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# Viral-mimicking protein nanoparticle vaccine for eliciting anti-tumor responses

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ABSTRACT

The immune system is a powerful resource for the eradication of cancer, but to overcome the low immunogenicity of tumor cells, a sufficiently strong $CD8^+$ T cell-mediated adaptive immune response is required. Nanoparticulate biomaterials represent a potentially effective delivery system for cancer vaccines, as they can be designed to mimic viruses, which are potent inducers of cellular immunity. We have been exploring the non-viral pyruvate dehydrogenase E2 protein nanoparticle as a biomimetic platform for cancer vaccine delivery. Simultaneous conjugation of a melanoma-associated gp100 epitope and CpG to the E2 nanoparticle (CpG-gp-E2) yielded an antigen-specific increase in the $CD8^+$ T cell proliferation index and IFN-γ secretion by 1.5-fold and 5-fold, respectively, compared to an unbound peptide and CpG formulation. Remarkably, a single nanoparticle immunization resulted in a 120-fold increase in the frequency of melanoma epitope-specific $CD8^+$ T cells in draining lymph nodes and a 30-fold increase in the spleen, relative to free peptide with free CpG. Furthermore, in the very aggressive B16 melanoma murine tumor model, prophylactic immunization with CpG-gp-E2 delayed the onset of tumor growth by approximately 5.5 days and increased animal survival time by approximately 40%, compared to PBS-treated animals. These results show that by combining optimal particle size and simultaneous co-delivery of molecular vaccine components, antigen-specific anti-tumor immune responses can be significantly increased.

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## 1. Introduction

Recent years have brought an improved understanding of the interplay between cancer and the immune system, increasing clinical interest in immunotherapy [1]. The immune system possesses many unique advantages for targeted disease eradication [2,3], mediated primarily by robust $CD8^+$ cytotoxic T lymphocyte (CTL) responses [4,5]. Strategies for therapeutic vaccination against tumor-associated antigens (TAAs) have included administration of whole protein antigen [6], mature peptide epitopes [7], cell lysate [8], and adoptive transfer strategies [9,10]. Peptide vaccines, in particular, represent an attractive strategy by allowing for incorporation of multiple mature epitopes; however, suboptimal CTL responses are typically observed in clinical trials, prompting the need for enhanced approaches [11].

In contrast to peptide vaccines, viruses are effective inducers of CTL immunity [12]. They are generally comprised of one or a few protein monomers that self-assemble into symmetrical hollow structures packaged with genetic material [13]. Dendritic cells (DCs), perhaps the most potent antigen presenting cell (APC) for induction of adaptive immunity, have evolved sensing mechanisms (*e.g.*, Toll-like receptors; TLRs) to recognize common features of pathogens (*e.g.*, viruses) for activation and orchestration of CTL responses [14]. In addition, DCs are effective cross-presenters of exogenous antigens, such as those of viruses and

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cancers [15]. Therefore, mirroring the pathogenic features of viruses, sans virulence, with biomaterials represents a potentially effective approach of delivering TAA-derived epitopes [13]. While active targeting of DCs with biomaterials is an approach with demonstrated potential [16], an advantage of virus mimicry is the passive targeting and preferential accumulation within immunologically rich regions (*i.e.* lymph nodes) and interaction with DCs following immunization [17].

In particular, the viral size, repetitive structural features, and co-delivery of immune-inducing viral components are characteristics that have been attributed to the induction of an effective immune response [17]. The weak immune responses to peptide (and protein) TAA vaccines may be related to physical size, in which these components are typically well below the size range reported to be optimal for efficient delivery to APCs [17]. Cancer vaccine delivery vehicles such as synthetic nanostructured biomaterials (*e.g.*, liposomes, metals, and polymers) and natural systems (*e.g.*, viruses and exosomes) have been explored [18–20] as alternatives to traditional TAA peptide delivery platforms to enhance the efficacy of the anti-tumor immune response.

Since the first clinically approved virus-like particle (VLP)-based vaccine (Gardasil), many other nanoparticulate protein-based assemblies have been clinically developed as vaccines, primarily for infectious diseases [13,21], and in particular for induction of adaptive T cell responses. For example, VLP-based vaccines targeting influenza were previously shown to induce protective $CD8^+$ T cell responses following a single immunization [22]. In cancer therapy, Qβ VLPs have been undergoing clinical trials for vaccination against the Melan-A/MART1 melanoma-associated tumor antigen [23]. Autologous tumor-derived heat shock proteins, hypothesized to bind autologous TAAs, have been explored for cancer vaccination as well, supporting clinical interest in natural protein-derived nanoassemblies that carry antigens [24,25].

In this work, we examine the use of the E2 subunit of pyruvate dehydrogenase for cancer immunotherapy applications. The E2 nanoparticle is a self-assembling hollow protein cage with an approximately 30-nm diameter and high physical stability [26]. It is also of non-viral origin and has been shown to be amenable to functionalization in biomedical applications [26–29]. Our research group has previously demonstrated significantly enhanced activation and cross-presentation of a model antigen using the E2 nanoparticle for delivery to and activation of DCs [30]. This increased activation, mediated by virus-mimicking E2 nanoparticles, may allow the immune system to overcome the low immunogenicity or tolerance to tumor antigens. Based on this prior study with ovalbumin, we hypothesized that E2-mediated co-delivery of a repetitive TAA epitope, together with CpG packaged for endolysosomal release, would induce increased antigen-specific anti-tumor responses following immunization (relative to other tumor peptide vaccine formulations of the same epitope).

Our target epitope in this current work is the gp100 melanocyte differentiation protein, a TAA that is a tumor regression antigen and a clinically-pursued target in humans [31]. The antigen is highly conserved between human and mouse, enabling testing of human vaccines in a murine model [5]. While the full gp100 protein has been loaded to heat-shock proteins for vaccination in murine melanoma models [32], to our knowledge, clinically-applicable gp100 epitopes packaged with DC activators have not been previously tested using non-viral protein nanoparticle systems. This study examines the induction of $CD8^+$ T cell and anti-tumor responses that are specific to a gp100 peptide epitope and demonstrates that the viral-mimicking E2 nanoparticle platform may be a particularly effective delivery system for tumor antigens.

## 2. Methods

### 2.1. Materials

All buffer reagents were purchased from Fisher Scientific, unless otherwise noted. The oligodeoxynucleotide TLR9 ligand CpG 1826 (5′-tccatgacgttcctgacgtt-3′) (CpG) was synthesized with a phosphorothioated backbone and 5′ benzaldehyde modification by TriLink Biotechnologies. The KVPRNQDWL peptide ($gp100_{25-33}$, herein abbreviated as "gp100") was from Genscript, and the custom gp100 peptide (for conjugation to E2) was synthesized with an N-terminal cysteine by Thinkpeptides (Proimmune). Unless otherwise noted, cell culture media was comprised of RPMI 1640 (Mediatech) supplemented with 10% heat-inactivated fetal bovine serum (Hyclone), 1 mM sodium pyruvate (Hyclone), 2 mM L-glutamine (Lonza), 100 units/ml penicillin (Hyclone), 100 μg/ml streptomycin (Hyclone), 50 μM 2-mercaptoethanol (Sigma), and 0.1 mM non-essential amino acids (Lonza) (complete RPMI media). Carboxyfluorescein diacetate succinimidyl ester (CFSE), flow cytometry antibodies, and recombinant murine GM-CSF were purchased from eBioscience. Phytohemagglutinin (PHA-M) was from Gibco.

### 2.2. Mice and cell lines

All animal studies were carried out in accordance with protocols approved by the Institute for Animal Care and Use Committee (IACUC) at the University of California, Irvine. Female C57BL/6 mice and pmel-1 mice, which display transgenic T-cell receptors specific for the $gp100_{25-33}$ epitope in the context of $H2\text{-}D^b$ in a C57BL/6 background [5], were purchased from Jackson Laboratories and used at 6–12 weeks of age, unless otherwise noted. The B16-F10 murine melanoma cell line was purchased from ATCC and cultured in DMEM media supplemented with 10% FBS according to vendor instructions.

### 2.3. E2 purification and characterization

The D381C E2 protein nanoparticle (E2) was prepared and characterized as previously described [26,30]. D381C is an E2 mutant with a non-native cysteine introduced to the internal cavity of the nanoparticle at amino acid location 381 for site-specific conjugation. Briefly, proteins were expressed in *E. coli* and soluble cell lysates were applied to a HiPrep Q Sepharose anion exchange column (GE Healthcare) followed by a Superose 6 size exclusion column (GE Healthcare) for purification. The hydrodynamic diameter of the purified proteins was analyzed by dynamic light scattering (DLS; Zetasizer Nano ZS, Malvern). Electrospray ionization mass spectrometry and SDS-PAGE confirmed molecular weight and purity. Final protein preparations were stored in 50 mM potassium phosphate at pH 7.4 with 100 mM NaCl (phosphate buffer) at 4 °C for short-term and −80 °C for long-term storage. Residual LPS was removed using Triton X-114, and endotoxin levels were checked as previously described [30].

### 2.4. CpG and gp100 conjugation

Aldehyde-terminated CpG oligonucleotides were covalently packaged within E2, and cysteine-terminated peptide epitopes were displayed on the external surface of E2 as previously described [30]. Briefly, the cysteines in the E2 internal cavity were reduced with TCEP (Pierce), followed by incubation with N-(β-maleimidopropionic acid) hydrazide (BMPH) linker (Pierce) and removal of unreacted linker. Conjugation with the aldehyde-modified CpG 1826 involved overnight incubation and excess CpG removal. The number of conjugated CpG molecules was

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