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# VennVax, a DNA-prime, peptide-boost multi-T-cell epitope poxvirus vaccine, induces protective immunity against vaccinia infection by T cell response alone

Leonard Moise[a,b,*], R. Mark Buller[c], Jill Schriewer[c], Jinhee Lee[d], Sharon E. Frey[c], David B. Weiner[f], William Martin[a], Anne S. De Groot[a,b,e,*]

[a] EpiVax, Inc., Providence, RI, USA
[b] University of Rhode Island, Providence, RI, USA
[c] Saint Louis University School of Medicine, St. Louis, MO, USA
[d] University of Massachusetts Medical Center, Worcester, MA, USA
[e] Pediatric Infectious Disease, Brown University Warren Alpert Medical School, Providence, RI, USA
[f] Department of Pathology, Laboratory Medicine, University of Pennsylvania School of Medicine, Philadelphia, PA, USA

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ABSTRACT

The potential for smallpox to be disseminated in a bioterror attack has prompted development of new, safer smallpox vaccination strategies. We designed and evaluated immunogenicity and efficacy of a T-cell epitope vaccine based on conserved and antigenic vaccinia/variola sequences, identified using bioinformatics and immunological methods. Vaccination in HLA transgenic mice using a DNA-prime/peptide-boost strategy elicited significant T cell responses to multiple epitopes. No antibody response pre-challenge was observed, neither against whole vaccinia antigens nor vaccine epitope peptides. Remarkably, 100% of vaccinated mice survived lethal vaccinia challenge, demonstrating that protective immunity to vaccinia does not require B cell priming.

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## 1. Introduction

The CDC and NIH classify Variola major as a Category A pathogen because concerns remain that it, or closely related poxviruses, such as monkeypox, might be used to provoke fear and induce widespread morbidity and mortality in a bioterror attack [1,2]. Variola (smallpox) is a particularly dangerous biological threat because of its clinical and epidemiologic properties [3,4]. Smallpox virus can be manufactured in large quantities, stored for an extended period of time, and delivered as an infectious aerosol. Vaccinia (smallpox vaccine) has been used to protect against smallpox but many individuals have not been vaccinated (the vaccine was no longer required in the U.S. after 1980), thus case-fatality rates could be higher than 25% of the population if smallpox were released as a bioterrorist weapon [2]. In addition, human monkeypox is an emerging zoonotic disease and potential biowarfare agent for which prophylactic agents are needed, and against which vaccinia (smallpox) vaccination has been considered.

Mass vaccination, as was successfully implemented to eradicate smallpox worldwide, would be the logical course of protective action in response to deliberate dissemination of monkeypox and smallpox, but it poses a medical dilemma because the risks associated with vaccination using live-attenuated vaccinia are not negligible. The use of live attenuated vaccinia in immunization protocols where a significant percentage of the population is immunocompromised because of HIV infection, has raised some concern [6,7]. Data accumulated over the eradication campaign years showed that immunization with replication-competent, attenuated vaccinia was associated with serious adverse effects, such as encephalitis, vaccinia necrosum and eczema vaccinatum [8,9]. While their incidence was low at the time, today they could be significantly magnified because a greater proportion of the population is immunocompromised. Although the current US government stockpiled vaccine, ACAM2000, a vero-cell-culture derived vaccinia, has the advantage of limiting the risk of adventitious agents, the replicating virus has a similar adverse event profile compared to Dryvax [10]. As a result, development of safer smallpox vaccines has become a priority. Currently, modified vaccinia Ankara (MVA), a highly attenuated nonreplicating virus in mammalian cells, has a

* Corresponding authors at: EpiVax, Inc., 146 Clifford Street, Providence, RI, USA. Tel.: +1 401 272 2123; fax: +1 401 272 7562.
*E-mail addresses:* LMoise@EpiVax.com (L. Moise), AnnieD@EpiVax.com (A.S. De Groot).

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significantly limited adverse event profile and is currently in clinical trials [11].

The goal of our VennVax smallpox vaccine development program has been to demonstrate proof-of-principle that a genome-to-vaccine approach can be successfully applied to a potential bioterror agent. To develop VennVax, we systematically evaluated the vaccinia and variola genomes for conserved immunogenic HLA Class I and Class II epitopes and demonstrated that these epitopes possess properties essential to all successful vaccine antigens: (1) HLA binding and (2) ex vivo antigenicity in human subjects, (3) in vivo immunogenicity and (4) protection from lethal challenge. Previously, we reported immunoinformatic selection of 50 conserved and immunogenic variola/vaccinia Class II HLA epitope sequences, of which >80% were antigenic in ex vivo T cell assays performed with blood from Dryvax-exposed volunteers [12]. Here, we report that these T-cell epitopes are immunogenic and efficacious in an HLA transgenic mouse model of vaccinia infection when delivered as a heterologous DNA-prime/peptide-boost vaccine. Remarkably, vaccine-induced antibody production is not required for protection from challenge.

## 2. Methods

### 2.1. Multi-epitope DNA vaccine engineering

Epitope sequences were concatenated to form two multi-epitope genes, each containing 25 HLA Class II epitopes that were identified by immunoinformatics methods, as described previously [12]. Initially, epitopes were assembled in a random sequence. To avoid creation of novel epitopes at epitope junctions, an algorithm which iteratively re-orders epitopes to reduce junctional immunogenicity (VaccineCAD) was used to optimize epitope order [13]. In addition, where re-ordering by VaccineCAD did not sufficiently reduce potential junctional immunogenicity, Gly-Pro-Gly-Pro-Gly spacer sequences were engineered between epitopes to optimize epitope processing [14].

A Kozak sequence was engineered upstream of the coding sequence for efficient translation initiation. To target the immunogens to the Class II processing pathway, the tissue plasminogen activator (tPA) leader sequence (MQMSPALTCLVLGLALVFGEGSA) was placed upstream of epitope sequences to direct translation products to the secretory pathway. A histidine tag was incorporated downstream of the epitope sequences followed by two stop codons. Genes were synthesized by GeneArt and subcloned at predetermined flanking restriction sites into pVAX1 (Invitrogen), a DNA vaccine vector that accommodates FDA recommendations for construction of plasmid DNA vaccines [15].

### 2.2. Plasmid DNA vaccine preparation

High purity plasmids for immunizations were prepared by PureSyn, Inc. at pre-clinical grade. Each plasmid underwent quality control testing including spectrophotometric concentration and $A_{260}/A_{280}$ ratio determination (~1.9), restriction digest analysis to assure the presence of the multi-epitope genes, agarose gel electrophoresis determination of residual host RNA and DNA (none detected), and quantitative endotoxin testing (<24.9 EU/mg).

### 2.3. Peptide synthesis

Peptides were manufactured using 9-fluoronyl-methoxycarbonyl (Fmoc) chemistry by SynPep (Dublin, CA) and by New England Peptide (Gardner, MA). Master batch records indicate that peptides were purified to >80% as ascertained by analytical reversed phase HPLC and peptide mass was confirmed by MALDI-TOF mass spectrometry.

### 2.4. Peptide vaccine preparation

The constituent peptides of the DNA vaccine were formulated in liposomes with immunostimulatory CpG oligodeoxynucleotide (ODN) 1826 (5′-TCCATGACGTT CCTGACGTT-3′; InvivoGen, San Diego, CA) [16]. Sterically stable cationic liposomes were prepared from three lipid components: dioleylphosphatidylethanolamine, dimethylaminoethanecarbamol-cholesterol, and polyethylene glycol 2000-phosphatidyl-ethanolamine (Avanti Polar Lipids, Alabaster, AL). The lipids were mixed, dried in a rotary evaporator and re-suspended in PBS to make empty multi-lamellar vesicles. These vesicles were then sonicated five times for 30 s each at 4 °C to convert them into unilamellar liposomes. Unilamellar liposomes (10 nmol) were mixed with 1 mg/ml CpG ODN and peptides, flash frozen and freeze-dried overnight. To encapsulate CpG ODN and peptides in liposomes, the resulting powder was re-suspended with sterile distilled water and vortexed for 15 s every 5 min for 30 min at room temperature. PBS was added to yield a final liposome concentration of 10 mM lipid/mg ODN and peptides. Vesicles < 150 nm in diameter were produced by 20–30 cycles of extrusion through polycarbonate filters using a Liposofast extruder (Avestin). Liposome formulations were prepared fresh for each study, one day before the first peptide immunization and stored at 4 °C until a second peptide immunization two weeks later.

### 2.5. Mice

HLA DR3 transgenic mice were obtained from Dr. Chella David (Mayo Medical School) under commercial license. The mice express the HLA DR3α and β genes on a B.10-Ab$^0$ mouse Class II-negative background [17]. Experiments were conducted with mice 6–10 weeks old at the point of initiation. All studies were performed in full compliance with the standards of the University of Rhode Island and Saint Louis University Institutional Animal Care and Use Committees and in accordance with NIH publications entitled "Principles for Use of Animals" and "Guide for the Care and Use of Laboratory Animals."

### 2.6. Vaccinations

DNA-prime vaccine was administered to mice intramuscularly by needle stick injection with 50 μl of 50 μg naked DNA in sterile PBS injected into the quadriceps muscle of each leg. For peptide-boost immunizations, each mouse was anesthetized with ketamine/xylazine and administered a 30 μl liposome preparation aliquot (50 μg peptide) at 15 μl per nare, via micropipette.

### 2.7. ELISpot assay

The frequency of epitope-specific splenocytes was determined by IFN-gamma ELISpot assay using the Mabtech IFN-gamma ELISpot Kit according to the manufacturer's protocol (Mariemont, OH). Briefly, splenocytes were harvested from control and vaccinated mice. Pharmlyse (1X, BD Biosciences) was used to lyse red blood cells. The remaining lymphocytes were re-suspended in RPMI–10% fetal bovine serum–1% penicillin/streptomycin–1% L-glutamine–0.1% BME to a concentration of $4 \times 10^5$ cells/ml. Single cell splenocyte suspensions were transferred at $2.5 \times 10^5$/well to ELISpot plates pre-coated with anti-murine IFN-gamma by the manufacturer. Individual and pooled peptides were evaluated at 10 μg/ml in triplicate wells. Cells in RPMI 1640 media containing 10% FBS were also plated with a positive control Con A (2 μg/ml) or with no peptide as a negative control. ELISpot plates were incubated at 37 °C, 5% $CO_2$ for 2 days, incubated with a secondary HRP labeled anti-IFN-gamma antibody and developed by addition of TMB substrate. Spot counts were determined by Zellnet, Inc. using a Zeiss

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