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Clearance of depot vaccine SPIO-labeled antigen and substrate visualized using MRI

Kimberly D. Brewer[a,b], Kerry Lake[c], Nicole Pelot[c], Marianne M. Stanford[a,d], Drew R. DeBay[c], Andrea Penwell[a], Genevieve M. Weir[a,d], Mohan Karkada[a,d], Marc Mansour[a], Chris V. Bowen[b,c,e,*]

[a] Immunovaccine Inc., Halifax, NS, Canada
[b] School of Biomedical Engineering, Dalhousie University, Halifax, NS, Canada
[c] Biomedical Translational Imaging Centre (BIOTIC), Halifax, NS, Canada
[d] Department of Microbiology & Immunology, Dalhousie University, Halifax, NS, Canada
[e] Departments of Radiology and Physics, Dalhousie University, Halifax, NS, Canada

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ABSTRACT

Immunotherapies, including peptide-based vaccines, are a growing area of cancer research, and understanding their mechanism of action is crucial for their continued development and clinical application. Exploring the biodistribution of vaccine components may be key to understanding this action. This work used magnetic resonance imaging (MRI) to characterize the *in vivo* biodistribution of the antigen and oil substrate of the vaccine delivery system known as DepoVax™. DepoVax uses a novel adjuvanted lipid-in-oil based formulation to solubilise antigens and promote a depot effect. In this study, antigen or oil were tagged with superparamagnetic iron oxide (SPIO), making them visible on MR images. This enables tracking of individual vaccine components to determine changes in biodistribution.

Mice were injected with SPIO-labeled antigen or SPIO-labeled oil, and imaged to examine clearance of labeled components from the vaccine site. The SPIO-antigen was steadily cleared, with nearly half cleared within two months post-vaccination. In contrast, the SPIO-oil remained relatively unchanged. The biodistribution of the SPIO-antigen component within the vaccine site was heterogeneous, indicating the presence of active clearance mechanisms, rather than passive diffusion or drainage. Mice injected with SPIO-antigen also showed MRI contrast for several weeks post-vaccination in the draining inguinal lymph node. These results indicate that MRI can visualize the *in vivo* longitudinal biodistribution of vaccine components. The sustained clearance is consistent with antigen up-take and trafficking by immune cells, leading to accumulation in the draining lymph node, which corresponds to the sustained immune responses and reduced tumor burden observed in vaccinated mice.

1. Introduction

The study of immunotherapy in cancer has been invigorated by recent clinical successes [1]. This renewed enthusiasm has extended to peptide-based cancer vaccines, where novel delivery platforms, adjuvants and appropriate combination therapies are significant areas of development [2,3]. As preclinical research leads into clinical trials, exploring the potentially unique mechanism of action of these new immunotherapy candidates is critical to the interpretation of clinical responses and will aid in clinical trial design. While other studies have examined the immunological responses of both responder and regulatory T cells after immunization [4], exploring the biodistribution of the vaccine components itself may provide further understanding of the mechanisms leading to the overall response. Although some groups [5–8] have looked at the biodistribution of antigen and adjuvant components *ex vivo* using fluorometry, radiation and histological methods, a non-invasive *in vivo* method like magnetic resonance imaging (MRI) would allow for the acquisition of longitudinal information about the biodistribution and clearance of the vaccine components.

Abbreviations: SPIO, superparamagnetic iron oxide; ROI, region of Interest; SOI, site of injection; bSSFP, balanced steady state free precession; ANOVA, analysis of variance.

* Corresponding author at: Biomedical Translational Imaging Centre (BIOTIC), Suite 3900 - 1796 Summer Street, Halifax, NS, Canada B3H 3A7.
Tel.: +1 902 473 1858; fax: +1 902 470 6767..
E-mail address: Chris.Bowen@dal.ca (C.V. Bowen).

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Previously, MRI has been used for non-invasive *in vivo* longitudinal assessment of tumor growth. Immune changes were indirectly monitored *via* MRI by measuring volumetric changes in lymph nodes (LNs) adjacent or distant to the vaccination site [9]. In this study, we evaluated the clearance rate of components of DepoVax™, a lipid platform that does not require creation of an emulsion and was developed to enhance the potency of peptide-based vaccines. The lipid platform can be formulated with mixtures of peptide MHC class I and II antigen epitopes and an adjuvant of choice. The lipids carry incorporated antigens and adjuvant directly into an oil medium such as Montanide ISA51 VG. This entraps the vaccine ingredients in a form amenable to efficient uptake and processing/presentation by antigen presenting cells (APCs). Previous work has demonstrated that formulations containing model tumor antigens eradicate tumors in mouse HPV and melanoma models [10–13] and can stimulate increased effector T cell immune responses without the concomitant increases in $FoxP3^+CD4^+$ and IL10-secreting regulatory T cells induced by other vaccines [4].

By attaching superparamagnetic iron oxide (SPIO) to the antigen and formulating it in DepoVax™, it is possible to visualize the biodistribution of the antigen at both the site of vaccination and nearby LNs over time, and to evaluate the clearance of the antigen from the depot site [11,13,14]. In this study, we were able to differentiate clearance of the antigen from clearance of the oil substrate by differential labeling of either constituent.

Antigen persistence can influence the magnitude and duration of immune responses. Aqueous vaccine formulations release antigens quickly in the form of a bolus over a few hours to a week [5,7]. These formulations are usually associated with weak and short lived immune responses. Generation of a depot *in vivo* by delivering antigens in water-in-oil emulsions is a common strategy to increase antigen persistence. However, this type of depot may attract and trap antigen-specific T cells, reducing systemic immunity [15]. The DepoVax formulation generates a depot without an emulsion and has been demonstrated to induce strong and sustained systemic immune responses in pre-clinical and clinical trials [4,14]. Therefore, in this study we sought to understand the distribution patterns and kinetics of antigen release from a DepoVax vaccine depot *in vivo*.

In this preclinical study, naïve mice or those bearing C3 (HPV16 tumor model) tumors were utilized to evaluate the longitudinal clearance of the DepoVax vaccine components using SPIO conjugated to the antigen or associated with the oil. The immunogenicity of the vaccine was tested, and MRI was used for longitudinal *in vivo* visualization of the migration and clearance of both the HPV16E7 antigen and the oil substrate. This allowed us to evaluate: (1) how quickly the antigen and oil substrate cleared, and (2) whether the biodistribution and migration indicate active *versus* passive clearance processes.

2. Materials and methods

2.1. Mice

Female C57BL/6 mice were obtained from Charles River Labs (Saint-Constant, QC, Canada). Mice were used at 4-6 weeks of age with average body weight of 20 g ± 2 g. Mice were maintained following Canadian Council on Animal Care guidelines and supplied with food and water ad libitum. All procedures were approved by the ethics committee at Dalhousie University (Halifax, NS, Canada).

2.2. Cell lines

The C3 tumor line was provided by Dr. Martin Kast, and is derived from the C57BL/6 mouse embryo cell line and transformed to express HPV16E7 [16]. Cells were maintained in Iscove Modified Dulbecco's Medium (Sigma, St. Louis, MO) supplemented with 10% heat-inactivated fetal calf serum (Sigma), 2 mM L-glutamine (Gibco, Burlington, ON), 50 mM 2-mercaptoethanol (Gibco), 100 U/mL penicillin and 100 μg/mL streptomycin (Gibco). Cells were incubated at 37 °C and 5% CO_2.

2.3. Vaccine formulation

All peptides were synthesized by NeoMPS (San Diego, CA, USA) at >90% purity. Vaccines containing either $HPV16E7_{49–57}$ (RAHNIVTF; R9F) or SPIO-R9F were formulated using the proprietary DepoVax™ (DPX) formulation [4]. Briefly, an appropriate volume of R9F (or SPIO-R9F), F21E, and polynucleotide adjuvant were added to a mixture of 30% *t*-butanol in sterile water (v/v). DOPC/Cholesterol (10:1 w/w) (Lipoid GmBH, Germany) was added and the mixture was shaken for 30 min at 200 rpm. The solution was filled into vials (1.60 mL fill volume), lyophilized and resuspended in Montanide ISA51 VG (0.52 mL) (SEPPIC, France), a biodegradable mineral oil commonly used in vaccines, just prior to vaccination. The final composition in a 50 μL dose was DOPC/Cholesterol (6.6 μg/dose), R9F (5 μg/dose), F21E (5 μg/dose) and polynucucleotide adjuvant (20 μg/dose).

To label the vaccine components, a 20 nm superparamagnetic iron oxide (SPIO) particle with amphiphilic polymer coating and functional surface group of carboxylic acid (Ocean NanoTech, Arkansas, USA) was conjugated to the R9F peptide [13,16] using the procedure recommended by Ocean NanoTech [17]. Each vaccine dose contained 5 μg of R9F, and vaccines containing the conjugated SPIO-R9F (hereafter referred to as SPIO-antigen) had ~11 μg of iron per dose. For labelling the oil component of the vaccine, 20 nm hydrophobic SPIO particles coated in oleic acid (Ocean NanoTech) were resuspended in Montanide after chloroform evaporation at ~5 μg iron/dose (hereafter referred to as SPIO-oil). Oleic acid was chosen as the ligand for SPIO modification since the oil carrier contains DOPC, a phospholipid composed of oleic acid.

2.4. Tumor experiments

Twenty C57BL/6 mice were implanted with 5×10^5 C3 tumor cells subcutaneously (s.c.) into the left flank. Five days post-implantation (Study Day 0) mice were vaccinated (five per group) with; (i) DepoVax with R9F (unlabeled vaccine), (ii) DepoVax with SPIO-R9F (SPIO-antigen), (iii) DepoVax containing SPIO-oil with R9F (SPIO-oil) or (iv) PBS. Vaccine formulations were delivered *via* a single 50 μL s.c. contralateral immunization (right flank). Tumor sizes were measured every 4–7 days with calipers and volume calculated using the formula: longest measurement × (shortest measurement)^2 divided by 2. In an additional experiment, eight non-tumor bearing mice also received SPIO-antigen or SPIO-oil formulated vaccinations, and were imaged alongside the tumor bearing mice as controls for general immunogenic effects.

2.5. IFN-γ ELISPOT

An IFN-γ ELISPOT assay (BD Bioscience, San Diego, CA) was used to detect antigen-specific cytotoxic T-cells from splenocytes harvested from immunized C57BL/6 mice as described in [13]. A brief description can also be found in Supplemental Materials.

2.6. Data acquisition and MR imaging

All data were acquired on a 3T magnet equipped with 21 cm ID gradient coil (Magnex Scientific, Oxford, UK) interfaced with an Agilent DD Console (Agilent Inc., Santa Clara, CA). A 25 mm ID quadrature transmit/receive RF coil (Doty Scientific, Col., SC), was