 95% resection of the T1-gadolinium enhancing component of the tumor. Prior to vaccination all patients had received at least standard of care external beam radiation. Patients were not enrolled if they progressed immediately after radiation or were dependent on steroids above physiologic levels at the time of the first vaccination. In the first clinical trial (VICTORI) conducted at Duke University Medical Center, PEPvIII-KLH/CDX-110 was loaded onto autologous DCs, which were matured and used for immunization. Thirteen patients who were not screened for EGFRvIII expression received 3 vaccinations 2 weeks apart. EGFRvIII expression was not able to be obtained in a post-hoc fashion for this trial. The median time to progression (TTP) was 10.2 months and the median overall survival (OS) was 22.8 months.

A second clinical trial, A Complimentary Trial of an Immunotherapy Vaccine Against Tumor Specific EGFRvIII (ACTIVATE), was conducted at both Duke University Medical Center and M.D. Anderson Cancer Center. In this trial, 18 newly diagnosed EGFRvIII-positive GBM patients were treated with PEPvIII-KLH/CDX-110 given intradermally in GM-CSF without

accompanying DCs. The vaccination regimen consisted of 3 vaccines 2 weeks apart followed by monthly vaccinations until progression. Toxicity was minimal and there was no evidence of induced autoimmunity. Both humoral and cytotoxic EGFRvIII immune responses were enhanced in patients vaccinated with PEPvIII-KLH/CDX-110. In vaccinated patients the median TTP was 14.2 months which compared favorably to a historical control group matched for entry criteria and failure to progress after radiation (6.3 months) ($p=0.0102$). OS was 26 months compared to 15 months for the historical controls ($p<0.0001$)[112,113].

To evaluate the effectiveness of vaccination in combination with standard of care temozolomide (TMZ) chemotherapy, in a third clinical trial (ACTII), the vaccine was given in coordination with concurrent daily TMZ in monthly cycles after completion of radiation. Patients were enrolled sequentially into two groups based on the dose of TMZ given during monthly cycles and vaccinated as described for ACTIVATE. Patients in group A received TMZ at a dose of 200 mg/m^2 for 5 days of a 28 day cycle and those in group B received TMZ at a dose of 100mg/m^2 for 21 days of a 28 day cycle. Patients were vaccinated on day 21 of each cycle until progression. All patients enrolled in ACT II vaccinated in coordination with monthly cycles of TMZ have a median TTP of 15.2 months versus 6.4 months for historical controls ($p=0.0004$) and a median survival of 23.2 months versus 15.2 months for historical controls ($p=0.0004$). Patients treated in group A had a median survival of 33 months [115]. Monitoring of EGFRvIII-specific responses demonstrated that the sequential administration of CDX-110 with TMZ did not ablate immunological responses. We are currently investigating whether EGFRvIII-targeted vaccines produce responses that are strictly restricted to the EGFRvIII epitope or may be more broadly reactive.

7 Expert Opinion

In light of the documented expression of normal and fetal brain antigens on human glioma cell lines [116] and brain tumor tissue [117-120], active immunization with untested or unselected antigens risks inducing an uncontrolled autoimmune response against normal CNS antigens similar to EAE. Myelin basic protein (MBP) is the most common known antigenic trigger, but myelin proteolipid protein [121,122], myelin oligodendrocyte glycoprotein [123], glial fibrillary acidic protein, and S-100β [124] are also sufficient antigens for the induction of EAE, and many other antigens remain unidentified, which would be present in tumor homogenates and acid eluted peptides used in some types of vaccinations strategies. Humans are susceptible to the induction of EAE [125-129] and EAE can be induced in monkeys after repeated injections of homogenized CNS tissue [130] and in the various species with adjuvants [131].

Given the range of protocols that routinely use immunization with CNS tissue, the induction of such autoimmunity is a concern. Although no cases of EAE were reported in some human studies [132-135]; a careful review of the studies by Bloom *et al*. [136] and Trouillas [137] reveal one possible case of EAE in each study. Thus, the risk of EAE, or other similar and potentially lethal autoimmune responses, may limit the optimization and efficacy of active immunotherapy for CNS tumors if antigens are not selected carefully for tumor-specificity.

Although the specificity of tumor-specific vaccination therapies, such as those targeting EGFRvIII, may have the possible advantage of minimizing autoimmune complications, the heterogeneity of malignant brain tumors may limit the effectiveness of vaccinations targeting only one tumor-specific antigen. Furthermore, cross-presentation of non-targeted antigens could also lead to deleterious autoimmune responses. Conversely, multi-antigenic vaccines in this patient population have demonstrated robust immunologic responses and encouraging clinical results without the induction of autoimmunity, and catastrophic autoimmune responses have not been reported to date. Although recent human vaccine studies have not demonstrated evidence of autoimmunity, they have also not formally demonstrated any evidence of efficacy.

If more potent vaccines are required for clear evidence of efficacy, the risk of autoimmunity might become more evident.

In both the ACTIVATE and ACT II clinical trial, upon tumor progression, in the vast majority of recurrent patients, the EGFRvIII expression on the GBM was lost. This was similarly seen in our preclinical model system [25]. Furthermore, not all newly diagnosed GBM patients express the EGFRvIII target. CMV is an endemic β-*Herpesvirus* that does not usually cause significant clinical disease in adults but has been implicated in a number of human malignancies [138,139]. GBMs, but not the normal surrounding brain, express highly immunogenic *Cytomegalovirus* (CMV) antigens [140-143] which can be used as tumor-associated targets. The cellular arm of the immune system surveys and eradicates virally-infected cells, even within the “immune privileged” CNS, through the induction of well-characterized cytolytic mechanisms [144-147]. Thus, CMV-directed immunotherapy may effectively elicit the selective killing of CMV-infected tumor cells in patients with GBM. Near universal detection of the CMV immunodominant proteins pp65, glycoprotein B (*UL55*), and immediate early gene 1 protein (IE1) in GBM has been confirmed by a variety of approaches [140,148-150] including electron microscopy [140]. The subclinical reactivation of CMV has been shown in critically ill and immunocompromised patients [151]. Interestingly, astrocytic cell lines are some of the few cell lines that support CMV propagation *in vitro* [152], and a patient with AIDS associated CMV infection and malignant astrocytoma was found to have widespread infection of malignant astrocytes by CMV at autopsy with little to no spread in adjacent normal brain, indicating a preferential trophism of CMV [153]. The potential relevance of targeting CMV antigens in GBM was recently highlighted in a New England Journal of Medicine publication demonstrating the potent induction of anti-CMV immune responses in a patient immunized with tumor-lysate pulsed DCs. This patient’s tumor was subsequently shown to exhibit strong expression of CMV antigens including pp65 by immunohistochemistry [154]. Thus, vaccination to cytomegalovirus (CMV) represents an alternative therapeutic target in the scenario of GBM patients who fail to express EGFRvIII.

At the core of the development of more effective immunotherapeutic strategies against brain tumors is the simultaneous stimulation of a more potent immune response against the tumor while overcoming immunosuppressive mechanisms induced by the tumor itself. An optimal approach for modulating or suppressing the T_{reg} population for therapeutic purposes is an area of controversy. Overcoming T_{reg} immune suppression can be achieved via a variety of approaches including Ontak (denileukin diftitox; a recombinant protein of diphtheria toxin and IL-2)[155], cyclophosphamide [156], anti-CD25 antibody (targets the receptor for IL-2 [157]), cytotoxic T-lymphocyte-associated protein (CTLA)-4 blockade (inhibits co-stimulation)[158], signal transcription and activator of translation (STAT)-3 blockade agents (blocks the transcriptional activation of FoxP3)[159], by inhibiting the T_{reg} trafficking (i.e. inhibition of CCL2) with temozolomide [160], or non-specifically with lymphodepletion to augment immunological responses, which has been described in both murine model systems [156] and in human cancer patients [161]. The enhanced anti-tumor responses after lymphodepletion may be secondary to the removal of competition at the surface of antigen-presenting cells [162], enhanced availability of cytokines that augment T cell activity (such as IL-7 and IL-15)[163], and/or the depletion of the immune inhibitory T_{regs} [164]. Temozolomide can inhibit the proliferation of lymphocytes and deplete T_{regs} [165] and inhibit trafficking of T_{regs} into the glioma microenvironment [160] and in combination with PEPvIII-KLH/CDX-110 has encouraging clinical results [115]. It is possible that regulatory T cell depletion by temozolomide may be responsible for the synergy between immunotherapy and chemotherapy; however, regulatory T cells have not been rigorously quantitated before and after temozolomide therapy.

Several of the aforementioned agents are being considered in combination with PEPvIII-KLH/CDX-110 vaccine. In preclinical animal models, inactivation of T_{regs} with an anti-CD25 antibody in murine glioma models has been shown to enhance vaccination-induced anti-tumor immune responses and result in the eradication of intracerebral astrocytomas without induction of autoimmunity [157]. Similar results were also obtained in this murine model system with systemic CTLA-4 blockade. The CTLA-4 blockade reversed the $CD4^+$ T cell deficit, similarly seen in malignant glioma patients and normalized the ratio of T_{regs} in tumor-bearing mice [158]. While eliminating the suppression of endogenous antitumor immune responses through the elimination of T_{regs} may enhance tumor immune clearance, there is a potential risk of inducing autoimmunity, although that was not found in the murine models. It is likely that strategies that induce Th17 responses and not necessarily the inhibition of T_{regs} are more likely to induce CNS autoimmunity [166].

In the ACTIVATE and ACT II clinical trials, only GBM patients with gross total resections of the T1-gadlinium enhancing component were eligible for enrollment. The purpose of which was to minimize the profound immune suppressive influences of the local tumor microenvironment. However, many, and likely the majority of cancer patients, can't achieve this state of minimally residual disease. Agents that can counteract the immune suppressive influence of a bulky tumor are essential - especially those that can control multiple, redundant immune suppressive mechanisms. The signal translation and activator of transcription (STAT)-3 pathway, which is induced in diverse tumor-infiltrating immune cells [167-169], is a highly immune suppressive pathways. Activation of STAT-3 suppresses macrophage activation [170-172], limits inflammatory responses [173], reduces cytotoxicity by natural killer (NK) cells and neutrophils, reduces the expression of MHC II, CD80, CD86, and IL-12 on DCs rendering them unable to stimulate T cells and generate antitumor immunity [167-169], reduces CNS microglia/macrophage activity [89] but induces T_{regs} [159,174,175].

We have shown that an orally bioavailable, small molecule inhibitor of the STAT-3 pathway achieves excellent CNS penetration with minimal systemic toxicity [89] and has marked in vivo activity against established intracerebral syngeneic murine models of tumor. In vivo efficacy is mediated by a combination of enhanced tumor cytotoxicity and T_{reg} inhibition [159,175]. The potential of using STAT-3 inhibitors in the treatment of CNS gliomas and metastasis is evident, and especially in combination with other immune therapeutics. Since PEP-3-KLH/CDX-110 is effective in the treatment of intracerebral tumors in both murine models [25] and in GBM patients [113,114,176] by a combination of EGFRvIII-specific humoral and cytotoxic responses, synergistic activity would be anticipated with STAT-3 inhibitors since the later inhibit T_{regs}, enhance cytotoxic responses, and reverse immune suppression in the tumor microenvironment. Additionally, since many of the known inhibitors of T_{reg} activity (anti-CD25, CTLA-4 etc) have cross-reactivity to other T effector populations, the selective STAT-3 inhibitors may be superior to optimizing immune activation since T cell effector activation is a STAT-5 dependent process. Thus, the combination approach of PEPvIII-KLH/CDX-110 with STAT-3 inhibition may be beneficial to GBM patients with bulky disease who are unable to undergo surgical resection.

In summary, vaccination with PEPvIII-KLH/CDX-110 has been proven to be safe and immunogenic in multiple clinical trials for newly diagnosed GBM patients. TTP and OS in vaccinated patients is encouraging and a larger Phase II trial is ongoing (ACT III) sponsored by Celldex Therapeutics and a Phase III trial is planned (ACT IV). Other common malignancies such as breast and lung cancer also express EGFRvIII [177], so the implementation of this type of promising vaccination approach may be considered.

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