

Immunological targeting of cytomegalovirus for glioblastoma therapy

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Human cytomegalovirus (CMV) is purportedly present in glioblastoma (GBM) while absent from the normal brain, making CMV antigens potentially ideal immunological anti-GBM targets. We recently demonstrated that patient-derived CMV pp65-specific T cells are capable of recognizing and killing autologous GBM tumor cells. This data supports CMV antigen-directed immunotherapies against GBM.

Despite aggressive surgery, radiation and chemotherapy, *glioblastoma multiforme* (GBM), the most common primary brain cancer, remains incurable. The limitations of conventional therapies are non-specificity and damage to surrounding normal brain tissue. Immunotherapy is a promising alternative to these non-specific cytotoxic therapies by specifically targeting tumor cells and leaving surrounding normal tissue intact. Moreover immunotherapy results in systemic immune surveillance that can control tumor recurrence. In addition, T cells can eradicate large tumors and tumors that reside within the immunologically privileged central nervous system. Immune-based recognition of tumor cells is achieved by specific targeting of antigens that are expressed solely by, or overexpressed on, tumor cells but not on normal cells. However such exquisitely tumor-specific antigens are rare and many remain unidentified. To this end, immunotherapy strategies targeting the entire GBM antigen repertoire has been explored with unfractionated tumor antigens in the form of tumor lysates, total tumor RNA, and tumor peptides. These studies have shown safety, feasibility, capacity to elicit

immune responses and promising efficacy. However, these approaches are limited by the requirement of patient primary tumor tissue as the source of antigen, which can be limiting.

Cobbs et al. first reported the detection of cytomegalovirus (CMV) antigens in GBM in 2002.¹ We published the first confirmatory report of this association in 2008.² We have demonstrated the presence of CMV nucleic acids and proteins in primary and recurrent GBM specimens using immunohistochemistry (IHC), RNA and DNA in situ hybridization (ISH), qualitative and quantitative real-time PCR coupled with viral DNA sequencing, and western blot (WB) analysis. We have ruled out laboratory contamination as a source of viral detection through DNA sequencing of distinct viral isolates from GBM specimens, and detection in formalin-fixed, paraffin-embedded (FFPE) tumor samples by IHC and ISH. We have also ruled out the detection of latent virus through demonstration of early and late viral gene products by WB analysis on tumor samples but not in normal brain lysates, and the inability to detect CMV proteins or nucleic acids in samples from age-matched normal volunteers,

or patients undergoing craniotomy at Duke University Medical Center for non-malignant diseases. Independent laboratories have now confirmed CMV expression in GBM.³

While the role of CMV in GBM oncogenesis is still under investigation, its presence in neoplastic tissue allows for the potential development of a targeted immunotherapeutic. To this end, we set out to determine the feasibility of targeting the CMV antigen pp65 as an immunological target.⁴ To demonstrate relevance and therapeutic potential all immune assays were conducted in vitro using an autologous set-up with GBM patients' peripheral blood mononuclear cells (PBMCs) and tumor cells. Our first task was to demonstrate feasibility of generating dendritic cells (DCs) in vitro from patient PBMC-derived adherent monocytes. The in vitro generated immature DCs were transfected with CMV pp65 encoding mRNA and then matured using the standard maturation cocktail of GM-CSF and IL-4 with TNF α , IL-1 β , IL-6, and PGE₂.⁵ This DC-RNA platform was chosen based on our prior studies showing that RNA-transfected DCs are potent antigen-presenting cells and are very effective at

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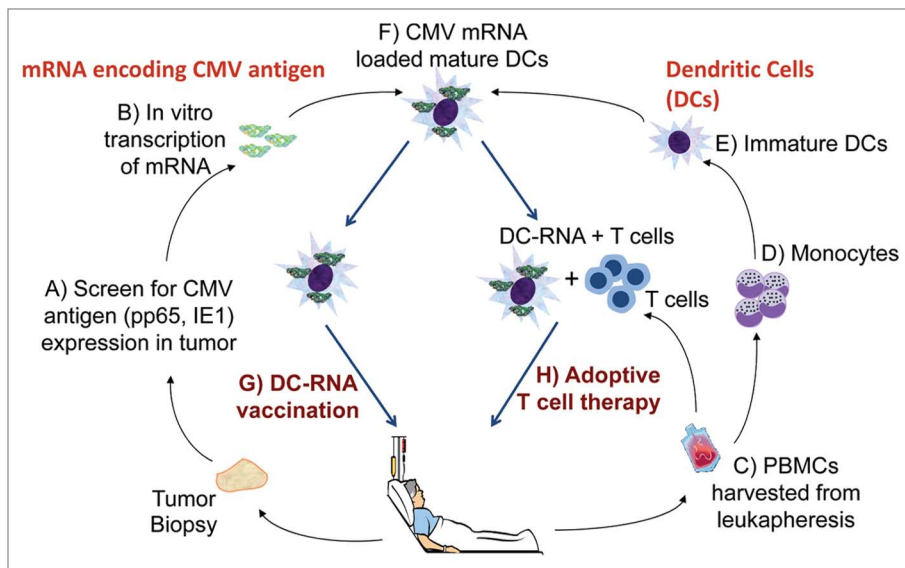


Figure 1. GBM immunotherapy using CMV RNA-loaded dendritic cells. Clinical immunotherapy trial for patients with *glioblastoma multiforme* (GBM). (A) Tumor specimens are screened for expression of cytomegalovirus (CMV) antigens. (B) mRNA encoding CMV antigens is generated by in vitro transcription. (C) Peripheral blood mononuclear cells (PBMCs) are harvested from patient for production of autologous cellular therapy products. (D) Monocytes are isolated from PBMCs for dendritic cell (DC) generation. (E) Immature DCs are differentiated from monocytes using standardized clinical DC generation protocols. (F) DCs are electroporated with CMV RNA followed by maturation. (G) DCs are used in vaccination strategies in combination with standard treatment. (H) DCs are used to stimulate autologous T cells for ex vivo expansion of CMV-specific T cells for adoptive T cell therapy.

inducing antitumor immunity in vivo and in vitro.⁶ CMV pp65 RNA-transfected, mature DCs were consistently generated from GBM patient derived PBMCs.

To determine if CMV pp65 could serve as an immunological target in GBM immunotherapy, we used CMV pp65 RNA-transfected, mature DCs to stimulate autologous T cells (isolated from patient PBMCs) in vitro. We demonstrated the induction of CMV pp65-specific immune responses using tetramer analysis and analysis of IFN- γ production. To provide additional support of immunologic competence in GBM patients, we demonstrated that the functional response of CMV pp65-specific T cells derived from GBM patient PBMCs were comparable to those derived from a healthy donor.

The objective of this study was to demonstrate that CMV could serve as an immunological target in GBM. In particular, we wanted to determine if CMV-specific T cells could specifically target the endogenous low levels of CMV expressed in GBM. We therefore

examined the cytotoxic activity of CMV pp65-specific T cells on autologous patient-derived, primary GBM tumor cells. Using cells from 4 patients with GBM, we demonstrated that CMV pp65-specific T cells recognize and lyse autologous GBM tumor cells. To ascertain specific cytotoxicity of CMV-specific T cells toward GBM tumor cells, we used autologous DCs transfected with RNA encoding the following antigens as cellular targets; survivin RNA, Flu M1 RNA, or total cellular RNA as control targets and CMV pp65 RNA or GBM tumor-derived total RNA as test targets. As such DCs transfected with GBM tumor-derived total RNA also serve as effective surrogate tumor targets in the absence of primary tumor cells, as we have previously demonstrated.⁷ Total cellular RNA refers to RNA derived from autologous PBMCs or DCs. The in vitro generated CMV-specific T cells only lysed CMV pp65- and GBM total tumor RNA-loaded DCs and not any of the control antigen RNA-loaded DCs. As an additional control we generated

Flu M1-specific T cells (targeting the influenza virus matrix (M1) protein), and demonstrated that Flu M1-specific T cells lysed only Flu M1-expressing DC targets with no reactivity against DCs pulsed with GBM total tumor RNA or autologous GBM tumor cells.

To further investigate if CMV is an immunological target in GBM, we generated GBM tumor-specific T cells in vitro using total tumor RNA-transfected autologous DCs as stimulators. The induction of CMV-specific cytotoxic reactivity was measured in the GBM tumor-specific T cell population in a cytotoxicity assay with autologous DCs transfected with either CMV pp65 RNA or Flu M1 RNA as cellular targets. We demonstrated that GBM tumor-specific T cells only lyse DCs expressing CMV pp65 RNA but not Flu M1 RNA. We also demonstrated expansion of CMV pp65 tetramer+ CD8+ T cells after stimulation with total tumor RNA-pulsed DCs. Importantly, no expansion of CMV pp65 tetramer+ T cells was observed when stimulated by Flu M1 RNA-pulsed DCs.

In this study, we demonstrate the physiologic relevance of CMV as an immunotherapeutic target in GBM. This study supports the rationale for CMV-directed immunotherapy for the treatment of GBM.⁸⁻¹⁰ CMV-directed immunotherapy for patients with GBM is the subject of investigation in 3 Phase I clinical trials at Duke University Medical Center (Fig. 1). Two studies are investigating the use of DCs transfected with CMV pp65 RNA as a GBM vaccine (NCT00639639, NCT00626483). One study is investigating the use of adoptive T cell therapy in patients with GBM using CMV pp65-specific T cells generated ex vivo using autologous CMV pp65 RNA-transfected DCs (NCT00693095).

We are cognizant of the fact that CMV directed immunotherapy is impacted by other factors, including heterogeneous CMV expression in GBM tumors, immunosuppression encountered by T cells in the tumor microenvironment, suppression mediated by inhibitory immune receptors on T cells, such as CTLA-4 and PD-1, and the effect of prior standard-of-care therapies on the tumor and the host. Ultimately, a combination

therapy and an understanding of how to sequence these combination therapies will be required to fully realize the potential of CMV-directed immunotherapy.

Disclosure of Potential Conflicts of Interest

S.K.N. is a co-inventor on the patent describing the use of dendritic cells transfected with tumor antigen encoding RNA that has been licensed by Argos Therapeutics (Durham, NC) through Duke University. S.K.N. has no financial interest in Argos Therapeutics and is not compensated by Argos Therapeutics. J.H.S. and D.A.M. hold a patent related to technologies disclosed in this work.

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