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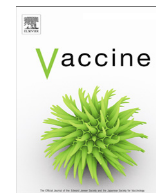

Retinaldehyde dehydrogenase 2 as a molecular adjuvant for enhancement of mucosal immunity during DNA vaccination

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ABSTRACT

In order for vaccines to induce efficacious immune responses against mucosally transmitted pathogens, such as HIV-1, activated lymphocytes must efficiently migrate to and enter targeted mucosal sites. We have previously shown that all-trans retinoic acid (ATRA) can be used as a vaccine adjuvant to enhance mucosal $CD8^+$ T cell responses during vaccination and improve protection against mucosal viral challenge. However, the ATRA formulation is incompatible with most recombinant vaccines, and the teratogenic potential of ATRA at high doses limits its usage in many clinical settings. We hypothesized that increasing *in vivo* production of retinoic acid (RA) during vaccination with a DNA vector expressing retinaldehyde dehydrogenase 2 (RALDH2), the rate-limiting enzyme in RA biosynthesis, could similarly provide enhanced programming of mucosal homing to T cell responses while avoiding teratogenic effects. Administration of a RALDH2- expressing plasmid during immunization with a HIVgag DNA vaccine resulted in increased systemic and mucosal $CD8^+$ T cell numbers with an increase in both effector and central memory T cells. Moreover, mice that received RALDH2 plasmid during DNA vaccination were more resistant to intravaginal challenge with a recombinant vaccinia virus expressing the same HIVgag antigen (VACVgag). Thus, RALDH2 can be used as an alternative adjuvant to ATRA during DNA vaccination leading to an increase in both systemic and mucosal T cell immunity and better protection from viral infection at mucosal sites.

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1. Introduction

The majority of pathogenic infections are transmitted via mucosal surfaces. Vaccines designed to prevent viral transmission via mucosal sites would optimally induce both systemic and mucosal T cell immunity thus reducing or eliminating viruses that evade mucosal control [1,2]. While an oral or rectal route of vaccination may be the optimal route to provide immunity at the gut-associated mucosal tissue, these vaccines face the same host defenses as do microbial pathogens: they are diluted in mucosal secretions, captured in mucus, attacked by proteolytic enzymes and nucleases, and excluded by epithelial barriers. Thus, relatively large doses of the vaccine are required to induce an immune response and these antigens generally induce immune tolerance [2,3]. The route and type of vaccination also plays an important role in mucosal protection. Some studies have shown that intramuscular immunizations with live replication attenuated or defective recombinant vaccine vectors such as modified vaccinia Ankara or adenovirus type 5 are able to elicit mucosal responses in mice [4,5], and macaques [6] but immunization safety is an issue [7]. On the other hand, the enhanced safety, stability, and accelerated product development offered by DNA vaccination make it an appealing option for developing prophylactic and therapeutic vaccines against mucosal pathogens [8]. DNA vaccination via the muscle or skin is currently being used in clinical trials to protect against HIV infection, as it is a simple and effective approach for the stable delivery of pathogen proteins for recognition by the immune system [9,10]. While this method generally fails to provide adequate mucosal protection, co-expression of adjuvants during DNA vaccination can possibly induce such immunity with systemic inoculation.

The vitamin A metabolite all-trans retinoic acid (ATRA) has been shown to function as an effective vaccine adjuvant in enhancing mucosal protection from viral challenge [11–13]. *In vivo*,

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retinoic acid (RA) upregulates the expression of the gut-homing receptors α4β7-integrin and the chemokine receptor CCR9 by $CD4^+$ and $CD8^+$ T cells. CCR9 binds to CCL25 produced by epithelial cells of the small intestine and α4β7-integrin binds to mucosal vascular addressin cell-adhesion molecule 1 (MAdCAM-1) which is expressed by the vascular endothelium of the gastrointestinal tract [14–19]. Retinoic acid also regulates T cell function, including apoptosis and differentiation between Th1 and Th2 responses [14,20]. Synthesis of retinoic acid from retinol occurs in two steps. In the first reaction, alcohol dehydrogenases are needed to convert retinol, the storage form of vitamin A, to retinal. In the second reaction retinaldehyde dehydrogenases (RALDH) further catalyze the conversion of retinal to RA with RALDH2 being the rate-limiting enzyme in RA biosynthesis. RA enters the nucleus and binds to RA receptors (RARs) and retinoid X receptors (RXRs), which form a heterodimer and bind to a sequence of DNA known as the RARE (RA-response element). This binding activates transcription of the target gene with up-regulation of mucosal homing molecules and production of RA [14,15]. Dendritic cells (DCs) from the gut-associated lymphoid organs produce RA and imprint T cells with gut-homing potential [14,21]. The administration of ATRA, the major metabolic derivative of vitamin A, has been shown to act physiologically to induce expression of gut-homing receptors on lymphocytes [11]. Unfortunately, ATRA is practically insoluble in water, which precludes its inclusion in many vaccines [22].

In this study we are transfecting epithelial cells with a RALDH2 DNA construct using an intramuscular-electroporation regime with the final objective of increasing mucosal-homing T cells and protection against viral challenge. We hypothesized that RA produced by transfected muscle epithelial cells could imprint DCs that traffic to the site of immunization with a mucosal phenotype (i.e. the ability to produce RA themselves). Alternatively, transfected DCs can be conditioned to produce RA and present the vaccine antigen in draining lymph nodes, the RA they produce should then instruct the responding lymphocytes to migrate to mucosal tissues.

Our results demonstrate that RALDH2 immunization increases systemic responses and protects from future mucosal viral challenge. A similar effect has been previously observed when ATRA is used as an adjuvant [11].

2. Materials and methods

2.1. Recombinant plasmids

2.1.1. WLV151M

WLV151M (pHIVgag) is a DNA expression vector encoding a fusion of the HIV-1 HXB2 gag-pol gene under control of the human cytomegalovirus immediate early promoter and bovine growth hormone polyadenylation signal [23]. The parental WLV151 has a Wyeth vaccine backbone vector and has been previously shown to provide a robust immune response to multiple HIV-1 derived antigens in rhesus macaques [8].

2.1.2. pMAX-PRO-RALDH2

The DNA sequence of human RALDH2 (GenBank Nucleotide Accession number AB015226) was codon-optimized for expression in human cells by GeneArt. The optimized sequence was subcloned into pMAX-PRO using *Kpn*-I and *Xho*-I.

2.2. RARE-luc reporter assay

Fifty thousand Human muscle RD cells or human embryonic epithelial (HEK-293) cells/well were plated in 96-well opaque wall plates (Nunc, Rochester, NY). The next day, individual wells of cells were transfected using 270 nl/well of Fugene 6 transfection reagent (Roche, Basil Switzerland) mixed with 8 ng of the pRARE-Luc plasmid (Panomics) and the indicated amount of the empty plasmid or the RALDH2 expressing plasmid. Twenty-four hours later, all cells were lysed and assessed for luciferase production by light emission using a Bright-Glo Luciferase kit (Promega, Madison, WI). Light emission was measured using a DTX 880 Multimode Detector (Beckman Coulter, Brea, CA). RA was used as a positive control. For this, control cells transfected with the pRARE-Luc plasmid received 10 μM RA (Sigma-Aldrich) six hours after transfection.

2.3. Animals and immunization

Six-week old female C57BL/6 mice were purchased from Jackson Laboratories and maintained at Arizona State University following a protocol approved by the Institutional Animal Care and Use Committee. Mice were immunized by intramuscular injection into both quadriceps muscles (50 μl/leg) with PBS containing 10 μg of the pHIVgag vaccine plasmid and either with or without 10 μg of the mucosal homing plasmid pMAX-PRO-RALDH2. Immediately after DNA delivery, muscle electroporation was used to increase the immunogenicity as previously described [24]. Quadriceps were subjected to six pulses of 100 V lasting 50 ms each, with 200 ms intervals between pulses using a BTX model 830 electroporation generator with a two needle probe. Control mice received one dose of ATRA at 300 μg in 100 μl delivered via intraperitoneal (i.p.) injection on day −1 and day +1 after pHIVgag vaccination. Mice were immunized three times on days 0, 14 and 28.

2.4. Challenge experiments

Recombinant vaccinia virus HIVgag (VACVgag) Western Reserve (WR) strain was used for challenge experiments. Viral challenge was performed via intravaginal infection with VACVgag (3×10^7 PFU) on anesthetized mice 6 weeks following the last immunization. Five days prior, mice received a 3 mg dose of medroxyprogesterone (Sigma-Aldrich) subcutaneously for menstrual synchronization.

2.5. Reagents

ATRA (Sigma-Aldrich) was dissolved in dimethyl sulfoxide (DMSO) at 40 mg/ml and stored as aliquots in the dark at −80 °C. A working solution of ATRA (3 mg/ml) was prepared by dilution in soybean oil (Sigma-Aldrich) and administered through i.p. injection as previously mentioned. Fluorochrome-conjugated monoclonal antibodies CD4 (GK 1.5), CD8α (53-6.7), CD44 (IM 7), CD62L (MEL-14), interferon gamma (IFN-γ) (XMG 1.2), tumor necrosis factor alpha (TNF-α) (MP6-XT22), interleukin-2 (IL-2) (JES6-SH4), CCR9 (1.2), α4β7 (DATK-32) and Foxp3 (FJK-16S) were all purchased from eBioscience, Inc. San Diego, CA. Foxp3 staining buffer kit (eBioscience, Inc.) was used for Foxp3 staining following manufacturer's instructions. The HIV_{SQV} tetramer (SQVTNSATI) was purchased from the Fred Hutchinson Cancer Research Center.

2.6. Lymphocyte isolation

2.6.1. Blood

A total of 100 μl of blood was collected from the submandibular vein two weeks following the last immunization. Blood was collected into 4% citrate buffer and RPMI complete media (10% FBS) was added to each sample. Mixture was purified using Histopaque 1077 (Sigma-Aldrich) and lymphocytes were resuspended in RPMI complete media until plating.

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