DUKE CANCER INSTITUTE

A National Cancer Institute-designated Comprehensive Cancer Center

I- ATTAC:
Improved Anti-Tumor Immunotherapy Targeted Against Cytomegalovirus in Patients with Newly-Diagnosed WHO Grade IV Unmethylated Glioma (NCT03927222)

Sponsor:	PI - Duke Cancer Institute	
Funding Source:	NIH Grant	
Protocol Source:	PI - Duke Cancer Institute	
Duke IRB#:	Pro00090683	
IND#:	16301	
Principal Investigator Mustafa Khasraw, M.D. [redacted]	**Sub-Investigator(s)** Annick Desjardins, M.D. [redacted] Henry Friedman, M.D. [redacted] Margaret Johnson, M.D. [redacted] Katherine Peters, M.D. [redacted] Kristen Batich [redacted] John Sampson, M.D., Ph.D. [redacted] Daniel Landi, MD [redacted] David Ashley, MBBS, Ph.D. [redacted]	**Statistician** James E. Herndon II, Ph.D. [redacted] **Lead Study Coordinator** Nicole Cort [redacted] **Regulatory Coordinator** Chasiety Turner [redacted] **Therapeutic Material Manufacture Manager** Gary Archer, Ph.D. [redacted] **Data Manager** Eric Lipp [redacted]

	Version Date	Description of Changes
Original version:	20180108	
Amended version:	20180205	Title page for Dr. Ashley's degrees; amended objectives/endpoints for grant submission (Sections 8 & 15); amended total number of subjects enrolled and updated number of subjects for endpoint evaluations (Sections 9 & 12); included a CBC with all immune monitoring time points (Section 12); clarified Td pre-conditioning dose and toxicity definition (Section 9); definition for progression & DC migration better described, as well as subclinical automimmunity included (Section 12).
Amended version	20180316	In response to FDA information request: For Grade 3 toxicity management, immunization will be withheld until NCI CTC toxicity improves to a Grade 1 or less (Section 9.1.3);
Amended version	20190318	CTCAE version number updated to 5, Updated the missing footnote 8 (Table 4. Schedule of Events), Title page changed the Lead clinical and regulatory coordinator, updated Sub-Investigator list, specified the size of the blood draws in the footnotes of the table (Table 4. Schedule of Events), all mentions of CBC specified auto differential, We added a specification that if leukapheresis 2 must occur earlier than scheduled, that there will be a blood draw performed at that scheduled time point, added an interim analysis in Section 15.8, added a section on Adverse events of Special Interest (Section 13.2)
Amended version	20190529	Updated the study schema (Section 6), Updated the acceptable steroid dosage, Modified the inclusion criteria to reflect radiation requirements for eligibility (Section 11), Updated the MGMT testing details to include use of CARIS services (Section 12.1).Clarified Td booster IM can be given on the day or before vaccine #1 as long as it is given prior to vaccine#1 (Table 4. Schedule of Events).Updated criteria for early withdrawal to >4 weeks of RT (Section 12.6.1). Included details of companion leukapheresis study that can likely be used for DC vaccine manufacture (Section 10.1 12.2); Vaccines 4-10 will be given every 35 (±7) days after third vaccine.
Amended version	20191009	Dr. Mustafa Khasraw, M.D will now be the PI for the study. The PI change is reflected throughout the protocol. Clarified statements in Table 4. Schedule of Events footnote.
Amended version	20200212	Subject eligibility criteria updated to make it consistent across studies in the center. KPS ≥ 70%, Serum creatinine level was changed to ≤ 3 times institutional upper limit of normal for age, serum aspartate aminotransferase (AST) ≤ 3 times institutional upper limit of normal for age (Section 11).
Amended version	20200409	Updates to title page. Added description of the use of bevacizumab (reduced dose) to treat inflammatory reactions secondary to the immune response expected with DC vaccination (Sections 9.1, 9.1.3). Change in immune monitoring blood draws (amounts, tube types, timing) (Sections 12.2, 12.7.5). Table 4 in Section 12 has been completely revised. Change in how many weeks RT should be started after surgery and PI discretion language added (Section 9.1). Change in hemoglobin and bilirubin inclusion criteria (Section 11). Change in timing of MMSE at baseline (Section 12.1). Revised language regarding initial tissue testing (Section 12.1). Change in timing for early withdrawals being replaced (Section 12.6.3). Curran Status removed (Section 12.7.2). Formatting changed and clarification made in Section 15.6.5.
Amended version	20220204	Updates made to staff on the face page. Removed ^{111}In-labeled DCs and associated SPECT/CT scan at Vaccine #4 as objective already met in an earlier completed study (see Schema in Section 6, Table 4, and Sections 12.7.4 and 15.6.1). Slight revision to exclusion criteria related to current/recent participation in another study in Section 11. Revise exclusion criteria in Section 11 to remove mention of SPECT

		scan and to allow for patients with MRI-compatible devices. Schema in Section 6 re-done so now an editable picture.

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4 LIST OF ABBREVIATIONS

Ab	Antibody
ABC	Automated Blood Count
ACD	Acid Citrate Dextrose
ACLS	Advanced Cardiac Life Support
ACTH	Adrenocorticotropic Hormone
AE	Adverse Event
AIDS	Acquired Immune Deficiency Syndrome
ALT	Adoptive Lymphocyte Transfer
APAAP	Alkaline Phosphatase Antialkaline Phosphatase Complex
ANC	Absolute Neutrophil Count
AST	Aspartate Aminotransferase
AT	Ambient Temperature
AUC	Area Under the Curve
BCR	B Cell Receptor
β-HCG	Beta-Human Chorionic Gonadotropin
BMT	Bone Marrow Transplant
BTIP	Brain Tumor Immunotherapy Program
BTSC	Brain Tumor Stem Cells
Ca^{++}	Calcium
caBIG	Cancer Biomedical Informatics Grid
CAP	College of American Pathologists
CBC	Complete Blood Count
CCL3	Chemokine (C-C motif) ligand 3
cDNA	Complimentary Deoxyribonucleic Acid
CFA	Complete Freund's Adjuvant
CFC	Cytokine Flow Cytometry
cGMP	Current Good Manufacturing Practice
CLIA	Clinical Laboratory Improvement Act
CLN	Cervical lymph Nodes
Cmax	Maximum Concentration of Drug in Plasma or Serum
CMP	Comprehensive Metabolic Panel
CMV	Cytomegalovirus
CNC	Clinical Neurologic Change
CNS	Central Nervous System
COI	Conflict of Interest
Con-A	Concanavalin A
CPC	Cancer Protocol Committee
CT	Computed Tomography
CTL	Cytotoxic T-Lymphocyte
DAR	Drug Accountability Record
DC	Dendritic Cell
DCI	Duke Cancer Institute
DI-TMZ	Dose-Intensified Temozolomide
DLT	Dose Limiting Toxicity
DNA	Deoxyribonucleic Acid
DOB	Date of Birth
DSMB	Data Safety Monitoring Board
DSMP	Data Safety and Monitoring Plan
DTH	Delayed-type Hypersensitivity

DTPA	DiethyleneTriamine Penta-acetic Acid
DUHS	Duke University Health System
eCRF	Electronic Case Report Form
IEC	Institutional Ethics Committee
EAE	Experimental Autoimmune Encephalomyelitis
EBRT	External Beam Radiation Therapy
ELISA	Enzyme-Linked ImmunoSorbent Assay
ELISPOT	Enzyme-linked Immunospot
EGFR	Epidermal Growth Factor Receptor
EGFRvIII	Epidermal Growth Factor Receptor variant type III
EGFRvIII-KLH	EGFRvIII conjugated to Keyhole Limpet Hemocyanin
FACS	Fluorescence Activated Cell Sorting
FDA	Federal Drug Administration
FEV	Forced Expiratory Volume
FFPE	Formalin-Fixed, Paraffin-Embedded
GMP	Good Clinical Practice
GBM	Glioblastoma
GM-CSF	Granulocyte Macrophage Colony Stimulating Factor
HAMA	Human Anti-Murine Antibody
H&E	Hematoxylin and Eosin
HIV	Human Immunodeficiency Virus
HLA	Human Leukocyte Antigen
HMO	Health Maintenance Organization
HRPP	Human Research Protection Program
I.C.	Intracerebral
ICF	Informed Consent Form
ICH	International Conference on Harmonisation
ICS	Investigational Chemotherapy Services
ID	Identification
I.D.	Intradermal
IDH	Isocitrate Dehydrogenase
IE1	Immediate Early Gene 1 Protein
IFN-γ	Interferon-gamma
IgG	Immunoglobulin G
IgM	Immunoglobulin M
IHC	Immunohistochemistry
IL-2	Interleukin-2
IL-4	Interleukin-4
IL-12	Interleukin-12
IL-13	Interleukin-13
I.M.	Intramuscular
In	Indium
IND	Investigational New Drug
iRANO	Immunotherapy Response Assessment in Neuro-Oncology
IRB	Institutional Review Board
ISH	In Situ Hybridization
IT	Information Technology
IUD	Intra-Uterine Device
I.V.	Intravenous
KLH	Keyhole Limpet Hemocyanin
KPS	Karnofsky Performance Status
LAMP	Lysosomal-associated Membrane Protein
Lf	Flocculation unit

LIMS	Laboratory Information Management System
MAb	Monoclonal Antibody
MFI	Median Fluorescence Index
MG	Malignant Glioma
MGMT	Methylguanine Methyltransferase
MHC	Major Histocompatibility Complex
mL	MilliLiter
MMSE	Mini-Mental Status Examination
MPACT	Molecular Products and Cellular Therapies
MRI	Magnetic Resonance Imaging
mRNA	Messenger Ribonucleic Acid
MTD	Maximally Tolerated Dose
NA	Non-adherent
NCI CTC	National Cancer Institute Common Toxicity Criteria
NCI CTCAE	National Cancer Institute Common Toxicity Criteria of Adverse Events
ng	NanoGram
NIH	National Institutes of Health
NK	Natural Killer
OARC	Office of Audit, Risk and Compliance
OS	Overall Survival
OT-I	Ovalbumin-specific T cells
OVA	Ovalbumin
PBLs	Peripheral Blood Lymphocytes
PBMC	Peripheral Blood Mononuclear Cells
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reactions
PD	Progressive Disease
PHA	Phytohemagglutinin
PFS	Progression Free Survival
PI	Principle Investigator
PO	By Mouth
PRTBTC	Preston Robert Tisch Brain Tumor Center
PT	Prothrombin Time
PTT	Partial Thromboblastin Time
QA/QC	Quality Assurance/Quality Control
RDSP	Research Data Security Plan
RIO	Research Integrity Office
RNA	Ribonucleic Acid
RT	Radiation Therapy
RT-PCR	Reverse Transcriptase Polymerase Chain Reaction
SAE	Severe Adverse Event
S.C.	Subcutaneous
SFC	Spot-Forming Cells
SOC	Standard of Care
SOCOMM	Safety Oversight Committee
SOP	Standard Operating Procedure
SPECT	Single-Photo Emission Computerized Tomography
TCR	T cell Receptor
TD	Tetanus-Diphtheria
TGF-β	Transforming Growth Factor-β
T_H2	T helper type 2
TILs	Tumor-Infiltrating Lymphocytes
TMZ	Temozolomide

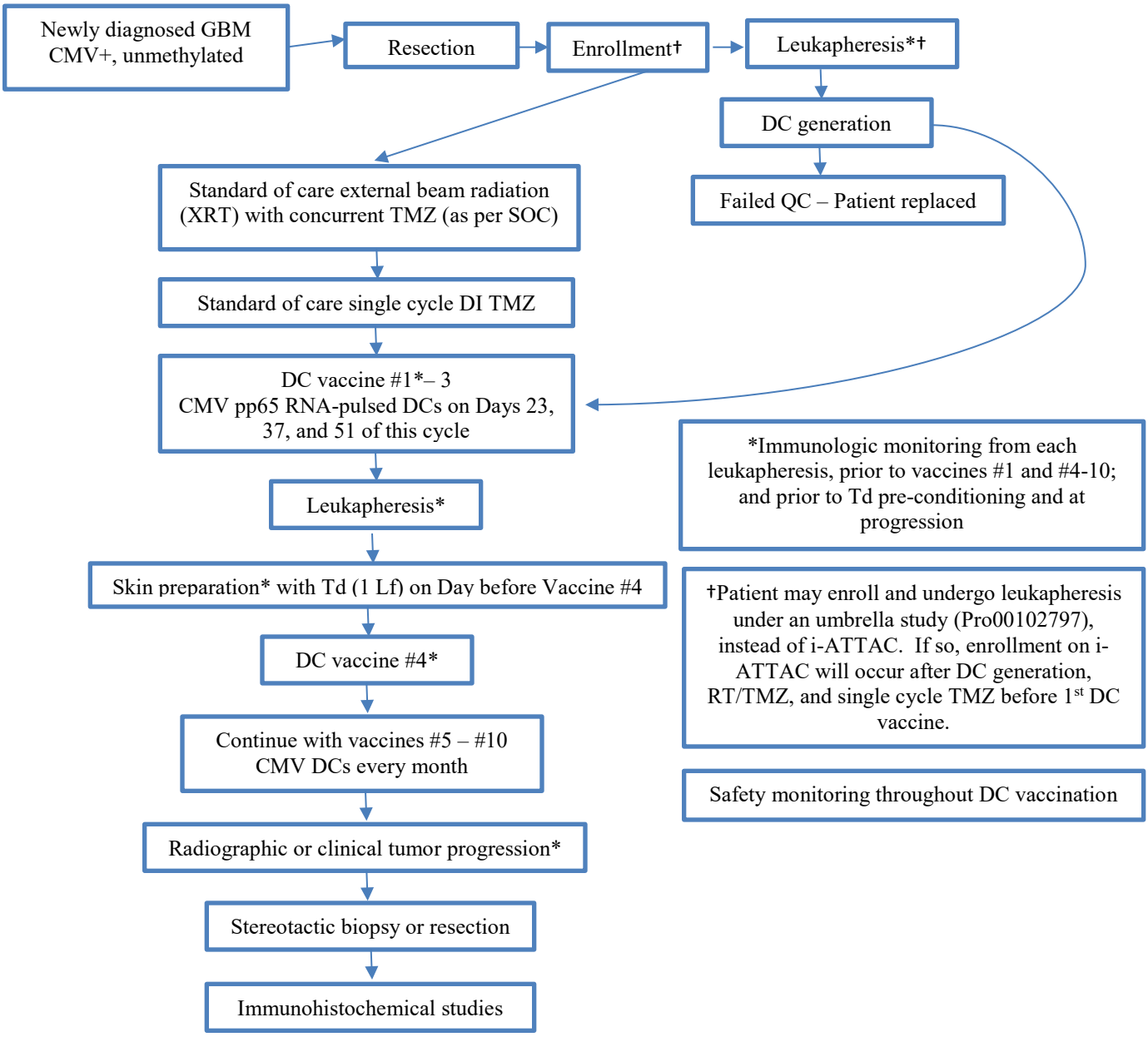
TNF-α	Tumor Necrosis Factor-α
T_{Regs}	Regulatory T cells
TTP	Time to Progression
TTRNA	Total Tumor mRNA
VDLNs	Vaccine-site Draining Lymph Nodes
WBI	Whole Body Irradiation
WBRT	Whole Brain Radiation Therapy
WHO	World Health Organization

5 PROTOCOL SYNOPSIS AND RESEARCH SUMMARY

Research Summary is available upon request.

6 STUDY SCHEMA

Figure 1. Study Schema



7 BACKGROUND AND SIGNIFICANCE

7.1 Study Disease

Malignant primary brain tumors are more common than Hodgkin's disease and account for more human deaths than melanoma or than cancer of the bladder or kidney. Despite aggressive, computer-guided tumor resection [1], high-dose external beam radiation therapy (XRT), and multi-mechanistic chemotherapy delivered at toxic doses, most patients with malignant primary brain tumors live < 15 months from the time of diagnosis, and patients with recurrent tumors usually survive < 12 weeks [2-7]. 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 mention 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, conventional therapy for patients with malignant brain tumors is the most expensive medical therapy per quality-adjusted life-years (QALYs) saved currently provided in the United States [8, 9]. Moreover, the non-specific nature of standard of care therapy for brain tumors often results in incapacitating damage to surrounding normal brain and systemic tissues [10, 11]. Thus, in order to be more effective, therapeutic strategies need 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 urgent need for more specific and less toxic therapy.

7.1 Study Agent

Human *Cytomegalovirus* pp65- Lysosomal-Associated Membrane Protein mRNA-Pulsed Autologous Dendritic Cell Vaccines Containing Granulocyte Macrophage-Colony Stimulating Factor

Dendritic cells (DCs) are potent immunostimulatory cells that continuously sample the antigenic environment of the host and specifically activate CD4+ and CD8+ T cells and B cells [12, 13]. They are at the crossroads of many of the elegant networks of the immune system, and DCs represent the most promising contemporary biologic entity for realizing the promise of immunotherapy. Potent immune responses and encouraging clinical results have been seen in Phase I and II human clinical trials in systemic cancers [14-30].

Adjuvants frequently used with vaccination include Freund's incomplete adjuvant, bacilli Calmette-Guerin, QS-21, and diphtheria toxoid. Supplemental cytokines have been used as well for the adjuvant immunological effects [31]. Granulocyte macrophage-colony stimulating factor (GM-CSF) has been commonly used, as it is commercially available and well tolerated. GM-CSF is capable of stimulating macrophage function, inducing proliferation and maturation of DCs, and is able to enhance T cell stimulatory function. Intradermal administration of GM-CSF enhances the immunization efficacy at the site of administration in a dose-dependent fashion at an optimal dose of 125 μg [32]. Significant anti-tumor immunity has been demonstrated in preclinical murine studies in which irradiated, stably transfected tumor cell lines secreting GM-CSF have protected against subsequent tumor challenge, especially against intracerebral tumors [33, 34]. Furthermore, the potency of GM-CSF has been demonstrated in a Phase I clinical trial in melanoma patients vaccinated with irradiated autologous melanoma cells engineered to secrete GM-CSF [35]. The

immunization sites were intensely infiltrated with T cells, DCs, macrophages, and eosinophils in 100% of evaluable patients. Extensive tumor destruction was seen in 11 of 16 patients. Both cytotoxic T-cell and antibody responses were associated with this tumor destruction. Hence, GM-CSF has an extensive track record both as a growth factor and an adjuvant, is commercially available and has an acceptable toxicity profile. In a recently published study from our laboratory, we reported that immediately following a course of dose-intensified temozolomide (DI-TMZ), given over 21 consecutive days, the administration of three sequential *Cytomegalovirus* (CMV)-specific DC vaccines resulted in significantly increased CMV-specific cellular responses and superior overall survival (OS). Median progression free survival (PFS) and OS for this cohort were 25.3 months [95% confidence interval (CI), 11.0–∞] and 41.1 months (95% CI, 21.6–∞), exceeding projected survival using recursive partitioning analysis (RPA) and matched historical controls. Moreover, four patients remained progression-free at 59 to 64 months from diagnosis. No known prognostic factors (i.e. age, KPS, IDH-1/2 mutation, and MGMT promoter methylation) were biased to predict more favorable outcomes for the patients in this cohort [36].

Human CMV is an endemic β-Herpesvirus that does not usually cause significant clinical disease [37]. During primary maternal infection, however, human CMV can cause severe encephalitis in fetuses and lead to congenital brain defects. Human CMV disease is also a significant problem in immunocompromised adults such as organ transplant recipients or patients with Acquired Immune Deficiency Syndrome (AIDS) [37]. Herpesviruses have also been implicated in a number of human malignancies including lymphoma, nasopharyngeal cancer, cervical cancer, and Kaposi's sarcoma [38, 39]. Recently, expression of proteins unique to human CMV has been reported within a large proportion of malignant tumors including colorectal carcinoma, prostate cancer, and malignant astrocytomas [40-42]. Universal detection of the human CMV immunodominant protein pp65, immediate early gene 1 protein (IE1), and several other early antigens was demonstrated using immunohistochemistry (IHC) in Grade II-IV astrocytomas [42]. Presence of the virus in these samples was confirmed with *in situ* hybridization (ISH), polymerase chain reactions (PCR) for human CMV-specific glycoprotein B (UL55), electron microscopic detection of intact virions [42], and direct detection of the virus from fresh operative samples in the shell vial assay (unpublished data). Notably human CMV antigens were not detected in surrounding normal brain samples, meningiomas, or brains affected by ischemia, Alzheimer's disease, paraneoplastic encephalitis, or *Cryptococcal cerebritis*.

The presence of highly-immunogenic human CMV antigens within malignant gliomas (MGs) affords a unique opportunity to target these tumors immunologically. There is a vast amount of experience with both the safety and efficacy of immunotherapy targeting human CMV [37], and the presence of this virus within brain tumors may allow this experience to be leveraged toward the effective eradication of MG expressing human CMV antigens. Adoptive T cell therapy has been used to safely and successfully protect against CMV reactivation in myelodepleted bone marrow transplant (BMT) patients [43-46]. In addition, T cell mediated immunotherapy has proven highly effective in the treatment of CMV-associated disease within the central nervous system (CNS) [47] and in the treatment of acute CMV infections [44, 48]. Tumors associated with other human Herpesviruses, such as Epstein-Barr virus-associated lymphoma, including tumors within the CNS, have also been effectively treated and even large tumors have been cured by immunotherapy [49-54]. More recently, a vaccine directed against the potent viral antigens of

human papilloma virus has also been shown to reduce the incidence of human papilloma virus-related cervical intraepithelial neoplasia in a prospective, randomized, double-blind trial [55].

The potential for non-specific targeting of normal tissues is thought to be minimal in seropositive patients. After initial infection, CMV establishes lifelong latency in the infected individual, with cells of the myeloid lineage constituting a major reservoir for persistence of the virus. Virus can be detected within myeloid progenitors in the bone marrow, with a small portion of these cells demonstrating viral deoxyribonucleic acid (DNA) replication without any detectable gene expression [56, 57]. Also a small proportion (typically 1 in 1,000 to 1 in 10,000) of peripheral blood monocytes can be found to contain CMV DNA, while detection of viral ribonucleic acid (RNA) (gene expression) is not detected [58-60].

Vaccination specifically against CMV[61-65] has effectively reduced the risk of viral infection and transmission to fetuses in animal models[66-68] and in clinical trials [61, 65, 69-72]. Human clinical trials have also demonstrated some benefit of administering neutralizing antibodies in the treatment of human CMV infection [73-76], highlighting the importance of the development of vaccination strategies that elicit both cellular and humoral immune responses. DCs strongly activate both T cell and B cell responses *in vivo* [13], and DCs pulsed *in vitro* with CMV antigens have been shown to be potent inducers of CMV-specific cytotoxic T-lymphocyte (CTL) responses in several studies [77-81], in addition to our own work which is outlined below.

The use of RNA to encode tumor antigens for DCs was pioneered at Duke University in Dr. Gilboa's laboratory, but the ability of RNA-loaded DCs to stimulate potent antitumor immunity has been independently confirmed in murine and human systems [82-87]. In fact, there is accumulating evidence that RNA transfection represents a superior method for loading antigens onto DCs [84, 88]. This novel and innovative approach to DC antigen loading has multiple conceptual advantages over other forms of antigen delivery as well. RNA-based antigen loading does not require knowledge of major histocompatibility complex (MHC) restriction, and responses are not restricted to single MHC haplotypes or to a narrow B or T cell repertoire. This diversity increases the likelihood of inducing effective and sustained antitumor immune responses by simultaneous activation of both CTLs and helper T-cells [89-91]. Furthermore, in direct comparisons, RNA-loaded DCs have been found to be better stimulators of antigen-specific T-cells than other approaches[88]. Finally, RNA also carries a significant safety advantage, not possessed by other nucleic acid or viral vectors, in that it cannot be integrated permanently into the host genome. In addition to the preliminary data we present below, Kobayashi *et al.*[92] have demonstrated that tumor mRNA-loaded DCs can elicit a specific CD8+ CTL response against autologous tumor cells in patients with MG.

Temozolomide (TMZ)

TMZ, a methylating agent with superior blood-brain barrier penetration, has recently been shown to increase survival by a small, but statistically significant, 2.5 months in a subset of patients with newly-diagnosed GBM if given in conjunction with XRT following initial tumor resection of the tumor [93-95]. Leukopenia, diminished white blood cell counts in the host, is essentially the only known human toxicity of TMZ treatment. Although initially counter-intuitive, this TMZ-induced leukopenia may actually be advantageous in treating patients with immunotherapy due to the

subsequent homeostatic proliferation of newly generated lymphocytes that it induces. In this protocol, patients will receive standard of care six week course of TMZ concurrent with XRT following initial resection of the tumor, followed by a dose-intensified 21-day cycle of TMZ prior to DC vaccination. We believe that the lymphopenia induced by therapeutic TMZ treatment, if carefully timed before vaccination, will actually enhance the proliferation and maintenance of these tumor-specific T-cells through the natural forces that drive T cell homeostatic proliferative recovery. Thus, this combination strategy will uniquely exploit the toxicity of one effective therapy for MG to enhance another already promising therapy, immunotherapy. In preparation for this protocol, we have evaluated, in animal models, TMZ and sublethal whole body irradiation (WBI), as a positive control, as methods for induction of treatment-induced lymphopenia in order to determine the ability of TMZ-induced lymphodepletion to enhance active vaccination and adoptive immunotherapies. These studies were initially performed in a murine T cell receptor (TCR) transgenic model in which the antigen-specific T-cells can be followed *in vivo* in mice receiving adoptive transfer of lymphocytes. In this model system, we found that adoptive lymphocyte transfer (ALT) coupled with DC vaccination (DC + ALT) is a potent mechanism for inducing antigen-specific T-cell expansion after TMZ treatment. TMZ was found to be an effective agent for inducing homeostatic proliferation of transferred CD4+ and CD8+ T cells and for enhancing DC or peptide vaccinations with or without ALT (Figure 2).

Markedly elevated levels of antigen-specific T-cells were achieved and maintained in mice receiving ALT after therapeutic TMZ-induced lymphodepletion compared to normal hosts receiving ALT. The precursor frequency of ovalbumin (OVA)-specific CD8+ T cells in the peripheral blood of untreated mice receiving OVA peptide vaccine after transfer of OVA antigen-specific (OT-I) transgenic T cells was 1.5%. Mice pretreated with TMZ and subsequent OVA peptide vaccination had achieved a mean precursor frequency of 54%, exhibiting markedly increased levels of OVA-specific T-cells in the blood ($P < 0.0001$). Similar results were found in mice receiving DC vaccines, with untreated animals having an average precursor frequency of 3.6% and TMZ pretreated animals achieving an average of 11.9% OVA-specific CD8+ T-cells after a single vaccination. These results demonstrate that active and adoptive immunotherapy administered during hematopoeitic recovery from TMZ treatment may be a very effective way to enhance the efficacy of immunotherapy.

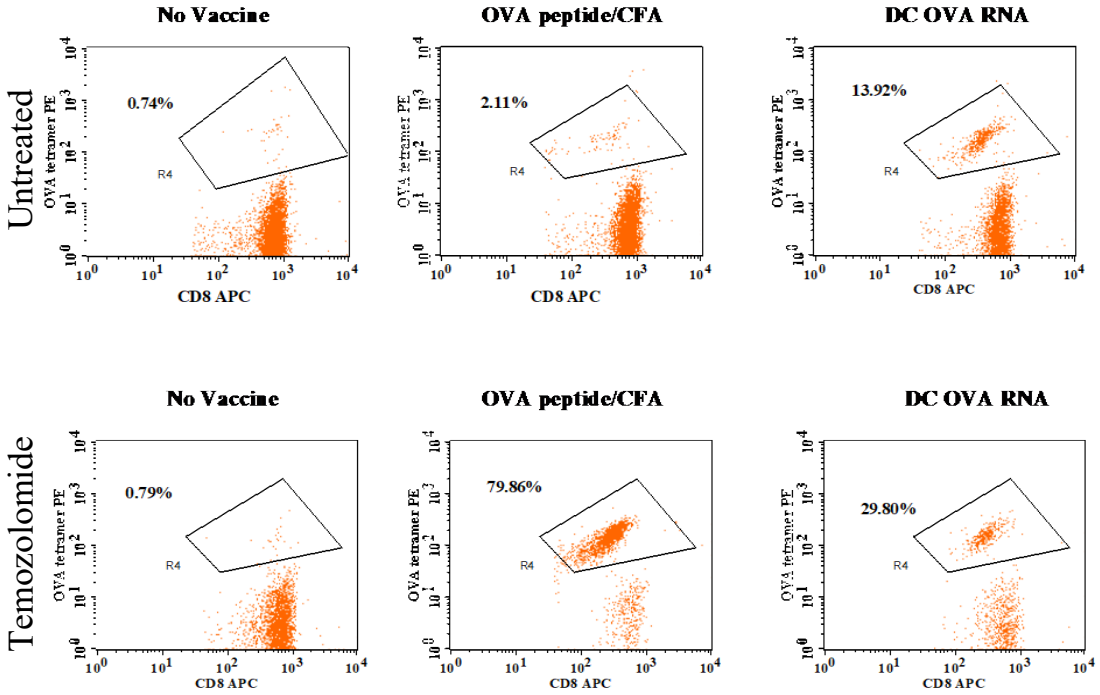


Figure 2: Homeostatic Lymphodepletion Induced by Temozolomide Enhances Antigen-Specific Immune Responses. Untreated mice or mice receiving non-myeloablative high-dose temozolomide treatment (5 days x 60 mg/kg) were administered a mixture of OVA-specific and normal lymphocytes intravenously and received either no vaccine, vaccination with DCs transfected with OVA RNA, or OVA peptide vaccination in complete Freund's adjuvant. The peripheral blood of treated animals was collected and analyzed 6 days after vaccination for the expansion of OVA-specific T cells in the circulation using αCD8 monoclonal antibodies and a specific tetramer. As shown above, mice treated with temozolomide prior to ALT demonstrated markedly increased T cell expansion after vaccination compared to untreated hosts receiving ALT.

^{111}Indium-labeling of Cells for *in vivo* Trafficking Studies

The *in vivo* distribution of DCs will be evaluated using ^{111}Indium (^{111}In)-labeled pp65-lysosomal-associated membrane protein (pp65-LAMP) messenger RNA (mRNA)-loaded mature DCs. ^{111}In-labeling has been used extensively for evaluation of adoptively transferred tumor infiltrating lymphocytes, natural killer cells, granulocytes, DCs, and whole blood leukocytes, for *in vivo* localization studies in humans [96-99]. DCs will be labeled at the Duke Radiopharmacy Lab according to standardized protocols. Briefly, 2 x 10^7 pp65-LAMP mRNA-loaded mature DCs will be labeled with ^{111}In (50 µCi / 5 x 10^7 DCs) (Nycomed-Amersham, Chicago, IL) prior to injection. Intradermal DC injections sites will be carefully marked at 10 cm from the groin crease. Gamma camera (dual-headed) images will then be taken immediately after injections (0 hour), and at 24 and 48 hours after the DC vaccination to compare DC migration from the inguinal intradermal injection sites to the inguinal vaccine site-draining lymph nodes (VDLNs). Regions of interest will represent the quantity of ^{111}In signal and be gated on VDLNs with calculations corrected for isotope decay. The quantity of DC uptake in VDLNs will serve as the migration efficiency of the cellular vaccine, and these migration rates will be analyzed for correlation with OS in these patients. This will serve to address the secondary objective if migration rates of DC vaccines given after Td pre-conditioning correlate with OS of patients receiving this therapy. Beginning with

protocol version 20220204, ^{111}Indium-labeling of cells has been discontinued as the objective has already been met in a previously completed study entitled ELEVATE (Pro00054740).

Tetanus-Diphtheria Toxoid (Td)

The current use of Td toxoid is for active immunization in children and adults against infection with the bacteria *Clostridium tetani* and *Corynebacterium diphtheria*. Tetanus infection is manifested primarily by neuromuscular dysfunction caused by a potent exotoxin released by *C. tetani*. Diphtheria is an acute toxin-mediated infectious disease caused by toxigenic strains of *C. diphtheriae*. Protection against disease is due to the development of neutralizing antibodies to the diphtheria toxin. Td toxoids adsorbed are readily available as several approved administrations [i.e. Daptacel (DTaP), Infanrix (DTap), Tenivac (Td adult), Boostrix (Tdap)][100, 101]. Protection against disease is due to the development of neutralizing antibodies to the tetanus toxin. A serum tetanus antitoxin level of at least 0.01 IU/mL, measured by neutralization assays, is considered the minimum protective level. A level ≥0.1 IU/mL by ELISA has been considered as protective[102]. A serum diphtheria antitoxin level of 0.01 IU/mL, measured by neutralization assays, is the lowest level giving some degree of protection; a level of 0.1 IU/mL by enzyme-linked immunosorbent assay (ELISA) is regarded as protective. Diphtheria antitoxin levels ≥1.0 IU/mL by ELISA have been associated with long-term protection[103].

Following deep subcutaneous/intramuscular (s.c./i.m.) administration of the tetanus toxoid vaccine, toxoid molecules are taken up at the vaccination site by immature DCs, which are professional antigen-presenting cells. Within these cells, they are processed through the endosomal pathway (involving the phagolysosome) where they are bound to MHC type II molecules on the surface of DCs. The MHC II:toxoid complex then migrates to the cell surface. This cellular processing is similarly thought to occur for native immature DCs at the skin site and for vaccinated DCs. Upon encounter with tetanus toxoid, the now activated mature DC at the vaccine site migrates along lymph channels to the VDLN where they encounter naive T_H2 cells, each with their own unique T cell receptor (TCR). Identifying and then binding of the MHC II:toxoid to the specific T_H2 receptor then activates the naive T cell, causing it to proliferate. Simultaneously, toxoid molecules not taken up by DCs pass along lymph channels to the same draining lymph nodes where they come into contact with B cells, each with their own unique B cell receptor (BCR). Binding to the B cell through the specific immunoglobulin receptor that recognizes tetanus toxoid results in the internalization of toxoid, processing through the endosomal pathway and presentation on the cell surface as an MHC II:toxoid complex, similarly to DCs undergoing the same process [104].

These two processes occur in the same part of the lymph node with the result that the B cell with the MHC II:toxoid complex on its surface now comes into contact with the activated T_H2 whose receptors are specific for this complex. The process, termed linked recognition, results in the T_H2 activating the B cell to become a plasma cell with the production initially of IgM, with a later switch to IgG antibodies produced. Additionally, a subset of these B cells becomes memory cells[104].

The novelty of using Td toxoid vaccination lies in the ability of this potent recall antigen to enhance antitumor responses as part of a cancer vaccination protocol. Td toxoid induces an inflammatory

milieu within the intradermal vaccine site, thereby promoting the migration of injected tumor-specific DCs. Additionally, in the context of vaccinating the host with tumor-derived peptides, conditioning the vaccine site with Td toxoid has demonstrated enhanced immunogenicity with these peptides.

Our data from the ATTAC clinical trial (NCT00639639) demonstrating the capacity to enhance DC migration to VDLNs via Td pre-conditioning of the vaccine site offer potential therapeutic interventions whereby we can enhance the immunologic responses to ultimately overcome the inherent challenges in faithfully eradicating established tumors. In a completed randomized clinical trial, we found that migration of injected DCs to VDLNs following vaccine site pre-conditioning with Td toxoid was significantly increased compared to controls and that the efficiency of DC migration was strongly associated with clinical outcomes of patients with newly-diagnosed GBM, the most fatal type of malignant brain tumors. To address this observation, we took our Td pre-conditioning platform back into the preclinical setting using transgenic mouse models and were able to corroborate the effects of Td pre-conditioning on increasing the lymph node homing of intradermally administered DCs. Moreover, Td pre-conditioning at a single vaccine site increased the migration of a bilateral DC vaccine to both inguinal lymph nodes. Regardless of the side of the Td intradermal skin prep, DC migration to bilateral inguinal VDLNs was increased to similar magnitudes, supporting a systemic response to recruit peripherally administered DCs.

Our Td pre-conditioning platform in the context of DC vaccination also elicited superior anti-tumor responses compared to controls receiving DC vaccines without Td pre-conditioning. In our clinical trial, patients with newly diagnosed GBM who were administered the Td skin prep before DC vaccination revealed significantly longer progression-free and overall survival rates compared to the control cohort. In evaluating the relationship between DC migration and clinical responses, we observed a modest positive association between levels of DC migration and survival. In our preclinical model, Td pre-conditioning prior to vaccination with tumor antigen-specific DCs dramatically suppressed the growth of established and highly aggressive B16-F10/OVA tumors. The use of Td with a DC vaccine increased antitumor responses in an antigen-specific manner, as non-specific DC vaccines were not potentiated with Td pre-conditioning. Furthermore, in a challenge setting, where mice are administered the treatment platform prior to challenge with tumor inoculation, Td pre-conditioning at the vaccine site induced a significant survival benefit compared to controls.

7.1.1 Pre-Clinical Experience

In our laboratories and those of others, systemic immunization using DCs co-cultured with uncharacterized tumor homogenate [105], whole tumor RNA [106], unidentified peptides eluted from tumor cells by gentle acid washing [107], or a distinct peptide encompassing the tumor-specific epidermal growth factor receptor variant type III (EGFRvIII) mutation [108], have been shown to induce humoral and cell-mediated systemic immune responses and to prolong the survival of rodents with brain tumors.

In our laboratory [105], inbred VM/Dk mice received three or four weekly intraperitoneal injections of autologous bone marrow-derived DCs transiently co-cultured with tumor homogenate. The homogenate was derived from a syngeneic murine astrocytoma cell line derived from a spontaneously occurring astrocytoma in the inbred VM/Dk mouse strain. Splenocytes from

mice immunized in this way were able, in vitro, to lyse the astrocytoma cell line that was used to generate the tumor homogenate. They were also able to lyse other astrocytoma cell lines derived from the same inbred mouse strain, but they had no effect against syngeneic fibroblasts. Similarly, these immunized mice also demonstrated a significantly increased antibody titer against the astrocytoma cell line used to generate the homogenate. In addition, mice immunized with DCs transiently co-cultured with tumor homogenate that were subsequently challenged with a lethal dose of this astrocytoma cell line intracerebrally were found to have a median survival >160% longer than those immunized with DCs cultured without tumor homogenate (P = 0.016). In addition, 50% of the mice treated with the tumor homogenate-supplemented DCs survived long-term without any evidence of tumor growth and also survived a rechallenge of tumor cells indicating that a sustained antitumor immune response had been established. These findings are especially significant in light of the fact that the astrocytoma cell line used is known to secrete the immunosuppressive agent transforming growth factor-β (TGF-β) which is secreted by most human gliomas [109-113].

In another report from our laboratory [106], C57BL/6 mice received three weekly intraperitoneal injections of autologous bone-marrow derived DCs co-cultured with tumor homogenate or whole tumor RNA derived from the poorly immunogenic B16F10 melanoma cell line. Standard *in vitro* cytotoxicity assays again revealed that splenocytes harvested from mice immunized with DCs transiently co-cultured with either tumor-derived homogenate or whole tumor RNA were able to lyse B16F10 melanoma cells but not unrelated tumor cells from the same MHCbackground. In this experiment, mice immunized with autologous bone-marrow derived DCs co-cultured with tumor homogenate or whole tumor RNA increased median survival by > 233% (P = 0.0006) and 48% (P = 0.0001), respectively, relative to mice immunized with DCs co-cultured with tumor homogenate or whole tumor RNA derived from an unrelated tumor with the same MHC background. In addition, 8/13 (61.5%) in the specific homogenate group and 4/10 (40%) in the specific RNA group survived beyond the endpoint of the study without evidence of tumor. Immunization of mice with pre-existing tumors with specific tumor homogenate also demonstrated the potency of this immunization approach by increasing survival by 62.5% relative to controls. In these mice, an inflammatory infiltrate composed of mononuclear cells and polymorphonuclear leukocytes was identified, only in mice treated with DCs co-cultured with tumor homogenate that matched the intracerebral tumor challenge.

In a recent report from another laboratory [107], the survival of tumor-bearing rats injected subcutaneously with autologous bone marrow-derived DCs co-cultured with peptides eluted from tumor cells with a gentle acid wash was significantly prolonged compared to tumor-bearing rats receiving equivalent numbers of DCs co-cultured with peptides acid-eluted from normal astrocytes (P < 0.05). Median survivals in these groups were 35 and 22 days, respectively. In addition, three of the twelve rats (25%) treated with DCs co-cultured with acid-eluted tumor peptides remained alive at the end of the experiment. In addition, immunohistochemical analysis of five animals from each group in this experiment documented an increased peritumoral and intratumoral infiltration of CD8+ T-cells, and to a lesser extent CD4+ T cells and macrophages, in the group treated with DCs co-cultured with peptides acid-eluted from tumor cells when compared to controls.

7.1.2 Clinical Experience

Prior Experience in Patients with GBM Receiving GM-CSF-Containing DC Vaccines

In two previous studies conducted under Investigational New Drug (IND)-12839 and one under IND-13240, we have vaccinated patients with newly-diagnosed GBM with DCs loaded with mRNA encoding the human CMV matrix protein pp65. To date, a total of 59 patients have received 545 vaccinations with pp65-loaded DCs.

One patient in the ATTAC-GM trial (NCT00639639, IND-12839), which utilized pp65-specific DC vaccines admixed with GM-CSF, had a severe Grade 3 immunologic reaction after receiving vaccine #8 [114]. The patient developed flushing of the face, reddening of the eyes, nausea, tunnel vision, headache, raised red bumps on the chest, back, antecubital spaces of both arms, and large red swollen areas in both injection sites. Allergy and Immunology was consulted and recommended testing the individual components of the vaccine for hypersensitivity. The components were separated into two parts: GM-CSF and DCs pulsed with pp65 RNA. Immunologic workup for this patient revealed sensitization to the GM-CSF component of the vaccine and the production of high levels of anti-GM-CSF autoantibodies during vaccination (Figure 3, Figure 4). Removal of GM-CSF from the DC vaccine allowed continued vaccination (total of 10 vaccines) without incident for this patient. No other study drug AEs were detected in this study. The allergic reaction is detailed in the MEDWATCH submitted May 16, 2011, IND 12839 serial number 34.

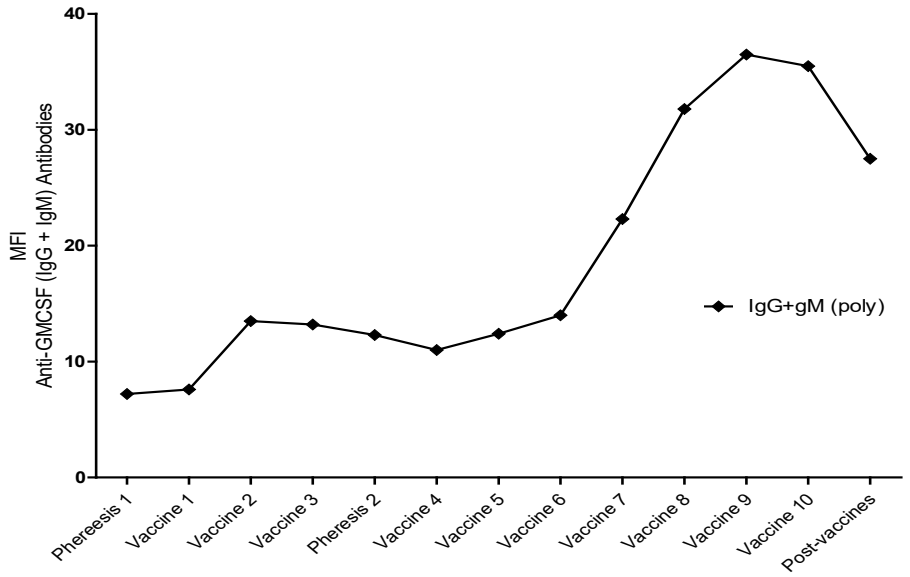


Figure 3: Polyclonal Antibody Median Fluorescence Index (MFI) Against GM-CSF is Plotted Over Time Coinciding With Vaccine and Apheresis Administrations in One subject. Anti-GM-CSF Antibodies (IgG+IgM) Increases Over Time With Repeated Vaccinations Using Recombinant GM-CSF and Begin to Decrease After the Adjuvant Was Removed.

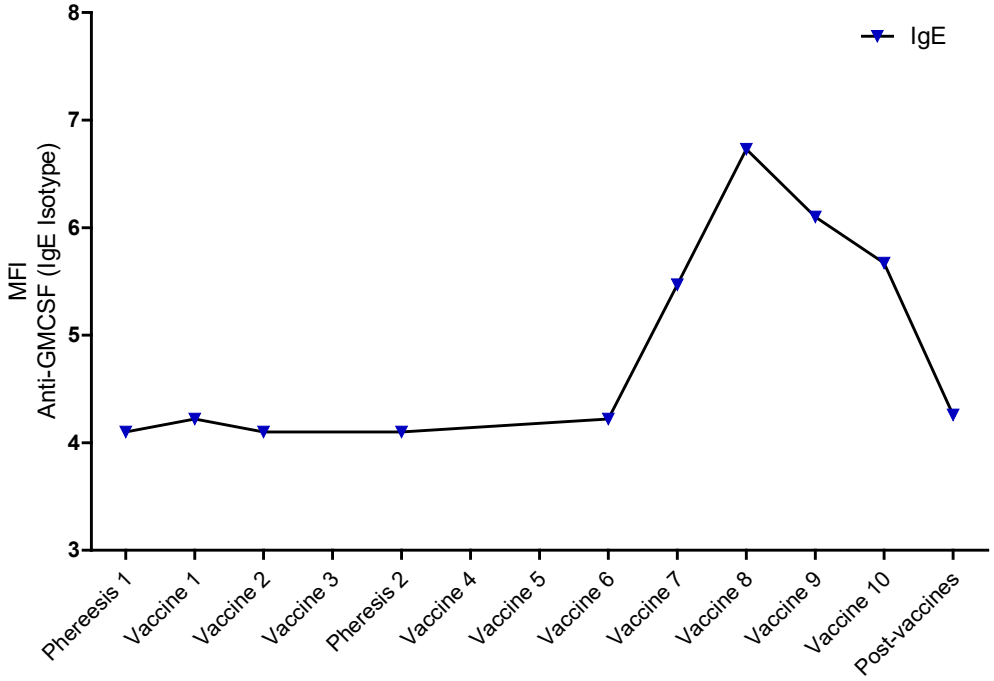


Figure 4: IgE Antibody Median Fluorescence Index (MFI) Against GM-CSF is Plotted Over Time Coinciding With Vaccine and Apheresis Administrations in One Subject. IgE Antibody Fluorescence Against GM-CSF Peaks at Vaccine#8, Consistent With the Subject's Anaphylactic Episode at This Time Point Before Slowly Decreasing Back to Baseline After Adjuvant Removal.

Intradermal, intravenous and intralymphatic migration studies using ^{111}In labeled DCs have been successfully done in both animal models and patients with no adverse effects [99, 115-117].

Under IND-12839, we have used labeled DCs with ^{111}In and used single-photo emission computerized tomography (SPECT) imaging to track the migration to the VDLNs. This work was published in *Nature* [118].

There is a vast amount of experience with both the safety and efficacy of immunotherapy targeting human CMV [37]. Vaccination specifically against CMV has effectively and without toxicity, reduced the risk of viral infection and transmission to fetuses in clinical trials [61, 65, 69-72]. Adoptive T-cell therapy has been used to safely and successfully protect against CMV reactivation in myelodepleted bone marrow transplant patients [43, 44, 46, 119]. In addition, T cell-mediated immunotherapy has proven highly effective in the treatment of CMV-associated disease within the central nervous system [47] and in the treatment of acute CMV infections [44, 48]. Tumors associated with other human Herpesviruses, such as Epstein-Barr virus-associated lymphoma, including tumors within the CNS, have also been effectively treated and even large tumors have been cured by immunotherapy [49-52, 54, 120]. The pp65 minimal epitope peptide (495-503) was shown to be safe in doses up to 10mg in hematopoietic stem cell transplant patients, with grade 1 adverse events being most commonly reported in approximately 10-15% of the participants and was successful in expanding CMV reactive T-cells [121].

7.2 Study Purpose/Rationale

Our Overall Goal in this project is to extend our substantial pre-clinical and clinical findings and verify these in a larger cohort. We hypothesize that CMV pp65-loaded DCs given with Td and GM-CSF to a larger group of patients with TMZ-induced lymphodepletion will confirm our previous findings by inducing significant DC migration to draining lymph nodes, producing high levels of Chemokine (C-C motif) ligand 3 (CCL3) and polyfunctional CMV pp65-specific T cells systemically, and confirming extra-ordinary PFS and OS.

We will conduct a larger Phase 2 trial of CMV pp65-loaded DC vaccination in patients with GBM. We will confirm our observations from smaller studies, in order to determine if a Phase 3 trial is warranted and to estimate patient numbers that might be required. Patients with CMV positive, newly-diagnosed GBM will receive serial vaccines with CMV pp65-loaded DCs with Td and GM-CSF vaccine site pre-conditioning after standard of care treatment with concurrent external beam radiation and TMZ chemotherapy. Only patients that are methylguanine-DNA methyltransferase (MGMT) unmethylated will be enrolled because adjuvant TMZ shows very little benefit in that population so it can be avoided after the initial treatment that induces transient lymphodepletion.

Next, we will also confirm predictive biomarkers of survival. In our prior studies, DC migration to draining lymph nodes and systemic CCL3 and CMV pp65-specific polyfunctional T cells predicted PFS and OS. Here we will collect samples to confirm these predictive biomarkers prospectively.

Strikingly, Td-treated patients had systemically enhanced DC migration to VDLNs and significantly improved PFS and OS compared to controls (median PFS 18.5 vs 10.8 months, OS 25.7 vs 18.5 months) with half the Td cohort living > 44.1 months from diagnosis. Our murine studies corroborated that Td pre-conditioning systemically enhanced DC migration and suppressed tumor growth. In the proposed clinical study, we aim to enhance the migration of CMV pp65 RNA-pulsed DCs with Td and adjuvant GM-CSF and will assess if increased migration using these strategies can be predictive of patient survival outcomes.

8 OBJECTIVES AND ENDPOINTS

Table 1. Objectives and Endpoints

	Objective	Endpoint	Analysis
Primary	Determine if Td preconditioning of pp65 loaded DC vaccination with GM-CSF extends overall survival.	Median survival from initiation of dose intensive adjuvant temozolomide.	See Section 15.5
Secondary	Assess the association between migration of CMV pp65 RNA-pulsed DCs with GM-CSF to site-draining inguinal lymph nodes after Td pre-conditioning and subsequent survival. (PLEASE NOTE: With Protocol v.20220204, this objective has been removed as it was already addressed in a previous study.)	For survival after vaccine 4, the hazard ratio associated with a 1 unit increase in migration, defined as the maximum percentage of ^{111}In-labeled DCs reaching inguinal nodes during the 48 hours after the 4th vaccination.	See Section 15.6.1
Secondary	Assess the association between CCL3 measured post-Td pre-conditioning and subsequent survival.	For survival after vaccine 4, the hazard ratio associated with a 1-unit increase in CCL3 measured post-Td pre-conditioning at vaccine 4.	See Section 15.6.2
Secondary	Assess the association between fold changes in T cell polyfunctionality and subsequent survival.	Mean fold change from baseline in the frequency of pp65 antigen-specific CD8$^+$ T cells producing three or more cytokines (IFNγ, CCL3, IL-2, TNFα, CD107a) at leukapheresis 2 14± (4) days after vaccine 3. Mean fold change from baseline in the frequency of pp65 antigen-specific CD8$^+$ T cells producing three or more cytokines (IFNγ, CCL3, IL-2, TNFα, CD107a) at vaccine 6. For survival after vaccine 4, the hazard ratio associated with a 1-fold increase in the frequency of pp65 antigen-specific CD8$^+$ T cells producing three or more cytokines (IFNγ, CCL3, IL-2, TNFα, CD107a).	See Section 15.6.3
Secondary	Assess whether T_{Reg} levels remain low without adjuvant temozolomide.	Maximum mean peak increase from vaccine 1 in percent T_{Reg} of CD4+ T cells.	See Section 15.6.4
Secondary	Assess the safety of DI-TMZ treatment followed by DC vaccination.	Proportion of patients who experience unacceptable toxicity.	See Section 15.6.5
Exploratory	Assess the association CCL3 levels and migration.	Mean fold change in CCL3 between pre- and post-Td pre-conditioning. Correlation between CCL3 change from fold change in CCL3, and migration.	See Section 15.7.1
Exploratory	Assess the association between pp65-specific polyfunctional CD8+ T cells and increased DC migration.	Correlation between fold change from baseline (pre-vaccine 1) in polyfunctional CD8+ T cells and migration.	See Section 15.7.2
Exploratory	Assess the association between pp65-specific polyfunctional CD8+ T cells and increased serum CCL3.	Correlation between fold change in polyfunctionality and fold change in CCL3	See Section 15.7.3

9 INVESTIGATIONAL PLAN

9.1 Study Design

Approximately 64 patients with resected, newly-diagnosed WHO Grade IV glioma who are CMV+ and in which the MGMT is not methylated will be accrued to this study before standard of care radiation therapy (RT) and concurrent TMZ, with the goal of treating 48 patients with dose-intensified temozolomide and pp65 loaded dendritic cell vaccine after completion of standard RT and TMZ.

All enrolled patients will undergo a leukapheresis after resection for harvest of peripheral blood lymphocytes (PBLs) for generation of DCs. Patients will then receive approximately 6 weeks of standard of care radiation therapy (RT) and concurrent TMZ at a standard targeted dose of 75 mg/m^2/d (please see Package Insert uploaded in electronic IRB for standard therapy). Patients should start RT within approximately 7 weeks of surgery. [PI discretion can be used in this situation to allow patients outside of this window to enroll.] Patients who either experience progressive disease during radiation, are dependent on steroid supplements above 2 mg/day at time of first vaccination, are unable to tolerate TMZ, or whose DCs fail to meet release criteria will be withdrawn from the study and replaced. For patients whose initial leukapheresis yields less than 3 vaccines, repeat leukapheresis may be obtained a minimum of 2 weeks from the previous leukapheresis (and may be repeated as needed) if pre-apheresis blood work is within the Apheresis Center's parameters and as long as this does not cause a significant delay in treatment for the patient. At the post-RT clinic visit the patient's MRI and blood work is evaluated before starting a single post-RT cycle of dose intensified TMZ (100 mg/m^2/day for 21 days). On day 23 (± 2 days) of the cycle, patients will receive the first of 3 pp65 DC vaccines. DC vaccines #1-3 will be given every two weeks (± 2 days). All patients will receive up to a total of 10 DC vaccines, with vaccines administered every 35 days (± 7 days) after the third vaccine, given bilaterally at the groin site unless progression occurs with no further cycles of TMZ. DC vaccines will be given intradermally (i.d.) and divided equally to both inguinal regions. Before the first DC vaccination, patients will receive 0.5 mL of Td (tetanus and diphtheria toxoids adsorbed) intramuscularly into the deltoid muscle to ensure adequate immunity to the tetanus antigen. The Td vaccine will be obtained through Duke Investigational Chemotherapy Services (ICS) Pharmacy. Patients will undergo leukapheresis again for immunologic monitoring with specific assessment of baseline antigen-specific cellular and humoral immune responses if needed for further DC generations 14 (± 4) days after vaccine #3. Prior to pp65 DC vaccination #4, (3±1) weeks after leukapheresis 2 the vaccine site will receive a pre-conditioning intradermal injection of Td (1 flocculation unit (Lf), in 0.3 mL of saline for a total of 0.4 mL).

As part of standard care for these patients, upon tumor progression, participants may undergo stereotactic biopsy or resection. As this is not a research procedure consent will be obtained separately. However, if tissue is obtained, it will be used to confirm tumor progression histologically and to assess immunologic cell infiltration and pp65 antigen escape at the tumor site. No other treatment intervention is allowable while on study, unless the patient needs bevacizumab at 7.5 mg/kg IV approximately every 3 weeks per the "Special Considerations" in Section 9.1.3.

9.1.1 Definition of Unacceptable Toxicities

Toxicities will be graded according to the National Cancer Institute Common Toxicity Criteria of Adverse Events (NCI CTCAE) version 5 criteria. An unacceptable toxicity is defined as any Grade 3 or greater toxicity that is possibly, probably, or definitely attributed to the pre-conditioning agent Td or pp65 DC vaccine that does not resolve to baseline in 2-3 weeks; any Grade 3 hypersensitivity reactions or autoimmune toxicity requiring steroids or hormone replacement; and is not due to progressive disease, or any life–threatening event not attributable to concomitant medication, co-morbid event, or disease progression. Rules for monitoring unacceptable toxicity are provided in Section 15.6.5.

9.1.2 Dose Modification

TMZ will be administered concomitantly with standard external beam RT under the direction of the oncologist. Please see TMZ package insert uploaded in electronic IRB for standard of care treatment guidelines. The dose of the concomitant TMZ with RT may be adjusted at the discretion of the treating oncologist.

The study drug (CMV pp65 RNA-pulsed DCs with GM-CSF) dose will not be modified in this trial. Those subjects whose cells fail to meet release criteria will be removed and replaced.

9.1.3 Safety Considerations

Management of Toxicities

If a Grade 3 NCI CTC or greater toxicity is seen that is not attributable to a concomitant medication, co-morbid event, or disease progression that has been documented radiographically or clinically, the next immunization for that patient will be withheld for up to 2 months or until the NCI CTC toxicity improves to a Grade 1 or less. However, planned procedures requiring hospitalization, or long-term clinical decline that is now seen in patients years from whole brain radiation therapy (WBRT), which are clearly not related to study drug, but are the natural development common in this patient population, will not be considered an unacceptable toxicity nor will have immunizations withheld.

Special Consideration

Due to the impact of high dose steroids on the development of an optimal immune response, if a patient demonstrates neurologic or cerebral radiographic signs suggestive of a localized inflammatory reaction, secondary to the immune response triggered by DC vaccination, that requires an increase in dexamethasone dose, every effort should be made to not increase the dose above 4 mg per day at any time. Instead, patients should be treated with bevacizumab at the reduced dose of 7.5 mg/kg IV approximately every 3 weeks. If a patient requires planned treatment for their tumor with bevacizumab > 7.5 mg/kg approximately every 3 weeks, they will be considered off study and enter the follow-up phase. Neuroimaging (MRI) will be performed according to protocol schedule and, at that time, it will be assessed whether further treatment with bevacizumab is needed to control the cerebral inflammation. Bevacizumab will not be provided by the study. Every attempt should be made to reduce or discontinue dexamethasone, when clinically possible, so as not to mitigate immune response.

If there are AEs or other circumstances prohibiting the use of bevacizumab, corticosteroids, surgery, or other interventions deemed more appropriate for the patient by the treating physician will be used, if needed, to treat any localized inflammatory reaction secondary to DC vaccination.

Adverse Event Reporting and Documentation

An "Adverse Event" will be defined as any adverse change from the subject's pre-treatment baseline condition (which is assessed at the post-RT/TMZ clinic visit), including any clinical or laboratory test abnormality that occurs during the course of research. Adverse events will be categorized and graded in accordance with the NCI CTCAE (Version 5).

A "Serious Adverse Event" will be defined as an undesirable sign, symptom or medical condition which: 1) is fatal or life threatening; 2) requires inpatient hospitalization for > 24 hours or a prolongation of existing hospitalization; 3) results in persistent or significant disability/incapacity; 4) constitutes a congenital anomaly or a birth defect and/or; 5) medically significant such that it may jeopardize the subject, and may require medical or surgical intervention to prevent one of the outcomes listed above.

A summary of all adverse events (not just those considered related to study drug) that occur following RT/TMZ will be kept which will categorize the event by organ system, relationship to treatment, its grade of severity, and resolution. Periodic review by the primary investigator (PI) and weekly review at the Duke Preston Robert Tisch Brain Tumor Center (PRTBTC) Adverse Event (AE) meeting of the collective adverse events will occur with the intention of identifying any trends or patterns in toxicity. If any such trends are identified, depending on their severity and frequency, a protocol amendment will be considered.

All adverse events which are serious and unexpected should be reported immediately to Dr. Mustafa Khasraw M.D. at 919-684-5301 during regular business hours or at 919-2060493 after hours and to the Federal Drug Administration (FDA). Fatal or life-threatening, unexpected adverse events will be reported to the FDA by telephone, facsimile, or in writing as soon as possible, but no later than 7 calendar days after first knowledge by the sponsor followed by as complete a report as possible within 8 additional calendar days. Serious, unexpected adverse events that are not fatal or life-threatening will be reported to the FDA by telephone, facsimile, or in writing as soon as possible, but no later than 15 calendar days after first knowledge by the sponsor.

All adverse events that are considered serious, unanticipated, and related or possibly related to the research (as defined by 21CRF312.32[a]) will be reported to the Duke University Medical Center Institutional Review Board (IRB) using the appropriate serious adverse event (SAE) report form. At the time of the annual progress report to the Duke University Medical Center IRB, a summary of the overall toxicity experience will be provided.

9.1.4 Missed Doses

To ensure that repetitive DC vaccines will be given to patients, the initial 3 will be given every 2 weeks (± 2 days), and the monthly DCs will be given every 35 days (± 7 days). Therefore, with a margin of 2 days all patients will receive up to 10 DC vaccines or until progression, whichever comes first. At the discretion of the study PI, patients who miss vaccine administration for whatever reason will have their appointment re-scheduled to administer the vaccine as soon as possible and the study PI will assess whether this deviation needs to be filed with the IRB. For continued non-compliance with the scheduled vaccine appointments, the subject may be removed from the trial at the discretion of the study PI.

9.1.5 Concomitant Medications

Concomitant medications will be managed by the treating oncologist and recorded at each study visit by the study coordinator.

9.2 Rationale for Selection of Dose, Regimen, and Treatment Duration

In our previous randomized clinical trial of 12 patients with newly-diagnosed GBM, we evaluated the impact of vaccine site pre-conditioning with Td toxoid [118]. Patients randomized to Td showed increased dendritic cell migration bilaterally and significantly improved progression-free and overall survival. Furthermore, we observed a modest association between effective DC migration to VDLNs and clinical outcomes. Based on the small sample size of the initial study, we would like to conduct a validation study with Td pre-conditioning that is powered sufficiently to evaluate the impact of DC migration on clinical outcomes.

9.3 Rationale for Correlative Studies

Please see Section 12.7.4 ^{111}Indium-labeling of Cells for *in vivo* Trafficking Studies. PLEASE NOTE: With approval of Protocol v.20220204, this correlative study will no longer be conducted.

9.4 Definition of Evaluable Subjects, On Study, and End of Study

Subjects evaluable for the analysis of the primary endpoint of overall survival will include all patients who initiate DI-TMZ.

Once the patient signs an informed consent form (ICF), that subject will be considered "on study." Rationale for taking patient off protocol treatment will be documented.

9.5 Early Study Termination

This study can be terminated at any time for any reason by the PI-sponsor. If this occurs, all subjects on study should be notified as soon as possible. Additional procedures and/or follow up should occur in accordance with Section 12.6, which describes procedures and process for prematurely withdrawn patients.

10 STUDY DRUG

10.1 Names, Classification, and Mechanism of Action

Tetanus-Diphtheria Toxoid (Td Toxoid adsorbed)

Td is indicated for active booster immunization against tetanus, diphtheria, and pertussis as a single dose; substitute 1-time dose of Tdap for Td booster, then standardly boost with Td every 10 years. Please refer to Section 9.1 on the use of Td in this protocol.

DC Vaccine

Human CMV pp65-LAMP mRNA-pulsed autologous DCs with GM-CSF is the name of the study drug given with every vaccine. This vaccination will then consist of administration of 2 x 10^7 CMV pp65-LAMP mRNA loaded mature DCs as described above. In up to 16 patients, the fourth vaccine will be labeled with ^{111}In (50 μCi / 5 x 10^7 DCs) (Nycomed-Amersham, Chicago, IL) prior to injection. The class of action for all study drugs is a biological with the exception of the tetanus toxoid, which is an antitoxin.

GM-CSF (LEUKINE®; Sargramostim)

GM-CSF will be obtained from commercial supply and stored in the ICS, as a sterile, white, preservative-free powder lyophilized powder in a vial containing 250 mcg to be reconstituted in 0.5 mL of sterile water for injection.

Leukapheresis and Dendritic Cell Vaccine Generation

At least two leukaphereses may be performed on each patient enrolled on this protocol. The leukapheresis will be used for DC generation and immunologic monitoring. If the subjects were part of the companion study (Pro00102797) and have already undergone the leukapheresis procedure the DCs from that study will be used for the current study and any unused vaccine products will be handled as indicated by the patient in the companion study. All leukaphereses will be approximately a 4-hour leukapheresis. It is estimated that 10-12 L of blood will be processed during this leukapheresis. An additional 4-hour leukapheresis will be requested from patients with positive immunological responses to the therapy for additional immunologic monitoring studies or for patients who need additional DCs generated to ensure a total of 10 vaccines.

DCs will be generated from the first leukapheresis *in vitro* by 7-day culture with GM-CSF and IL-4. Peripheral blood mononuclear cells (PBMC) for *in vitro* generation of DCs will be obtained by leukapheresis at the Duke Apheresis Unit and transported to the Cell Processing facility. For patients without sufficient venous access for leukapheresis a temporary intravenous catheter may be inserted.

At the end of the 7 day incubation for generation of DC, a sample of the media is taken for mycoplasma testing, the cells are then harvested and electroporated with pp65-LAMP mRNA. The DCs are placed in a flask with AIM V media GM-CSF + IL-4 + TNF-α + IL-6 + IL-1β at 37^{O}C, 5% CO_2 for 18-20 hours for maturation. The cells are washed twice with PBS and frozen at 2-4 x 10^7cells/mL in 90% autologous human AB serum (Valley Biomedical, Winchester, VA 22602), 10% DMSO and 5% dextrose.

The DCs are then stored until needed at –135ºC. After freezing, an aliquot of cells is thawed for QA/QC. This testing will look at viability, (>70%) endotoxin content, (<5 E.U. /Kg B.W.) mycoplasma contamination (negative) and sterility testing for aerobic and anaerobic bacterial cultures (1 x 10^6 DCs) and fungal cultures (1 x 10^6 DCs).

For each vaccination, cells that have passed quality assurance/quality control (QA/QC) will be rapidly thawed at 37ºC, washed three times with phosphate buffered saline (PBS) and counted. The cell concentration will be adjusted to 5 x 10^7 cells/mL and DCs will be resuspended in preservative free saline and GM-CSF and placed into a sterile tuberculin syringe with a 25-gauge needle.

For <u>all</u> DC preparations, and ^{111}In-labeled DCs, from the final preparation a sample of cells will be sent for Gram stain and endotoxin testing prior to administration. DC vaccination will not be given until endotoxin testing has been passed (< 5.0 E.U/Kg) and the Gram stain has been found

to be negative. An aliquot of cells will also be sent for aerobic and anaerobic bacterial cultures (1 x 10^6 DCs) and fungal cultures (1 x 10^6 DCs).

The SOP *MPACT-QA-0017 Responding to Sterility Test Results* describes the roles, responsibilities, and actions that should be taken in the event of a positive, delayed, incomplete, or invalid sterility test result for a product manufactured in the MPACT facility after it has been administered to a study subject. Briefly, personnel informed of the result will notify MPACT Management who is responsible for notifying all relevant study personnel. The Principal Investigator or treating Neuro-Oncologist (or his or her designee) will notify the patient. The patient will be asked to be evaluated by a physician within 48 business hours. Based on the route of administration of the investigational product, appropriate cultures (aerobic, anaerobic, and fungal) will be ordered for the subject (blood cultures or wound cultures) along with CBC and CMP tests. If the patient has or develops a temperature ≥38.5°C or clinical evidence of infection, the patient will be treated expectantly with antibiotics based on the sensitivities of the organisms identified from the immunization product, as medically necessary. An independent infectious disease consultation will be obtained to guide further therapy if medically necessary. MPACT management and MPACT QA will perform an investigation into the sterility tst result. If there are remaining vials from the affected batch, a vial will be sent for repeat sterility testing, and the remainder of the batch will be quarantined until re-testing is complete with negative results. Administration of subsequent products from the affected batch will be held, and upcoming study visits should be rescheduled as necessary. Immunizations will proceed only if the patient fully recovers and subsequent samples are found to be sterile.

10.2 Packaging and Labeling of Study Agents

- For CMV pp65-LAMP mRNA-pulsed DCs and CMV pp65-LAMP mRNA loaded mature DCs labeled with ^{111}In (50 μCi / 5 x 10^7 DCs):

Vaccine Label
Patient Name / MRN
DOB / ALPS #
Trial Name and Number
Date
FOR AUTOLOGOUS USE ONLY
NOT EVALULATED FOR INFECTIOUS SUBSTANCES
Caution: New Drug Limited by Federal Law to Investigational Use

- For tetanus diphtheria toxoid used in pre-conditioning
  Name
  MRN
  DOB
  Drug: Td
- **Td™** (Tetanus diphtheria toxoid adsorbed); Stored at 4ºC used as Td booster vaccine
  Drug: Td
  Lot #
  Expiration Date

10.3 Supply, Receipt, and Storage

The DCs will be stored in a locked liquid nitrogen freezer in the Molecular Products and Cellular Therapies (MPACT) current Good Manufacturing Practice (cGMP) facility. The Nautilus

Laboratory Information Management System (LIMS) database will track receipt and storage location.

Table 2. Storage of Study Agents

MPACT Facility	Duke ICS Pharmacy	Duke Radiopharmacy
CMV pp65-LAMP mRNA-pulsed DCs	Td used for booster and for pre-conditioning	CMV pp65-LAMP mRNA loaded mature DCs labeled with ^{111}In

10.4 Dispensing and Preparation

The pp65 DC vaccines will be delivered from the MPACT facility directly to the clinic under the supervision of the trained research staff. Each labeled patient vaccine will be transported to the clinic in a separate cooler. DCs will be administered according to protocol. The patient's name, Study identification (ID), date of birth (DOB), and Duke history number will be double verified prior to DC administration as is standard Duke transfusion procedure.

The radiolabeled DC products used with the migration studies will be prepared from the pp65 DC vaccines sent from the MPACT facility to the Radiopharmacy and dispensed radiolabeled from the Duke Radiopharmacy.

Table 3. Dispensing of Study Agents

MPACT Facility	Duke ICS Pharmacy	Duke Radiopharmacy
CMV pp65-LAMP mRNA-pulsed DCs	Td used for booster and for pre-conditioning	CMV pp65-LAMP mRNA loaded mature DCs labeled with ^{111}In

10.5 Compliance and Accountability

All DC vaccines will be stored in the Molecular Products and Cellular Therapies (MPACT) cGMP Facility in a temperature controlled, locked access controlled storage unit. A drug log sheet will be used to track and document the drug. The products will be signed out and distributed by the MPACT personnel. The MPACT personnel use safe medication practices to reduce the risk of medication errors and adverse events when setting up study drug procedures. Investigational drugs are ordered, received, stored, and dispensed for MPACT protocols that are approved by the Duke University Health System (DUHS) IRB. Investigational drugs are stored separately from other drugs in an area of limited access and in accordance with special storage requirements. They are clearly labeled with the identity of the study drug and other control numbers. All drug transfers, receipts, and disposal are recorded in the Duke Nautilus system.

10.6 Disposal and Destruction

Radioactively labeled drug will be disposed of according to standard Duke radiation safety practices. Un-labeled drug will be autoclaved.

11 SUBJECT ELIGIBILITY

Inclusion Criteria

- Age ≥18 years of age.
- Newly diagnosed World Health Organization (WHO) Grade IV Glioma with definitive resection prior to consent, with residual radiographic contrast enhancing disease on the post-operative computed tomography (CT) or Magnetic Resonance Imaging (MRI) of <1 cm in maximal diameter in any plane.
- Able to receive standard of care radiation and chemotherapy for approximately 6 weeks duration and of more than 54GY
- MRI post RT does not show progressive disease outside the radiation field
- Enough tumor tissue available for determination of MGMT gene promoter status (must be unmethylated) or prior pathology report available confirming MGMT gene promoter status.
- CMV Seropositive.
- KPS of ≥ 70%.
- Hemoglobin ≥ 9.0 g/dl, absolute neutrophil count (ANC) ≥ 1,000 cells/µl, platelets ≥ 100,000 cells/µl prior to starting TMZ cycle 1 (patient must meet these criteria within 4 weeks after the end of XRT/TMZ to be eligible).
- Serum creatinine ≤ 3 times institutional upper limit of normal for age, serum aspartate aminotransferase (AST) ≤ 3 times institutional upper limit of normal for age and
- Bilirubin ≤ 1.5 times upper limit of normal prior to starting TMZ cycle 1 (*Exception: Patient has known Gilbert's Syndrome or patient has suspected Gilbert's Syndrome, for which additional lab testing of direct and/or indirect bilirubin supports this diagnosis. In these instances, a total bilirubin of ≤ 3.0 x ULN is acceptable.)*.
- Signed informed consent approved by the Institutional Review Board.
- Female patients must not be pregnant or breast-feeding. Female patients of childbearing potential (defined as < 2 years after last menstruation or not surgically sterile) must use a highly effective contraceptive method (allowed methods of birth control, [i.e. with a failure rate of < 1% per year] are implants, injectables, combined oral contraceptives, intra-uterine device [IUD; only hormonal], sexual abstinence or vasectomized partner) during the trial and for a period of > 6 months following the last administration of trial drug(s). Female patients with an intact uterus (unless amenorrhea for the last 24 months) must have a negative serum pregnancy test within 48 hours prior to first study procedure (leukapheresis).
- Fertile male patients must agree to use a highly effective contraceptive method (allowed methods of birth control [i.e. with a failure rate of < 1% per year] include a female partner using implants, injectables, combined oral contraceptives, IUDs [only hormonal], sexual abstinence or prior vasectomy) during the trial and for a period of > 6 months following the last administration of trial drugs.

Exclusion Criteria:

- Pregnant or breast-feeding.
- Women of childbearing potential and men who are sexually active and not willing/able to use medically acceptable forms of contraception.
- Patients with known potentially anaphylactic allergic reactions to gadolinium-diethylenetriamine penta-acetic acid (DTPA).

- Patients who cannot undergo MRI due to obesity or to having certain metal in their bodies (specifically non-MRI compatible pacemakers, infusion pumps, metal aneurysm clips, metal prostheses, joints, rods, or plates).
- Patients with evidence of tumor in the brainstem, cerebellum, or spinal cord, radiological evidence of multifocal disease, or leptomeningeal disease.
- Severe, active comorbidity, including any of the following:
    - Unstable angina and/or congestive heart failure requiring hospitalization;
    - Transmural myocardial infarction within the last 6 months;
    - Acute bacterial or fungal infection requiring intravenous antibiotics at the time of study initiation;
    - Chronic obstructive pulmonary disease exacerbation or other respiratory illness requiring hospitalization or precluding study therapy;
    - Known hepatic insufficiency resulting in clinical jaundice and/or coagulation defects;
    - Known Human Immunodeficiency Virus (HIV) and Hepatitis C positive status;
    - Major medical illnesses or psychiatric impairments that, in the investigator's opinion, will prevent administration or completion of protocol therapy;
    - Active connective tissue disorders, such as lupus or scleroderma that, in the opinion of the treating physician, may put the patient at high risk for radiation toxicity.
- Co-medication that may interfere with study results; e.g. immuno-suppressive agents other than corticosteroids.
- Prior, unrelated malignancy requiring current active treatment with the exception of cervical carcinoma in situ and adequately treated basal cell or squamous cell carcinoma of the skin. (Treatment with tamoxifen or aromatase inhibitors or other hormonal therapy that may be indicated in prevention of prior cancer disease recurrence, are not considered current active treatment.)
- Patients are not permitted to have had any other conventional therapeutic intervention other than steroids prior to enrollment outside of standard of care chemotherapy and radiation therapy. Patients who receive previous inguinal lymph node dissection, radiosurgery, brachytherapy, or radiolabeled monoclonal antibodies will be excluded.
- Current, recent (within 4 weeks of the administration of this study agent), or planned participation in an experimental anti-cancer drug study.
- Known history of autoimmune disease (with the exceptions of medically-controlled hypothyroidism and Type I Diabetes Mellitus).

12 SCREENING AND ON-STUDY TESTS AND PROCEDURES

12.1 Screening Examination

The screening examination will take place at the Duke PRTBTC clinic visit. An informed consent must be signed by the patient before any screening procedure takes place. Tumor MGMT promotor methylation status will be reviewed and only patients with non-methylated tumors will be enrolled. MGMT gene promoter methylation status is obtained at Duke using validated testing from LabCorp by PCR and/or Caris by pyrosequencing. If MGMT gene promoter methylation status has already been conducted outside of Duke, the results will be used as long as the testing was performed by a validated method/test.

Patients whose methylation status is inconclusive will not be eligible to participate in the study. The baseline physical and neurologic examination with KPS score along with standard of care blood work and CMV immune screen will be performed and documented by the neuro-oncology team and verified by the study team during this PRTBTC clinic visit. All subject data is standard of care evaluation that occurs for all patients being seen in the PRTBTC. If the subject is considered a screen failure prior to vaccine treatment, the source documents for electronic data entry will be obtained from the Duke electronic medical record.

Initial clinical evaluations will also include a baseline and study eligibility MRI (with and without contrast) of the brain per standard of care for comparison to subsequent MRI images. Mini-mental status examination (MMSE) will be performed at the post-RT/TMZ visit.

The patient must have had a definitive resection. Residual radiographic contrast enhancement on post-resection CT or MRI must not exceed 1 cm in diameter in any planes at time of consent. Patients with progressive disease after radiation will not be a candidate for the vaccine despite being previously consented and will be removed from the study and replaced (please see Section 12.7.4 for radiologic evaluations).

During the initial intake process within the Preston Robert Tisch Brain Tumor Center, all patients' tissue is tested for diagnosis and IDH-1/2 mutation analysis through either the Duke Pathology Department or CARIS. The study will collect this information in order to ensure baseline molecular diagnostics of the tumor for each patient is known.

At the discretion of the study investigator, for surgical blocks containing initial resection and biopsy at recurrence (if applicable), formalin-fixed paraffin-embedded slides may be requested for immunohistochemistry assays for the detection of CMV. These slides for CMV detection will not exceed 10% of the original specimen block, so as to preserve adequate tissue should patients prefer future diagnostic studies. Patient consent will be obtained for obtaining these slides in either scenario if a surgery/biopsy was performed at Duke University or at an external institution.

If resection is done outside of Duke University Hospital, formalin-fixed paraffin-embedded slides sectioned from outside blocks, not to exceed 10% of surgical specimen block tissue, prepared on Fischer Plus glass or Histostix coated slides when available will be mailed to:

Mustafa Khasraw M.D.,
Duke Brain Tumor Immunotherapy Program
Department of Neurosurgery
The Preston Robert Tisch Brain Tumor Center at Duke
DUMC Box 3624
047 Baker House, Trent Drive Duke University Medical Center
Durham, North Carolina 27710, USA

After patients have been consented, they will be entered into the electronic Research system.

12.2 Treatment Period

All enrolled patients will then undergo a leukapheresis for generation of DCs and for baseline immunological monitoring. If the patients were part of the companion study (Pro00102797) and

have already undergone the leukapheresis procedure the relevant DCs from that study will be used for the current study and any unused vaccine products will be handled as indicated by the patient in the companion study. Within 48 hours of leukapheresis, patients will have blood samples taken for the following tests as required by the Duke Apheresis Center: CBC with auto differential, CMP, ionized Calcium, and Beta-Human Chorionic Gonadotropin (β-HCG) (for females of child-bearing potential). Total estimated blood volume required for these evaluations is 12-15 mL. For patients without sufficient venous access for leukapheresis, a temporary central intravenous catheter may be inserted. To prevent the development of hypocalcemia from the citrate used for leukapheresis, all patients will be instructed to take oral Tums, 2 tablets three times a day and at bedtime the day before and the day of the leukapheresis procedure. Patients who have lower levels of calcium will be treated per Apheresis lab standard protocols under the direction of apheresis attending physician. This first leukapheresis will be approximately a 4-hour leukapheresis, and it is estimated that 10-12 L of blood will be processed during this leukapheresis. One red top tube 10 mL draw tube will be obtained prior to leukapheresis #1 for immune monitoring. Nine yellow ACD 8.5 mL tubes and 2 red top 10 mL draw tubes will be obtained prior to leukapheresis #2.

Table 4. Schedule of Events

	CMV Screening	Main Consent[11]	Leukapheresis #1[7]	RT/TMZ	Post-RT DI TMZ Cycle 1	Td Booster & Vaccine #1	Vaccine #2	Vaccine #3	Leukapheresis #2[7]	Td Pre-conditioning[8]	Vaccine #4		Vaccines #5-10	Progression[9]
Time and Range				Within 7 weeks of resection	Within 4 (-2) weeks of RT	Day 23 (+/-2 days) Td booster given before vaccine #1	2 weeks post Vaccine #1 (+/-2 days)	2 weeks post Vaccine #2 (+/-2 days)	14 (+/-4) days after vaccine #3	3 (+/-1) week after leukapheresis #2, 12-24 hours prior to vaccine #4	3 (+/-1) week after leukapheresis #2		Every 35 (+/-7) days after previous vaccine	
CMV Screening Consent	X													
Main Consent[10]		X			X									
Medical History/ Baseline Symptoms		X												
Tumor Pathology[2]	X[1]													X
Physical Exam, Neurological Exam, KPS, Vitals[3]	X[1]				X	X	X	X	X	X			X	
MMSE[3]					X									
MRI[4]	X[1]				X				X				X	X
CMV IgG	X													
CBC (with auto differential)[5]			X		X	X	X	X	X	X	X		X	
CMP[5]			X		X	X	X	X	X	X			X	
Beta HCG quantitative (WOCBP only)			X		X				X					
Ionized Calcium			X						X					
Immune Monitoring Blood[6]			X (1R)		X (9Y, 2R)	X (9Y, 2R)		X (9Y, 2R)	X (9Y, 2R)	X (2R)	X (9Y, 2R)		X (9Y, 2R)	X (9Y, 2R)
SOC XRT/TMZ				X										
Dendritic Cell Vaccine						X	X	X			X		X	

Con meds and AEs		X	X	X	X	X	X	X	X	X	X		X	X

[1]Can be performed prior to any ICF being signed as part of standard of care evaluation.
[2]Patients must have a WHO Grade IV Glioma with definitive resection prior to enrollment, with residual radiographic contrast enhancement on most recent CT or MRI of <1 cm in maximal diameter in any plane. Methylation status must be checked per SOC practices (via LabCorp, CARIS, etc.) and patient must be unmethylated in order to be eligible to proceed with signing consent to undergo leukapheresis.
[3]Clinical evaluations with each vaccine will include a general physical examination, complete neurologic examination, KPS rating, and vitals (prior to vaccine and 30 mins post vaccine). MMSE will be performed at the Post-RT/TMZ visit only.
[4]A baseline and study eligibility MRI (with and without gadolinium enhancement) of the brain per standard of care for comparison to subsequent MRI images will also be obtained. MRI or contrasted CT, every 8 ± 4 weeks per PRTBTC standard of care (may be adjusted by the treating neuro-oncologist).
[5]CBC (with auto differential) and CMP to confirm eligibility, as per standard of care, and as per this schedule of events. Whenever blood for immune monitoring is obtained CBC with auto differential and CMP will be done to assess absolute number of T_{Regs}.
[6]Blood work for immunologic monitoring will be drawn before leukapheresis #1 (1 red top 10mL tube prior to leukapheresis), on the day of Post-RT/TMZ visit (9 yellow ACD 8.5mL tubes and 2 red top 10mL tubes), on the day of but prior to vaccine #1 and vaccine #3 (9 yellow ACD 8.5mL tubes and 2 red top 10mL tubes), on the day of Td preconditioning but before the administration of Td (2 red top 10mL tubes), at leukapheresis #2 (9 yellow ACD 8.5mL tubes and 2 red top 10mL tubes) when leukapheresis is performed 14 (+/-4) days post vaccine #3, at the time of vaccine 4 (9 yellow ACD 8.5mL tubes and 2 red top 10mL tubes), and then at vaccines 5 through 10 (9 yellow ACD 8.5mL tubes and 2 red top 10mL tubes) and/or at progression (whichever comes first). If an additional leukapheresis must occur earlier than 14 (± 4) days after vaccine 3, then no immune monitoring will be drawn. All patients will undergo at least 2 leukapheresis procedures – prior to radiation (1 red top 10mL tube) and approximately 2 weeks post vaccine #3 (9 yellow ACD 8.5mL tubes, 2 red top 10mL tubes) See footnote 7. For subjects who complete all 10 study vaccines without progressing, an attempt will be made to obtain blood for immunologic monitoring 2-3 times a year at standard Duke Preston Robert Tisch Brain Tumor Center visits and at progression (whichever comes first).
[7]Leukapheresis #1 will be scheduled following consent. Per standard Duke Apheresis Protocol, all subjects will have CBC with auto differential, CMP, ionized Calcium, and βHCG (WOCBP only) within 48 hours prior to the procedure(s). Leukapheresis #2 will occur approximately 14 (± 4) days after third immunization to obtain PBMCs for Immunologic Monitoring and generation of additional DCs for continued vaccinations. Additional leukapheresis can be obtained up to every 2 weeks if needed according to the Duke Apheresis Center protocol, but these will likely be needed less than every 2 months throughout the study to generate enough DCs to continue vaccinations. No immune monitoring blood will be drawn at the additional leukapheresis procedures; only at leukapheresis #1 prior to radiation and at leukapheresis #2 approximately 2 weeks post vaccine #3. Additional leukapheresis following leukapheresis #1 will be represented as: Leukapheresis #1a, Leukapheresis #1b, etc. Additional leukapheresis following Leukapheresis #2 will be represented as: Leukapheresis #2a, Leukapheresis #2b, etc.
[8]On the day before the fourth DC vaccine, patients will receive vaccine site pre-conditioning strategy. A single dose of Td toxoid (1 flocculation unit, Lf) will be administered to a single side of the groin 12-24 hours prior to the fourth DC vaccine, which is always given bilaterally at the groin site. Standard of care blood for CBC (with auto differential) and CMP will be drawn during the pre-conditioning visit. 2 red top 10 mL draw tubes for immune monitoring will be obtained on the same day of the pre-conditioning visit but prior to patients receiving Td pre-conditioning.
[9]As part of standard care for these patients, upon tumor progression, participants may undergo stereotactic biopsy or resection. As this is not a research procedure consent will be obtained separately. However, if tissue is obtained, it will be used to confirm tumor progression histologically and to assess immunologic cell infiltration and pp65 antigen escape at the tumor site.
[10]When CMV and/or methylation status is unknown, patients may sign CMV and/or leukapheresis consent for the Umbrella study (Pro00102797). This allows patients to undergo CMV screening and/or leukapheresis prior to signing main consent for this study. If patients sign the leukapheresis consent for the Umbrella study prior to leukapheresis, they will sign main consent for this study at the Post-RT/TMZ visit.

12.3 End of Treatment

Vaccines are given as described above for a total of 10 or until progression (whichever comes first). Once the vaccinations are complete, the treatment phase of the study will be over and the follow-up period will begin.

12.4 Follow-up Period

Patients will be followed for survival, progression, and subsequent therapies only, and this data will be recorded by the research team. For recording of subsequent therapies, the type of therapy(ies) that the subject receives will be recorded with approximate start and stop date, if possible. For subjects who complete all 10 study vaccines without progressing, an attempt will be made to obtain blood for immunologic monitoring 2-3 times a year at standard Duke Preston Robert Tisch Brain Tumor Center visits. If the subject progresses in the follow-up period, blood for immunological monitoring will be drawn at the time of progression, if possible.

12.5 End of Study

Rationale for taking a patient off study will be documented (see Section 12.6.1). In the Follow-up Period, patients will be followed for survival, progression, and subsequent therapies only, and this data will be recorded by the research team.

12.6 Early Withdrawal of Subject(s)

12.6.1 Criteria for Early Withdrawal

Subjects may voluntarily withdraw from the study at any time. Subjects may be withdrawn by the PI and considered screen failures prior to vaccine therapy and the PI may also withdraw a subject from the study at any time based on his/her discretion. Reasons for PI-initiated withdrawal may include, but are not limited to the following:

- Inability to complete approximately >4 weeks of RT with TMZ as per standard of care.
- Inability to tolerate TMZ
- Patients with an active infection requiring treatment or having an unexplained febrile illness (Tmax > 99.5° F).
- Patients requiring an increase in corticosteroids, with the exception of nasal or inhaled steroid, such that at the time of first vaccination they require a dose above 2 mg of dexamethasone / day. Once vaccinations have been initiated, if patients subsequently require increased steroids, they will still be permitted to remain on the study, but every effort will be made to minimize steroid requirements.
- Adverse events
- Abnormal laboratory values
- Abnormal test procedure results (DCs fail to meet release criteria; inability to tolerate leukapheresis)
- Protocol deviation
- Administrative issues
- Disease progression
- Pregnancy

12.6.2 Follow-up Requirements for Early Withdrawal

Consented subjects that are withdrawn prior to cycle 1 of post-RT DI TMZ for any of the aforementioned reasons will be considered eligibility failures and thus will not be followed for survival. All other subjects will be followed by the study coordinator until death or are lost to follow-up.

12.6.3 Replacement of Early Withdrawal(s)

As noted in Section 12.6.2, subjects who voluntarily withdraw prior to cycle 1 of post-RT DI TMZ will not be followed for survival, and will be replaced.

12.7 Study Assessments

12.7.1 Medical History

Medical history will be obtained from the Duke electronic system and from the subject and/or family at the screening visit and reviewed at each study visit. This data may include the following:

- All past medical and surgical history;
- Current medications;
- Changes in physical or neurologic symptoms;
- Any adverse events.

12.7.2 Physical Exam

Vital signs and physical and neurologic examinations will be assessed and recorded along with a KPS score prior to enrollment and at each visit.

12.7.3 Use of Antihistamines

Subjects will be advised to avoid antihistamine use 48 hours prior to each vaccine administration, the day of vaccine administration, and for 48 hours following each vaccine administration. If the subject has a pre-existing condition that requires antihistamine usage, the PI and the treating oncologist will decide if it is safe and appropriate for the subject's antihistamines to be held before and following vaccine administrations.

12.7.4 Radiologic Evaluations

Patients with newly diagnosed GBM will be imaged by MRI as per standard of care for eligibility and baseline measurements, and to assess progression prior to vaccine therapy; although the purpose of this study is not to detect tumor responses, any evidence of tumor response will be determined according to the Duke PRTBTC standard operating procedure (SOP). Immunotherapy Response Assessment in Neuro-Oncology (iRANO) criteria [122, 123] established by consensus conference, will be used for assessment of response and pseudoprogression following immunotherapy. If pseudoprogression is suspected, the subject will continue with vaccine therapy for a minimum of 2-3 subsequent months so long as subject remains clinically and radiographically stable compared to the MRI showing suspected pseudoprogression. If the subject continues to do well beyond the 3 months, they will continue on study as planned. Subjects demonstrating definitive progression will be removed from study. Tumor progression will need to be documented histologically, unless there are clinical contraindications, to exclude inflammatory responses presenting as radiographic or clinical changes, which could indicate potentially toxic or therapeutic responses and not tumor progression. If tissue is obtained through the Duke Brain Tumor Biorepository, it will be used to confirm tumor progression histologically and to assess immunologic cell infiltration and examine pp65 expression to evaluate antigen loss at the tumor

site. Upon progression, patients may be treated on other therapies as directed by the treating Oncologist.

12.7.5 Immunologic Assessments

Immunological response evaluations will be conducted as described in Table 4. All patients will undergo at least 2 leukapheresis procedures – prior to radiation (1 red top 10mL tube) and approximately 2 weeks post vaccine #3 (9 yellow ACD 8.5mL tubes, 2 red top 10mL tubes). If an additional leukapheresis must occur earlier than 14 (± 4) days after vaccine 3, then no immune monitoring will be drawn at this additional leukapheresis. For subjects who complete all 10 study vaccines without progressing, an attempt will be made to obtain blood for immunologic monitoring 2-3 times a year at standard Duke neuro-oncology visits and at progression (whichever comes first). The immune monitoring blood that is collected prior to the initiation of post RT-TMZ cycle 1 (on the same day) will be the baseline for T_{reg} analysis. The immune monitoring blood drawn at pre-vaccine #1 will be the baseline for polyfunctional T cell analysis. The blood drawn on the same day as (but prior to) Td preconditioning will be analyzed for the baseline CCL3. A comparison of pre-therapy lymphocyte functions to those at intervals after each immunization will be made. These tests may provide evidence for the development of immune responses following DCs immunization and will play an important role in the design of future DC-based clinical trials. Cellular immune responses to pp65 will be measured by polyfunctional T cell assays using 10-12 color panels previously optimized in our laboratory, which includes detection of CCL3 on T cells. The effect of treatment on cytokines and other soluble factors will be assayed in the serum/plasma. Serum/plasma will be measured to determine levels of CCL3 from the blood drawn at the Td preconditioning visit, and at the vaccine #4 visit. T_{Reg} kinetics will be measured by using a constellation of markers specific for T_{Regs} such as foxp3, CD25 and CD4. Whenever blood for immune monitoring is obtained, we will submit a CBC with auto differential to assess absolute number of T_{Regs}. These will be drawn with SOC blood draws whenever possible.

In addition, blood and tumor samples will be stored for possible future research in the Duke BTIP Laboratory where all other samples from this study are stored. The samples being stored are ONLY for this study. Our mouse studies being done in the BTIP Lab are identifying markers that may influence migration, therefore, as other markers are identified, we would like to investigate whether these same markers are identified in human subjects enrolled on this trial.

Polyfunctional Flow Cytometry

Peripheral blood will be drawn into 9 yellow (ACD) tubes containing acid citrate dextrose and 2 red top tubes for serum. PBMCs will be separated by density gradient centrifugation using Leukosep tubes.

Polyfunctional T cell analysis will be done in Dr. Sanchez-Perez's immunology laboratory Dr. Sanchez-Perez's immunology laboratory conducts all the clinical immune monitoring for the Duke Brain Tumor Center. Polyfunctional T cell panels have been developed in our laboratory and optimized for detection of CCL3 in T cells. This process involves the rapid early detection and analysis of the production of Tumor Necrosis Factor-α (TNF-*a*), CCL3, IFN-γ, interleukin-2 (IL-2), and CD107a (a marker of cell cytotoxicity) prior to cellular secretion following antigen-specific stimulation *in vitro*. The functional CD4 and CD8 immune response of the patients will be monitored using a 10-12-color assay. To detect an increase in polyfunctional T cells we will analyze the fold change of T cells secreting TNF-*a*, CCL3, IFN-γ, IL-2 and CD107a from the blood

samples from pre-vaccine 1, and second leukapheresis (or blood draw) occurring 14 ± (4) days after vaccine #3.

Isolated PBMCs will be stimulated for 6 hours with the pool of 138 peptides spanning the entire pp65 gene. The cells will be stained for the surface markers CD3 (to identify it as a T cell), CD4 (to define as specific helper cell), CD8 (to define antigen specific cytotoxic T cells) and then submitted for intracellular cytokine staining. This procedure allows for the highly sensitive detection of TNF-*a*, CCL3, IFN-γ, IL-2 and CD107a secreting cells, which are assumed to be specifically responding to the stimulating antigen.

The maturation state and the activation status of samples will be detected by polychromatic flow cytometry using optimized panels which will include CD3, CD4, CD8, CCR7, CD45RA, HLA-DR, CD69 and HLA-DR. Levels of naïve (TN – CD45RA+CCR7+), central memory (TCM – CD45RA-CCR7+), effector memory (TEM – CD45RA-CCR7-), or terminally differentiated effector memory (TEMRA – CD45RA+CCR7+) T cells .

Serology/Multiplex Platform

CCL3 and levels of other inflammatory soluble factors may be assayed by multiplex platform. The effect of treatment on CCL3 Serum/plasma will be assayed (2 red top 10 mL tubes) to determine if levels of CCL3 are impacted by Td pre-conditioning and will be drawn prior to the Td pre-conditioning as described above, and at the vaccine #4 visit (9 yellow ACD 8.5 mL tubes, , 2 red top 10 mL tubes) 24 hours after Td pre-conditioning. Antibody levels to the immunizing antigen may be measured.

T_{Reg} Kinetics

T_{Regs} will be analyzed by flow cytometry using a constellation of markers specific for T_{Regs} included CD4, CD25 and foxp3. For T_{Reg} analysis, the time points will be leukapheresis 1, before DI TMZ in the Post-RT TMZ, pre-vaccine 1, leukapheresis 2, pre-vaccine 4, pre-vaccine 5, & pre-vaccine 6. We will analyze the percent of Foxp3, CD25, CD4 T cells over these time points.

13 SAFETY MONITORING AND REPORTING

13.1 Adverse Events

An AE is any untoward medical occurrence in a subject receiving pre-conditioning or the study vaccine and which does not necessarily have a causal relationship with this treatment. For this protocol, the definition of AE also includes worsening of any pre-existing medical condition. An AE can therefore be any unfavorable and unintended or worsening sign (including an abnormal laboratory finding), symptom, or disease temporally associated with the use of the DCs, whether or not related to use of the DCs. Abnormal laboratory findings without clinical significance (based on the PI's judgment) should not be recorded as AEs. But, laboratory value changes that require therapy or adjustment in prior therapy are considered adverse events.

Adverse events will be collected from the time of administration of the first DC vaccine through 1 month after the subject's last vaccine, all AEs must be recorded in the subject's medical record and adverse events case report form.

AEs will be assessed according to the CTCAE version 5. If CTCAE grading does not exist for an AE, the severity of the AE will be graded as mild (1), moderate (2), severe (3), life-threatening (4), or fatal (5).

Attribution of AEs will be indicated as follows:

- Definite: The AE is clearly related to the study drug
- Probably: The AE is likely related to the study drug
- Possible: The AE may be related to the study drug
- Unlikely: The AE is doubtfully related to the study drug
- Unrelated: The AE is clearly NOT related to the study drug

Attribution of AEs will be determined for each study intervention individually (pp65 DC vaccine, pre-conditioning agent [Td], TMZ, bevacizumab).

13.1.1 Reporting of AEs

A summary of all adverse events (not just those considered related to the study drug) will be kept which will categorize the event by organ system, relationship to which treatment, its grade of severity, and resolution. Periodic review by the PI and monthly review at the PRTBTC Adverse Event meeting of the collective adverse events will occur with the intention of identifying any trends or patterns in toxicity. If any such trends are identified, depending on their severity and frequency, a protocol amendment will be considered.

13.2 Adverse Events of Special Interest

Adverse events will not be collected until the patient has received the DC vaccine, unless they are considered an Adverse Event of Special Interest related to the leukapheresis procedure. Only these special interest adverse events will be collected prior to the DC vaccine, all other events occurring prior to DC vaccine administration will not be recorded or monitored.
Special Interest Adverse Events that may occur during the leukapheresis procedure include:

- Allergic Reaction
- Anaphylaxis
- Pre-syncope
- Syncope
- Vasovagal reaction

Special Interest Adverse Events that may occur after the procedure, but may still be related to leukapheresis include:

- Vascular Access Complications
- Venous Injury

13.3 Serious Adverse Events

An AE is considered "serious" if in the opinion of the investigator it is one of the following outcomes:

- Fatal
- Life-threatening
- Constitutes a congenital anomaly or birth defect
- A medically significant condition (defined as an event that compromises subject safety or may require
- medical or surgical intervention to prevent one of the three outcomes above).
- Requires inpatient hospitalization or prolongation of existing hospitalization
- Results in persistent or significant incapacity or substantial disruption to conduct normal life functions.

13.3.1 Reporting of SAEs

All SAEs should be reported immediately to Dr. Mustafa Khasraw M.D. at 919-684-5301 during regular business hours or at (Pager: 919-206-0493) after hours and to the FDA. Fatal or life-threatening, unexpected adverse events will be reported to the FDA by telephone, facsimile, or in writing as soon as possible, but no later than 7 calendar days after first knowledge by the sponsor followed by as complete a report as possible within 8 additional calendar days. Serious, unexpected adverse events that are not fatal or life-threatening will be reported to the FDA by telephone, facsimile, or in writing as soon as possible, but no later than 15 calendar days after first knowledge by the sponsor.

All adverse events that are considered serious, unanticipated, and related or possibly related to the research (as defined by 21CRF312.32[a]) will be reported to the Duke University Medical Center IRB and the FDA using the appropriate SAE reporting process. At the time of the annual progress report to the Duke University Medical Center IRB and the FDA, a summary of the overall toxicity experience will be provided. Safety Oversight Committee (SOCOMM)

The Duke Cancer Institute (DCI) SOCOMM is responsible for annual data and safety monitoring of DUHS sponsor-investigator phase I and II, therapeutic interventional studies that do not have an independent data safety monitoring board (DSMB). The primary focus of the SOC is review of safety data, toxicities and new information that may affect subject safety or efficacy. Annual safety reviews includes but may not be limited to review of safety data, enrollment status, stopping rules if applicable, accrual, toxicities, reference literature, and interim analyses as provided by the sponsor-investigator. The SOC in concert with the DCI Monitoring Team (see Section 14.1 for Monitoring Team description) oversees the conduct of DUHS cancer-related, sponsor-investigator therapeutic intervention and prevention intervention studies that do not have an external monitoring plan, ensuring subject safety and that the protocol is conducted, recorded and reported in accordance with the protocol, SOPs, Good Clinical Practice (GCP), and applicable regulatory requirements.

13.4 External Data and Safety Monitoring Board

The Principal Investigator and Sub-Investigators must comply with applicable federal, state, and local regulations regarding reporting and disclosure of conflict of interest. Conflicts of interest (COI) may arise from situations in which financial or other personal considerations have the potential to compromise or bias professional judgment and objectivity. Conflicts of interest include

but are not limited to royalty or consulting fees, speaking honoraria, advisory board appointments, publicly-traded or privately-held equities, stock options, intellectual property, and gifts.

The Duke University School of Medicine's Research Integrity Office (RIO) reviews and manages research-related conflicts of interest. The Principal Investigator and Sub-Investigators must report conflicts of interest annually and within 10 days of a change in status, and when applicable, must have a documented management plan that is developed in conjunction with the Duke RIO and approved by the IRB/Institutional Ethics Committee (IEC).

Due to potential for COI in relation to proprietary interest in the pp65CMV DC vaccine, a Data Safety and Monitoring Board (DSMBplus) has been established. The Duke PRTBTC DSMBplus Charter is available upon request.

14 QUALITY CONTROL AND QUALITY ASSURANCE

14.1 Monitoring

The DCI Monitoring Team will conduct monitoring visits to ensure subject safety and to ensure that the protocol is conducted, recorded, and reported in accordance with the protocol, standard operating procedures, good clinical practice, and applicable regulatory requirements. As specified in the DCI Data and Safety Monitoring Plan, the DCI Monitoring Team will conduct routine monitoring after the third subject is enrolled, followed by annual monitoring of 1 – 3 subjects until the study is closed to enrollment and subjects are no longer receiving study interventions that are more than minimal risk.

Additional monitoring may be prompted by findings from monitoring visits, unexpected frequency of serious and/or unexpected toxicities, or other concerns and may be initiated upon request of DUHS and DCI leadership, the DCI Cancer Protocol Committee, the SOC, the sponsor, the Principal Investigator, or the IRB. All study documents must be made available upon request to the DCI Monitoring Team and other authorized regulatory authorities, including but not limited to the National Institute of Health, National Cancer Institute, and the FDA. Every reasonable effort will be made to maintain confidentiality during study monitoring.

14.2 Audits

The Duke University Office of Audit, Risk and Compliance (OARC) may conduct audits to evaluate compliance with the protocol and the principles of GCP. The PI agrees to allow the auditor(s) direct access to all relevant documents and to allocate his/her time and the time of the study team to the auditor(s) in order to discuss findings and any relevant issues.

OARC audits are designed to protect the rights and well-being of human research subjects. OARC audits may be routine or directed (for cause). Routine audits are selected based upon risk metrics generally geared towards high subject enrollment, studies with limited oversight or monitoring, Investigator initiated Investigational Drugs or Devices, federally-funded studies, high degree of risk (based upon adverse events, type of study, or vulnerable populations), Phase I studies, or studies that involve Medicare populations. Directed audits occur at the directive of the IRB or an authorized Institutional Official.

OARC audits examine research studies/clinical trials methodology, processes and systems to assess whether the research is conducted according to the protocol approved by the DUHS IRB. The primary purpose of the audit/review is to verify that the standards for safety of human subjects in clinical trials and the quality of data produced by the clinical trial research are met. The audit/review will serve as a quality assurance measure, internal to the institution. Additional goals of such audits are to detect both random and systemic errors occurring during the conduct of clinical research and to emphasize "best practices" in the research/clinical trials environment.

14.3 Data Management and Processing

14.3.1 Study Documentation

Study documentation includes but is not limited to source documents, case report forms, monitoring logs, appointment schedules, study team correspondence with sponsors or regulatory bodies/committees, and regulatory documents that can be found in the DCI-mandated "Regulatory Binder", which includes but is not limited to signed protocol and amendments, approved and signed informed consent forms, FDA Form 1572, College of American Pathologists (CAP) and Clinical Laboratory Improvement Act (CLIA) laboratory certifications, and clinical supplies receipts and distribution records.

Source documents are original records that contain source data, which is all information in original records of clinical findings, observations, or other activities in a clinical trial necessary for the reconstruction and evaluation of the trial. Source documents include but are not limited to hospital records, clinical and office charts, laboratory notes, memoranda, subjects' diaries or evaluation checklists, pharmacy dispensing records, recorded data from automated instruments, copies or transcriptions certified after verification as being accurate copies, microfiches, photographic negatives, microfilm or magnetic media, x-rays, subject files, and records kept at the pharmacy, at the laboratories and at medico-technical departments involved in the clinical trial. When possible, the original record should be retained as the source document. However, a photocopy is acceptable provided that it is a clear, legible, and an exact duplication of the original document.

14.3.2 Data Management

The subject's medical records will be the primary source document for the study. Source documents include all information in original records and certified copies of original records of clinical findings, observations, or other activities in a clinical investigation used for reconstructing and evaluating the investigation.[1] Source documentations may also include paper eligibility checklists, data flowsheets, patient reported outcomes and other paper documents. The PI, study coordinator, study research nurse, data management team and all associated study key personnel, are permitted to make entries, changes, or corrections in the source documents or database per the study delegation of authority log.

Errors on the source documents will be crossed out with a single line, and this line will not obscure the original entry. Changes or corrections will be dated, signed, initialed, and explained (if necessary). Database changes will be tracked via electronic trail automatically.

[1] In 21 CFR 312.62(b), reference is made to records that are part of case histories as "supporting data;" the ICH guidance for industry *E6 Good Clinical Practice: Consolidated Guidance* (the ICH E6 guidance) (available at http://www.fda.gov/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/default.htm) uses the term "source data/documents." For the purpose of this guidance, these terms describe the same information and have been used interchangeably.

14.3.3 Data Management Procedures and Data Verification

The DCI Information Technology (IT) Shared Resource has developed Title 21 CFR Part 11 compliant databases for cancer clinical trials. DCI IT has extensive expertise in database quality assurance, data standards, and use of the Cancer Biomedical Informatics Grid (caBIG) tools to support cancer researchers.

Data queries will be generated automatically by the electronic case report form (eCRF) system. These data queries signify the presence of data inconsistencies. The study and data management team will cross-reference the data to verify accuracy. Missing or implausible data will be highlighted for the PI requiring appropriate responses (i.e., confirmation of data, correction of data, completion or confirmation that data is not available, etc.).

The database will be reviewed and discussed prior to database closure, and will be closed only after resolution of all remaining queries.

14.3.4 Coding

All medical terms will be coded using CTCAE (version 5).

14.3.5 Study Closure

Following completion of the studies, the PI will be responsible for ensuring the following activities:

- Data clarification and/or resolution;
- Accounting, reconciliation, and destruction/return of used and unused study drugs;
- Review of site study records for completeness;
- Shipment of all remaining laboratory samples to the designated laboratories.

15 STATISTICAL METHODS AND DATA ANALYSIS

All statistical analysis will be performed under the direction of the statistician designated in key personnel. Any data analysis carried out independently by the investigator must be approved by the statistician before publication or presentation.

15.1 Study Design Overview

This single-arm phase II study will assess the impact of tetanus pre-conditioning and adjuvant GM-CSF on overall survival of newly diagnosed GBM patients who have undergone definitive resection, are CMV+ and unmethylated, and completed standard temozolomide and radiation treatment. After completing standard of care radiotherapy with concurrent temozolomide, patients will receive 1 cycle of dose intensified TMZ followed by pp65 DC vaccination beginning on day 23.

15.2 Analysis Sets

All patients who initiate dose-intensified TMZ after completion of standard of care RT and TMZ will be included in the primary efficacy analyses, and toxicity summaries.

Subject to the availability of data, analyses of correlative objectives will include all or some of the patients who undergo vaccine 4. For analyses of migration, the first 16 patients who reach vaccine 4 will undergo migration analyses. For CCL3, all patients who reach vaccine 4 will be included in statistical analyses. For analyses of polyfunctionality, the first 24 patients who reach vaccine 4 will be considered.

15.3 Patient Demographics and Other Baseline Characteristics

Summaries of clinical and socio-demographic characteristics will be generated within four groups of patients: (1) All patients who sign informed consent prior to the initiation of standard of care temozolomide and radiation, (2) All patients who start dose intensified TMZ, (3) All patients who receive at least one pp65 DC vaccination, and (4) All patients who receive vaccine 4. Categorical descriptors will be summarized using frequency distributions; whereas, interval variables will be summarized using percentiles, as well as means and standard deviations.

15.4 Treatments

Among patients who initiate dose intensified TMZ, a frequency distribution will be generated for the number of vaccines received by each patient.

15.5 Primary Objective

The primary objective of this study is to determine if Td preconditioning of pp65 loaded DC vaccination with GM-CSF extends survival relative to a historical benchmark.

15.5.1 Variable

Overall Survival (OS) is defined as the time between initiation of dose-intensified TMZ and death, or last follow-up if the patient remains alive at the time of analysis.

15.5.2 Statistical Hypothesis, Model, and Method of Analysis

The Kaplan-Meier estimator will graphically describe OS. Median OS will be estimated, with 95% confidence intervals, as well as 12, 18, 24, 36, and 48-month survival rates. A one-sample logrank test [124, 125] will compare this curve to that reported by Gilbert [126] in which the median OS from the start of adjuvant temozolomide treatment was 14.0 months (95% CI: 12.9 to 14.7 months) for patients with newly diagnosed GBM that are unmethylated. An intent-to-treat approach will be used in analyses.

The statistical hypothesis and power calculations are provided in Section 15.9.

Given the possibility that the characteristics of patients who have enrolled on this protocol differs from that described by Gilbert [127] for RTOG 0825, additional analyses will be conducted using the nomogram published by Gittlemen [128] for RTOG 0525 and 0825. For each patient, the probability of 24-month survival will be estimated from the nomogram based upon the patients' baseline characteristics. From these individualized estimates of 24-month survival, an overall estimate of 24-month survival will be generated with 95% confidence interval. That confidence interval will be compare to the actual 24-month survival confidence interval observed.

15.5.3 Handling of Missing Values, Censoring, and Discontinuation

Patients who withdraw from the study without an allowance for survival follow-up, or are lost to follow-up will have OS censored at the date that the patient was last know alive.

15.6 Secondary Objectives

This study has five secondary objectives. The first 3 secondary objectives examine the impact of functional measures of CMV pp65-loaded DC vaccine efficacy on survival post-vaccine. These measures include migration (Section 15.6.1), fold change in CCL3 (Section 15.6.2), and fold change in pp65 antigen-specific polyfunctionality (Section 15.6.3). Power calculations will be provided for each of the secondary objectives. Power calculations for the three functional predictors of survival post-vaccine 4 will be adjusted to account for multiple comparisons using a Bonferroni correction, and will used a type I error rate of 0.0167.

The other secondary objectives include an assessment of whether the percent of T_{Reg} of CD4+ T cells remains stable or decreases without additional temozolomide after vaccine 1 (Section 15.6.4), and an assessment of the safety of the protocol's treatment regimen (Section 15.6.5).

15.6.1 Secondary Objective #1: Migration and Survival

PLEASE NOTE: The study originally planned to address secondary objective #1 as described below. However, with the activation of Protocol v.20220204, the plans for examining the relationship between migration and survival have been dropped.

Objective	To assess the association between migration of CMV pp65 RNA-pulsed DCs with GM-CSF to site-draining inguinal lymph nodes after Td pre-conditioning and survival after vaccine # 4.
Analysis Set	All patients who undergo migration studies at vaccine 4. Due to budgetary considerations, only the first 16 patients who reach vaccine 4 will undergo migration studies.
Outcome	Survival from vaccine 4.
Hypothesis	Patients with greater migration will have longer survival.
Analysis Plan	The Cox proportional hazards model will assess the impact of migration on survival after vaccine #4. Migration is defined as the maximum percentage of ^{111}In-labeled DCs reaching inguinal nodes during the 48 hours after the 4th vaccination. The hazard ratio associated with a 1-unit change in migration will be estimated with 95% confidence intervals.
Power Calculations	Cox proportional hazards model will assess the impact of peak migration at 24 or 48 hours after vaccine 4 on subsequent survival, defined as the time between vaccine 4 and death, or last follow-up if the patient remains alive at the time of analysis. Data from ATTAC [129] show that the standard deviation (SD) for peak migration is 7.86 and that the hazard ratio associated with 1 unit increase in migration is approximately 0.86. Assuming SD=7.86, there would be 80% power to detect a hazard ratio of 0.86 assuming a two-tailed test (α=0.0167) within a Cox model after 8 deaths [130, 131]. Assuming the median OS post-vaccine 4 is 24 months, approximately 24 months after the 16th patient undergoes migration studies will 8 deaths be observed.

15.6.2 Secondary Objective #2: CCL3 and Survival

Objective	To assess the association between CCL3 measured post-Td administration and survival after vaccine # 4.
Analysis Set	All patients who undergo assessment of CCL3 after Td administration. All patients who reach vaccine 4 when Td is administered will be included in analyses. Forty (40) patients are anticipated.
Outcome	Survival from vaccine 4.
Hypothesis	Greater levels of CCL3 are associated with longer survival.
Analysis Plan	The Cox proportional hazards model will assess the impact of CCL3 on survival post-vaccine 4. The hazard ratio associate with a 1-unit increase in CCL3 will be estimated with 95% confidence intervals.
Power Analyses	Cox proportional hazards model will assess the impact of CCL3 on survival post-vaccine 4. Within ATTAC [129], the standard deviation for CCL3 was 12.9 and the hazard ratio for survival post-vaccine 4 was approximately 0.95 for a 1 unit increase in CCL3. Assuming SD=12.9, there would be 80% power to detect a hazard ratio of 0.95 with a two-tailed test (α=0.0167) within a Cox model under these assumptions after 24 deaths [130, 131]. If we assume that the median OS post-vaccine 4 is 24 months, then 24 deaths should be observed approximately 20 months after the 40th patient receives vaccine 4.

15.6.3 Secondary Objective #3: Polyfunctionality and Survival

Objective	To assess the association between fold changes in T cell polyfunctionality from baseline to pheresis 2, and survival after vaccine #4.
Analysis Set	All patients who have a baseline (pre vaccine 1) and polyfunctionality assessed at leukapheresis 2 who receive vaccine # 4. Polyfunctionality is assessed at leukapheresis so that results do not reflect the impact of Td-preconditioning post-vaccine 4 assessment of polyfunctionality. Due to budgetary considerations, only the first 24 patients who reach vaccine 4 will be analyzed.
Outcome	Survival from vaccine 4.
Hypothesis	Greater fold changes in polyfunctionality are associated with longer survival.
Analysis Plan	The mean fold change from baseline to leukapheresis 2 will be summarized for the frequency of pp65 antigen-specific CD8$^+$ T cells producing three or more cytokines (IFNγ, CCL3, IL-2, TNFα, CD107a). Cox proportional hazards model will assess the association between fold change increase between baseline and the leukapheresis 2 in the frequency of pp65 antigen-specific CD8$^+$ T cells producing three or more cytokines (IFNγ, CCL3, IL-2, TNFα, CD107a), and survival post-vaccine 4. The hazard ratio associate with a 1-unit fold change in polyfunctionality will be estimated with 95% confidence intervals.
Power Analyses	Cox proportional hazards model will assess the impact of fold change of pp65-specific polyfunctional CD8+ T cells between baseline and leukapheresis 2 on survival after vaccine #4. Data from Pro00000580 ERADICATE [132] shows that the SD for fold change in polyfunctionality among vaccinated patients is 1.589, and that the hazard ratio associated with 1 unit increase in fold change is

	approximately 0.609. Assuming SD=1.589, there would be 80% power to detect a hazard ratio of 0.6 assuming a two-tailed test (α=0.0167) within a Cox model after 16 deaths [130, 131]. If we assuming that the median OS post-vaccine 4 is 24 months, then 16 deaths should be observed approximately 38 months after the 24th patient receives vaccine 4.
Additional Analyses	Many of the 24 patients who have assessments at pre-vaccine 1 and leukapheresis 2 will also have an assessment of polyfunctionality at pre-vaccine 6. Among those patients who have all 3 assessments, a paired comparison of the fold change in polyfunctionality at pheresis 2 and vaccine 6 will be conducted using either a paired t-test or a Wilcoxon signed rank test. These analyses will assess whether levels of polyfunctionality are maintained in the absence of DI-TMZ. In addition, the Wilcoxon rank sum test will assess whether significant fold changes occur at pheresis 2.

15.6.4 Secondary Objective #4: T_{Regs}

Objective	To assess whether percent T_{Reg} levels increase further after vaccine 1
Analysis Set	All treated patients
Outcome	Maximum peak increase from vaccine 1 in percent T_{Reg} of CD4+ T cells
Hypothesis	With no additional temozolomide treatment after vaccine 1, the level of percent T_{Reg} will not significantly increase above vaccine 1 levels. Rather there should be no change or a reduction in T_{Reg} levels.
Analysis Plan	A one-tailed t-test will be conducted to assess whether the mean for the maximum change from vaccine 1 in percent T_{Reg} is significantly greater than 0. If the mean change is less than 0.71 (i.e. near 0 or a decrease), we will infer that levels of percent T_{Reg} do not increase after vaccine 1. Rather, they are either stable or decrease.
Power Analyses	In ATTAC-GM, the mean change in the percent T_{Reg} of CD4+ T cells between leukapheresis and vaccine 1, a period during which DI-TMZ is administered, was 5.77 (SD=4.52). Between vaccine 1 and the next cycle of DI-TMZ, the peak mean increase in percent T_{Reg} from vaccine 1 was -1.104 (SD=1.65). Assuming 48 patients and SD=1.65 for the mean change from vaccine 1 within I-ATTAC, there will be 90% power to detect an increase from vaccine 1 in the percent of T_{Reg} if the true mean increase is 0.71 or greater assuming a one-tailed test (α=0.05).
Additional Analyses	If the primary analysis that examines the maximum increase from vaccine 1 percent T_{Reg} of CD4+ T cells observed at any point after the initial vaccine detects such an increase, the timing of that increase is of interest. Hence, we will also examine the change from vaccine 1 to each of the follow-up assessments (leukapheresis 2, vaccine 4, vaccine 5, and vaccine 6). A generalized linear model may be used to explore these longitudinal changes. We may also use one-tailed t-tests to describe these changes. The change between vaccine 1 to leukapheresis 2 reflects the impact of vaccine 1, 2 and 3; whereas the change at vaccine 4 reflects the effect of the first 3 vaccines as well as T_d pre-conditioning.

	We will also conduct a t-test to examine the change in T_{Reg} levels observed before DI-TMZ and vaccine 1 to confirm that T_{Reg} levels are elevated with DI-TMZ, as well as the change between leukapheresis 1 and initiation of DI-TMZ.

15.6.5 Secondary Objective #5: Toxicity

Objective	Assess the safety of DI-TMZ treatment followed by DC vaccination.
Analysis Set	All patients who initiate DI-TMZ treatment.
Outcome	Proportion of patients with unacceptable toxicity as defined in Section 9.1.1.
Hypothesis	The toxicity profile associated with DI-TMZ followed by DC vaccination is safe.
Monitoring Plan	

Table A: Toxicity Monitoring Rules

Number of patients accrued	Number of patients with unacceptable toxicity requiring accrual suspension
≤5	≥2
6-11	≥3
12-17	≥4
18-23	≥5
24-29	≥6
30-34	≥7
35-39	≥8
≥40	≥9

Given that both long- and short-term toxicities are of interest in this study, it is not feasible to suspend accrual while toxicity is assessed as is often done in phase I trials. If the criteria in Table A are satisfied or there are other reasons for concern about the safety of patient treatment (e.g., treatment-related toxic death), accrual will be suspended and data will be carefully reviewed to determine if accrual should be permanently terminated or the protocol modified. Table A provides conditions under which accrual will be temporarily suspended and data carefully reviewed to determine the appropriate action, including permanent study termination, continuation with patient accrual after appropriate amendment, or continuation with patient accrual with no modification of the protocol. These guidelines have not been adjusted for differential length of follow-up of accrued patients.

If a death occurs within 30 days of DC vaccine administration, that is not attributable to progressive disease or other obvious non-study related cause (i.e. motor vehicle accident), enrollment of new subjects and all vaccinations will be suspended until review by the PI and IND sponsor has been completed and the FDA notified. If attribution of death is determined by the PI, IND sponsor or FDA to be possibly, probably, or definitely related to trial drug, then continued treatment of all enrolled subjects with trial drug will be suspended until the review is completed and recommendations for the study continuation have been

<table>
<tr><td></td><td>issued and approved by FDA. Any death attributed to trial drug, regardless of the timeline with respect to last treatment, will result in suspension of enrollment and suspension of continued treatment for enrolled subjects until review by FDA is complete and recommendations for study continuation issued and approved.

<table>
<tr><th colspan="2">TABLE B: Probability of Accrual Suspension</th></tr>
<tr><td>Underlying unacceptable toxicity Rate</td><td>Probability of accrual suspension</td></tr>
<tr><td>0.05</td><td>0.036</td></tr>
<tr><td>0.10</td><td>0.200</td></tr>
<tr><td>0.15</td><td>0.502</td></tr>
<tr><td>0.20</td><td>0.784</td></tr>
<tr><td>0.25</td><td>0.936</td></tr>
<tr><td>0.30</td><td>0.987</td></tr>
</table>

The probability of accrual suspension as a function of the true unacceptable toxicity rate is provided in Table B. These statistics were generated assuming toxicity outcome was known at the time of accrual, and ignored issues such as time to toxicity, accrual rate, and length of follow-up.

Every 6 months from the time the first patient received vaccine #1, the toxicity experienced by patients accrued to this study will be summarized and reviewed regardless of the number of patients accrued to determine whether the overall toxicity profile of treatment is unacceptable or not.</td></tr>
<tr><td>Analysis Plan</td><td>The proportion of patients who experience an unacceptable toxicity as defined in Section 9.1.1</td></tr>
<tr><td>Additional Analyses</td><td>Adverse events will be summarized in several other forms to satisfy scientific and monitoring needs, as well as various regulatory reporting needs (e.g. FDA, DCI Safety Oversight Committee, and ClinicalTrials.gov). For these summaries, the frequency of adverse events will be tabulated by the maximum grade experienced.</td></tr>
</table>

15.7 Exploratory Objectives

This study has 3 exploratory objectives: (1) To assess the relationship between migration and serum CCL3, (2) To assess the relationship between pp65-specific polyfunctional CD8+ T cells and migration, and (3) To assess the relationship between pp65-specific polyfunctional CD8+ T cells and serum levels of CCL3.

15.7.1 Exploratory Objective #1: Migration and CCL3

Objective	To assess the relationship between serum CCL3 levels and increased DC migration.
Analysis Set	All patients who undergo migration studies who have CCL3 levels measured.
Outcome	Correlation coefficient between fold change of CCL3 and migration.
Hypothesis	We hypothesize that fold change in CCL3 will be highly correlated with increased migration.

Analysis Plan	A Spearman rank correlation coefficient will assess the association between CCL3 change from pre to post-vaccine 4, and migration.
Additional Analyses	As an additional exploratory analysis of CCL3, a one-sample t-test or a Wilcoxon rank sum test will assess whether fold change in CCL3 between pre- and post-Td pre-conditioning differs from 0.

15.7.2 Exploratory Objective #2: Polyfunctionality and Migration

Objective	To assess the relationship between pp65-specific polyfunctional CD8+ T cells and DC migration.
Analysis Set	All patients who undergo migration studies who have polyfunctional levels measured.
Outcome	Correlation coefficient between fold change in polyfunctionality and migration
Hypothesis	We hypothesize that increased pp65-specific polyfunctionality CD8+ T cells will be highly correlated with increased DC migration.
Analysis Plan	A Spearman rank correlation coefficient will assess the association between fold change in polyfunctionality and migration.

15.7.3 Exploratory Objective #3: Polyfunctionality and CCL3

Objective	To assess the relationship between pp65-specific polyfunctional CD8+ T cells and serum CCL3.
Analysis Set	All patients who undergo polyfunctional studies and have CCL3 levels measured.
Outcome	Correlation coefficient between fold change in polyfunctionality and fold change in CCL3.
Hypothesis	We hypothesize that increased pp65-specific polyfunctionality CD8+ T cells will be highly correlated with increases in serum CCL3 after Td preconditioning.
Analysis Plan	A Spearman rank correlation coefficient will assess the association between fold change in polyfunctionality and fold change in CCL3.

15.8 Interim Analysis

As described in Section 15.9, we anticipate that at the time of the primary study analysis 33 of the patients treated with DI-TMZ will have died. An interim analysis for futility will be conducted after approximately 17 patients have died, assuming that occurs before accrual is completed. The α-spending function that approximately an O'Brien-Fleming boundary will defined critical values that will be used in making that assessment [133]. Based upon these analyses and available correlative data, a decision will be made concerning continued accrual without modification, continued accrual with modifications to the protocol, or termination of patient accrual.

15.9 Sample Size Calculation

Approximately 64 patients will be accrued to this study, with the goal of initiating DI-TMZ in 48 of these patients.

To evaluate the observed OS within this study of pp65 DC vaccination with tetanus pre-conditioning and adjuvant GM-CSF, we will consider Gilbert's study of dose-dense temozolomide for newly diagnosed glioblastoma. Gilbert reports a median OS from initiation of adjuvant

treatment among methylated patients to be 14.0 months (95% CI: 12.9 to 14.7 months).

Though the median OS from initiation of adjuvant DI-TMZ treatment in the ATTAC-GM pilot is 37.7 months (95% CI: 18.2, ∞), we hypothesize for power calculations that the true median OS for our novel treatment regimen is approximately 24 months. Assuming accrual of 64 patients over a period of 24 months, 28 months follow-up after the last patient initiates adjuvant treatment, and 12 patients who initiate vaccine treatment, a two-sided, a one-sample logrank test has 80% power at a 0.05 significance level to detect a hazard ratio of 0.6125 (i.e. = 14.7 / 24) when the median survival of the historic control group is 14.7 months, the upper bound of the 95% confidence interval for median survival reported by Gilbert [124, 125, 131]. We anticipate that at the time of the primary study analysis 33 of these patients will have died.

16 ADMINISTRATIVE AND ETHICAL CONSIDERATIONS

16.1 Regulatory and Ethical Compliance

This protocol was designed and will be conducted and reported in accordance with the International Conference on Harmonization (ICH) Harmonized Tripartite Guidelines for Good Clinical Practice, the Declaration of Helsinki, and applicable federal, state, and local regulations.

16.2 DUHS Institutional Review Board and DCI Cancer Protocol Committee

The protocol, informed consent form, advertising material, and additional protocol-related documents must be submitted to the DUHS IRB and DCI Cancer Protocol Committee (CPC) for review. The study may be initiated only after the Principal Investigator has received written and dated approval from the CPC and IRB.

The Principal Investigator must submit and obtain approval from the IRB for all subsequent protocol amendments and changes to the informed consent form. The CPC should be informed about any protocol amendments that potentially affect research design or data analysis (i.e. amendments affecting subject population, inclusion/exclusion criteria, agent administration, statistical analysis, etc.).

The Principal Investigator must obtain protocol re-approval from the IRB within 1 year of the most recent IRB approval. The Principal Investigator must also obtain protocol re-approval from the CPC within 1 year of the most recent IRB approval, for as long as the protocol remains open to subject enrollment.

16.3 Informed Consent

The informed consent form must be written in a manner that is understandable to the subject population. Prior to its use, the informed consent form must be approved by the IRB.

The Principal Investigator or authorized key personnel will discuss with the potential subject the purpose of the research, methods, potential risks and benefits, subject concerns, and other study-related matters. This discussion will occur in a location that ensures subject privacy and in a manner that minimizes the possibility of coercion. Appropriate accommodations will be made available for potential subjects who cannot read or understand English or are visually impaired. Potential subjects will have the opportunity to contact the Principal investigator or authorized key

personnel with questions, and will be given as much time as needed to make an informed decision about participation in the study.

Before conducting any study-specific procedures, the Principal Investigator must obtain written informed consent from the subject. The original informed consent form will be stored with the subject's study records, and a copy of the informed consent form will be provided to the subject. The Principal Investigator is responsible for asking the subject whether the subject wishes to notify his/her primary care physician about participation in the study. If the subject agrees to such notification, the Principal Investigator will inform the subject's primary care physician about the subject's participation in the clinical study.

16.4 Privacy, Confidentiality, and Data Storage

The Principal Investigator will ensure that subject privacy and confidentiality of the subject's data will be maintained. Research Data Security Plans (RDSPs) will be approved by the appropriate institutional Site Based Research group.

To protect privacy, every reasonable effort will be made to prevent undue access to subjects during the course of the study. Prospective participants will be consented in an exam room where it is just the research staff, the patient and his family, if desired. For all future visits, interactions with research staff (study doctor and study coordinators) regarding research activities will take place in a private exam room. All research related interactions with the participant will be conducted by qualified research staff who are directly involved in the conduct of the research study.

To protect confidentiality, subject files in paper format will be stored in secure cabinets under lock and key accessible only by the research staff. Subjects will be identified only by a unique study number and subject initials. Electronic records of subject data will be maintained using a Clinical database, which is housed by the DCI. Access to electronic databases will be limited to the Principal Investigator, key personnel, statisticians, the Radiolabeled Pharmacy personnel, and the PRTBTC data manager. Data stored on portable memory devices will be de-identified. The security and viability of the IT infrastructure will be managed by the DCI and/or Duke Medicine.

Upon completion of the study, research records will be archived and handled per DUHS Human Research Protection Program (HRPP) policy.

Subject names or identifiers will not be used in reports, presentations at scientific meetings, or publications in scientific journals.

16.5 Data and Safety Monitoring

Data and Safety Monitoring will be performed in accordance with the DCI Data and Safety Monitoring Plan. For a more detailed description of the DSMP for this protocol, refer the separate upload in electronic IRB.

16.6 Protocol Amendments

All protocol amendments must be initiated by the Principal Investigator and approved by the IRB prior to implementation. IRB approval is not required for protocol changes that occur to protect the safety of a subject from an immediate hazard. However, the Principal Investigator must inform the IRB and all other applicable regulatory agencies of such action immediately.

Though not yet required, the CPC should be informed about any protocol amendments that potentially affect research design or data analysis (i.e., amendments affecting subject population, inclusion/exclusion criteria, agent administration, etc.).

16.7 Records Retention

The Principal Investigator will maintain study-related records for the longer of a period of:

- at least two years after the date on which a New Drug Application is approved by the FDA
- at least two years after formal withdrawal of the IND associated with this protocol
- at least six years after study completion (Duke policy).

16.8 Conflict of Interest

The Principal Investigator and Sub-Investigators must comply with applicable federal, state, and local regulations regarding reporting and disclosure of conflict of interest. Conflicts of interest may arise from situations in which financial or other personal considerations have the potential to compromise or bias professional judgment and objectivity. Conflicts of interest include but are not limited to royalty or consulting fees, speaking honoraria, advisory board appointments, publicly-traded or privately-held equities, stock options, intellectual property, and gifts.

The Duke University School of Medicine's RIO reviews and manages research-related conflicts of interest. The Principal Investigator and Sub-Investigators must report conflicts of interest annually and within 10 days of a change in status, and when applicable, must have a documented management plan that is developed in conjunction with the Duke RIO and approved by the IRB/IEC.

16.9 Registration Procedure

After patients have been enrolled, subject registration will be entered into the Duke eResearch system and the subject's visits associated in the Duke Epic Maestro Care system with this protocol which is entered after Duke IRB approval.

17 REFERENCES

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18 APPENDICES

18.1 Standard Radiation Therapy

Radiotherapy typically begins within ≤ 5 weeks of surgery. One treatment of 1.8-2.0 Gy/fraction will be given daily 5 days per week for a total of 59.4-60.0 Gy over <7 weeks. 3D conformal and intensity-modulated RT is permitted. All portals should be treated during each treatment session. Doses are specified as the target dose that shall be to the center of the target volume.

The gross target volume (GTV) for both the initial volume (GTV1) and the conedown volume (GTV2) should be based on the postoperative CT/MRI (and preferably the MRI; the preoperative scans may be used if postoperative scans are not available). This initial target volume (GTV1) should include the contrast-enhancing lesion (and should include the surgical resection cavity) and surrounding edema (if it exists) demonstrated on CT/MRI plus a 2.0-cm margin (this 2.0-cm margin-extended volume will be considered the initial planning target volume, or PTV1). The initial target volume should be treated to 46 Gy at 2Gy/fraction or 45-50.4 Gy at 1.8Gy/fraction. If no surrounding edema is present, the initial planning target volume (PTV1) should include the contrast-enhancing lesion (and should include the surgical resection cavity) plus a 2.5-cm margin. Please note that clinical judgment may be used to modify PTV1 to exclude sensitive structures such as the optic chiasm, non-cranial contents, or anatomic regions in the brain where natural barriers would likely preclude microscopic tumor extension, such as the cerebellum, the contralateral hemisphere, directly across from the tentorium cerebri, the ventricles, etc. After 46 Gy, the tumor volume (GTV2) for the conedown treatment should include the contrast-enhancing lesion (without edema) on the pre-surgery CT/MRI scan plus a 1.5-2-cm margin (PTV2). Treat to 14 Gy at 2Gy/fraction or 14.4-9.0 Gy at 1.8Gy/fraction to a total of 60.0 or 59.4Gy, respectively.

Dose is prescribed to the isodose line such that at least 95% of the target volume receives he prescribed dose. The optic apparatus should be limited to a maximum of 54Gy and no more than 5% of the volume of the brainstem should receive >54Gy.

Radiation should be delayed or interrupted if the platelet count is < 20,000. Radiation should not begin or resume until the platelet count is ≥ 20,000. Hematologic toxicities should be rated on a scale of 0-5 as defined in the NCI Common Terminology Criteria for Adverse Events (CTCAE) version5.0. If radiotherapy has to be temporarily interrupted for technical or medical reasons unrelated to the temozolomide administration, then treatment with daily temozolomide should continue. If radiotherapy has to be permanently interrupted then treatment with daily temozolomide should stop. The following should be recorded at entry into this study: daily treatment record, all isodose distributions (in color), dose volume histograms including the cumulative dose to the target volumes, optic chiasm, optic nerves and brain stem, and the radiotherapy summary.

18.2 Temozolomide Therapy

Please refer to the current package insert for Temodar®.