Published in final edited form as:

Vaccine. 2016 November 4; 34(46): 5629–5635. doi:10.1016/j.vaccine.2016.09.013.

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

Susan A. Holec hek^{a,b,c}, Megan S. McAfee^{a,b}, Lizbeth M. Nieves^{a,b}, Vanessa P. Guzman^{a,b}, Kavita Manhas^{a,b}, Timothy Fouts^d, Kenneth Bagley^d, and Joseph N. Blattman^{a,b,*}

^aBiodesign Center for Infectious diseases and Vaccinology, Biodesign Institute, Arizona State University, Tempe, AZ 85287-5401, United States

^bSchool of Life Sciences, Arizona State University, Tempe, AZ 85287-4501, United States

^cSimon A. Levin Mathematical, Computational and Modeling Sciences Center, Arizona State University, Tempe, AZ 85287-3901, United States

^dProfectus BioSciences, Inc., Baltimore, MD 21224, United States

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.

*Corresponding author: Joseph N. Blattman, Joseph.Blattman@asu.edu, School of Life Sciences, CIDV/Biodesign Institute, Arizona State University, PO Box 875401, Tempe, AZ 85287-5401, Phone +1 480 965-2909.

Conflict of interest statement: Conflict of interest: none

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

Keywords

RALDH2 adjuvant; HIV DNA vaccine; retinoic acid; mucosal protection

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*, retinoic acid (RA) upregulates the expression of the gut-homing receptors $\alpha 4\beta 7$ -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 $\alpha 4\beta 7$ -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

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].

pMAX-PRO-RALDH2: The DNA sequence of human RALDH2 (NCBI Nucleotide accession 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 Eugene 6 transfection reagent (Roche, Basel 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 μ M 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 μ l/leg) with PBS containing 10 μ g of the pHIVgag vaccine plasmid and either with or without 10 μ g 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 μ g in 100 μ l 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. Mice were treated 5 weeks and a half later with progesterone to synchronize mucosal surfaces for 5 days, followed by intravaginal infection with VACVgag at 3×10^7 PFU.

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), CD8 α (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), $\alpha 4\beta 7$ (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

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

Peyer's patches—The small intestine was collected, stored in HBSS/HGPG buffer (HBSS, HEPES, Pen/Strep/L-Gln) and kept on ice. Peyer's Patches were isolated and lymphocytes were obtained by mechanically disrupting the tissues passing it through a 70-

µm cell strainer (BD Biosciences). Samples were maintained in RPMI complete media (10% FBS) and kept on ice until further analysis.

Lamina propria—Following the removal of Peyer's Patches and fatty tissue, the small intestines were cut into small pieces and incubated in EDTA buffer (1.3 mM EDTA, HBSS, HEPES) for 1 hour at 37°C. Samples were then digested with 100 IU/ml collagenase IV (Gibco) for 1 hour at 37°C and passed through a 70-µm cell strainer (BD Biosciences). Crude preparations were purified through 40% to 100% Percoll (GE Healthcare).

Spleen and inguinal lymph nodes—Samples were collected in cold HBSS media, mechanically disrupted, passed through a 70-µm cell strainer (BD Biosciences) and maintained in RPMI complete media as previously described. All lymphocytes were analyzed on a LSR Fortessa Instrument (BD Biosciences). Flow data were analyzed with FlowJo8.87 (Tree Star Inc.).

2.7. In vitro stimulation and Intracellular staining

Lymphocytes (1×10^6) were cultured with 1 µM HIV_{SQV} peptide in the presence of 1 µg/ml GolgiPlug (BD Pharmingen) at 37°C for 8h, washed, and stained for surface markers before fixation. Permeabilization of the cell membranes was done using Cytofix/Cytoperm at 4°C. Cells were subsequently washed with 1× Cytoperm/Cytowash buffer (BD Pharmingen) and stained with fluorochrome-conjugated antibodies for fluorescence-activated cell sorting (FACS) using an LSR Fortessa.

2.8. Virus plaque assay

Tissues were harvested from euthanized mice and homogenized as previously described [25]. All homogenates were subjected to three rounds of freezing (−80°C), slow thawing for 30 min on ice, and then quick thawing (37°C). Samples were then subjected to a 7-minute spin using a tabletop centrifuge at $700 \times g$ at 4°C and supernatants were retained for plaque assays on Rabbit kidney (RK) 13 cells. Plates were stained with crystal violet to determine the viral load within the tissue (PFU per organ).

2.9. Statistical analysis

Graphs were made with Prism5 software (GraphPad Software) with unpaired Student's *t* test for statistical analysis. Data represent means ± standard errors of the means (SEM) of data from two to five repeated experiments with 4 to 5 mice per group in each experiment. Statistical significant *p* values are indicated in each figure.

3. Results

3.1. Amount of effector CD8⁺ T cells recruited following immunization increases with the number of immunizations

The administration of ATRA during priming immunization has been shown to enhance CD8⁺ T cell responses in mucosal sites [11]. Verification of pRALDH2 activity was previously done by transfection into the human muscle cell line RD using a luciferase assay with the reporter plasmid pRARE-luc (Panomics) which has RARE sites upstream of a

luciferase gene (Fig. S1). In order to investigate if RALDH2 can enhance the *in vivo* production of RA and enhance the recruitment of effector CD8⁺ T cells, we evaluated the number of CD8⁺ T cells in mice following one or three immunizations using ATRA as a control. We observed that following a triple immunization the numbers of CD8⁺CD44⁺HIVgag⁺ cells increased across the examined tissues (blood, spleen, inguinal lymph nodes, Peyer's patches and lamina propria) (Figs. 1, 3 and S2).

3.2. Enhanced systemic gag-specific CD8⁺ T-cell responses upon intramuscular immunization with RALDH2 as an adjuvant

It has previously been shown that ATRA provision during priming immunization had no apparent effect on the function of CD8⁺ T cells isolated from the spleen and draining lymph nodes [11]. We evaluated the frequency of effector CD8⁺ T cells in these sites in the presence or absence of RALDH2 or ATRA. In mice that were primed either on day -1 or +1, ATRA provision did not alter the magnitude of vaccine-induced CD8⁺ T cells compared to mice immunized with gag only. However, immunization with HIVgag and RALDH2 did result in an altered tissue distribution of effector CD8⁺ T cells, with higher numbers in the spleen, compared to mice immunized with HIVgag alone ($p=0.0427$) or HIVgag and ATRA ($p=0.0350$) (Fig. 1A and 1B). Moreover, enhanced CD8⁺ T cell function was observed in the splenocytes following incubation with HIV_{5QV} peptide as determined by the higher frequency of LCMV-specific CD8⁺ T cells producing IFN- γ ($p=0.0394$) (Fig 1C).

3.3. RALDH2 immunization increases the frequency of central and effector memory CD8⁺ T cells in the spleen

We next examined the central (CD44^{hi}CD62L^{hi}) and effector memory (CD44^{hi}CD62L^{lo}) antigen-specific CD8⁺ T cells compartments in splenocytes. In contrast to what was observed in the groups of mice immunized with HIVgag alone or HIVgag with ATRA, mice co-immunized with HIVgag and RALDH2 displayed antigen-specific CD8⁺ T cells with central and effector memory phenotypes developing concomitantly following immunization. The central memory population in the group that was co-immunized with HIVgag and RALDH2 was statistically significantly higher than in the group immunized with HIVgag alone ($p=0.0018$) and the group immunized with HIVgag and ATRA ($p=0.0119$) (Fig. 2A). Similarly, the effector memory population was statistically significantly higher in the group immunized with HIVgag and RALDH2, compared to the group immunized with HIVgag alone ($p=0.0079$) and the group immunized with HIVgag and ATRA ($p=0.00196$) (Fig. 2B).

3.4. RALDH2 and ATRA provide similar immune responses in mucosal sites

Expression of RALDH2 is essential for the RA-producing capacity of dendritic cells in gut-associated tissues [16]. To explore if RALDH2 administration has a similar effect as ATRA, we analyzed the effect of this enzyme on CD8⁺ T cell responses against HIV-1 gag, the lamina propria and the Peyer's patches from mice immunized with HIVgag and/or RALDH2 were collected and analyzed in comparison to mice that received ATRA at day -1 and +1 post-immunization. ATRA and RALDH2 administration alone during priming immunization led to a similar percentage of effector CD8⁺ T cells in the Peyer's patches. No statistically significant difference was observed between the groups immunized with HIVgag and ATRA or HIVgag and RALDH2 in the Peyer's patches (Fig. 3A). No difference in the

percentage of effector CD8⁺ T cells was observed among the groups immunized with HIVgag alone, HIVgag and ATRA or HIVgag and RALDH2 in lamina propria (Fig. 3B).

3.5 ATRA or RALDH2 provision during immunization leads to enhanced protection from vaginal viral challenge

Following a triple immunization with either HIVgag and ATRA or HIVgag and RALDH2, we determined if mice would be protected against future viral challenges. Naïve and immunized mice were challenged intravaginally with a replication-competent VACVgag (3×10^7 PFU) and examined on day 6 post-challenge for viral loads in the ovaries and the uterus. This dose resulted in detectable virus in both sites, with a lower viral load being found in all immunized mice that received either ATRA or RALDH2 during vaccination. These experimental groups had significantly reduced viral titers in the ovaries ($p=0.0476$ for HIVgag and ATRA and $p=0.0476$ for HIVgag and RALDH2) (Fig. 4). Similar results were observed in the uterus ($p=0.0238$, for HIVgag and ATRA and $p=0.0079$ for HIVgag and RALDH2), compared to control mice immunized with only HIVgag (Fig. 4), suggesting that mucosal immune responses in these mice could contribute to reducing viral spread.

3.6 RALDH2 administration during systemic immunization leads to an increased number of regulatory T cells

We next determined if protection against challenge was due to T cell migration to mucosal sites. Due to the spleen being the site where most effector responses were observed, we evaluated the expression of CCR9 and $\alpha 4\beta 7$, lymphocyte surface receptors involved in their homing to mucosal sites [26-29]. The expression of CCR9 and $\alpha 4\beta 7$ on responding CD8⁺ T cells in the spleen was not significantly different between mice immunized with HIVgag only and mice immunized with HIVgag and either RALDH2, or RALDH2 and ATRA. Expression for $\alpha 4\beta 7$ was higher in the HIVgag and ATRA group (Fig. S3). We next analyzed if regulatory T cells (Tregs) CD4⁺CD25⁺Foxp3⁺ were recruited following immunization. Several studies have shown Tregs possessing different roles during infection [30-32]; they have been shown to induce early protective responses to local viral mucosal infections by coordinating the timely entry of immune cells into infected tissue [33]. The number of Tregs in the experimental group immunized with HIVgag and RALDH2 was statistically significantly higher ($p=0.0031$) compared to the group immunized with HIVgag alone. Additionally, the groups vaccinated with HIVgag and either ATRA or a combination of RALDH2 and ATRA showed an increased number of Tregs (Fig. S3).

4. Discussion

One of the main goals of designing protective vaccines against mucosal pathogens is the induction of memory T cells in the mucosa that are able to provide rapid and robust responses early during infection [2, 34]. Previous studies have shown that the provision of exogenous ATRA during systemic vaccination increases the number of effector and memory CD8⁺ T cells in mucosa-associated tissue and provides protection against mucosal viral challenge [11-13]. In [11], while the administration of ATRA to mice during in vivo priming with an adenovirus vector expressing the lymphocytic choriomeningitis virus glycoprotein (LCMVgp) (Ad5gp) enhanced the trafficking of CD8⁺ lymphocytes into mucosal tissues,

this technique of systemically administering ATRA is not clinically viable, due to potential teratogenicity as well as developmental abnormalities in fetuses from pregnant mice that received ATRA [22, 35]. The instability of ATRA as an adjuvant also makes it impractical for DNA vaccination [22]. In this study we evaluated the use of DNA vaccination as a safer and more easily delivered alternative to ATRA as a means of effectively increasing mucosal immunity. We used RALDH2 as a mucosal homing adjuvant for an HIV DNA vaccine with the idea that intramuscular administration of plasmids expressing the antigen and RALDH2 will trigger mucosal immune responses. We observed that (1) co-administration of plasmids expressing HIV antigens and RALDH2 induced mucosal antigen-specific immune responses similarly to those induced by ATRA in mice; (2) RALDH2 induces more robust systemic responses than ATRA and (3) using RALDH2 as an adjuvant for an HIV plasmid DNA vaccine improves protection against a mucosal challenge in the Vaccinia-HIV virus mouse model.

Most HIV-1 infections caused by a single strain are transmitted via a mucosal route, with 70 to 80% of infections [36]. Improving protection of the genital mucosa by enhancing immunological barriers via vaccination could potentially prevent the spread of the virus to other sites. Implementing vaccine-induced CD8⁺ T cells at the port of entry, the genital tract, would permit clearance of infected cells before viral spread to lymph nodes and other parts of the body [37]. Several HIV vaccine efforts have been done including the use of adenoviral vectors expressing Gag of HIV-1 and heterologous prime-boost regimens [37]. While originally implicated in gut homing, ATRA has shown to also increase the accumulation of vaccine-induced T cells at other mucosal sites such as the vagina by potentially the modulation of the same molecules [11].

Differential effects of ATRA (systemic) vs RALDH2 (local) may result in differential recruitment to the mucosal sites such as the Peyer's patches and lamina propria. While we anticipated an increase of CD8⁺Tet⁺CD44⁺ T cells with RALDH2 and ATRA in all the mucosal tissues, our results did not show a significant effect in the percentage of these cells in the lamina propria in contrast with the results observed in the Peyer's patches. This could be due to (a) a lower overall effect, (b) timing of vaccination and (c) preferential migration to Peyer's patches based on extra signaling for gut homing. Indeed, Peyer's patches but not the lamina propria express MAdCAM-1 (Mucosal Vascular Addressin Cell Adhesion Molecule 1) decorated with PNAd, a carbohydrate structure that mediates lymphocyte rolling via L-selectin, in their high endothelial venules. In this setting, L-selectin and $\alpha 4\beta 7$ could act synergistically in mediating homing to the Peyer's patches and this could explain the differential recruitment between both sites.

Our data did also demonstrate that provision of exogenous RALDH2 instead of ATRA during systemic vaccination leads to increased numbers of effector and memory CD8⁺ T cells in systemic sites and similar percentages as ATRA in mucosal sites with enhanced protection against mucosal viral challenge. Details of the effects of RALDH2 provision during priming on memory T cell differentiation remain to be fully resolved but the use of RALDH2 as an adjuvant during systemic vaccination induced both central memory and effector memory cells in the spleen. This should benefit the vaccinated host because the central memory cells with high proliferative potential will serve to replenish the memory

pool following infection, while effector memory cells would provide immediate protection. It has also been shown that while both central and effector memory cells contribute to recall responses, their relative contribution changes over time with effector memory cells being more prominent at early time post-infection and central memory cells having a more specific role at late times [38]. The increase in total number of both subsets of splenic memory T cells by RALDH2 treatment during vaccination suggest that RALDH2 is promoting a general improvement in vaccine efficacy and it could be a better adjuvant candidate than ATRA which has shown to only increase the central memory population [11]. Moreover, another route of protection against the mucosal challenge may have been conferred by an early protective response mediated by Tregs to a local viral infection possibly by allowing a timely entry of immune cells into infected tissue. This role of Tregs has been observed before during mucosal herpes simplex virus infection in which ablation of Tregs increased viral titers and impaired immune cell trafficking to the infected tissue [33]. Additionally, ablation of Tregs in mice infected with lymphocytic choriomeningitis virus (LCMV) have showed an increase in viral titers in the liver suggesting that Tregs play a role in both mucosal and systemic infections [39].

The importance of a rapid and a strong CD8⁺ T cell response against viral infections at mucosal sites has previously been emphasized for HIV vaccines [1, 34, 40-42] and our data suggest that RALDH2 should be further investigated as a potential adjuvant candidate for T-cell based HIV vaccines as an alternative to ATRA.

5. Conclusion

Our results show that the co-administration of a plasmid encoding RALDH2 and its delivery by intramuscular administration followed by electroporation in our mouse model of vaccination provides immunogenicity by increasing memory T cell responses and enhancing protection from viral infection at mucosal sites.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This study was supported by the National Institutes of Health (SBIR grant RAI089290A), Arizona State University and the Western Alliance to Expand Student Opportunities (WAESO) award (V13UR008/V2013ur0017).

References

1. Belyakov IM, Berzofsky JA. Immunobiology of mucosal HIV infection and the basis for development of a new generation of mucosal AIDS vaccines. *Immunity*. 2004; 20:247–53. [PubMed: 15030769]
2. Neutra MR, Kozlowski PA. Mucosal vaccines: the promise and the challenge. *Nature reviews Immunology*. 2006; 6:148–58.
3. Mayer L, Shao L. Therapeutic potential of oral tolerance. *Nature reviews Immunology*. 2004; 4:407–19.

4. Lin SW, Cun AS, Harris-McCoy K, Ertl HC. Intramuscular rather than oral administration of replication-defective adenoviral vaccine vector induces specific CD8+ T cell responses in the gut. *Vaccine*. 2007; 25:2187–93. [PubMed: 17229501]
5. Tatsis N, Lin SW, Harris-McCoy K, Garber DA, Feinberg MB, Ertl HC. Multiple immunizations with adenovirus and MVA vectors improve CD8+ T cell functionality and mucosal homing. *Virology*. 2007; 367:156–67. [PubMed: 17590405]
6. Kaufman DR, Liu J, Carville A, Mansfield KG, Havenga MJ, Goudsmit J, et al. Trafficking of antigen-specific CD8+ T lymphocytes to mucosal surfaces following intramuscular vaccination. *J Immunol*. 2008; 181:4188–98. [PubMed: 18768876]
7. Buchbinder SP, Mehrotra DV, Duerr A, Fitzgerald DW, Mogg R, Li D, et al. Efficacy assessment of a cell-mediated immunity HIV-1 vaccine (the Step Study): a double-blind, randomised, placebo-controlled, test-of-concept trial. *Lancet*. 2008; 372:1881–93. [PubMed: 19012954]
8. Luckay A, Sidhu MK, Kjekken R, Megati S, Chong SY, Roopchand V, et al. Effect of plasmid DNA vaccine design and in vivo electroporation on the resulting vaccine-specific immune responses in rhesus macaques. *Journal of virology*. 2007; 81:5257–69. [PubMed: 17329330]
9. Giri M, Ugen KE, Weiner DB. DNA vaccines against human immunodeficiency virus type 1 in the past decade. *Clin Microbiol Rev*. 2004; 17:370–89. [PubMed: 15084506]
10. Schertzer JD, Plant DR, Lynch GS. Optimizing plasmid-based gene transfer for investigating skeletal muscle structure and function. *Molecular therapy : the journal of the American Society of Gene Therapy*. 2006; 13:795–803. [PubMed: 16309967]
11. Tan X, Sande JL, Pufnock JS, Blattman JN, Greenberg PD. Retinoic acid as a vaccine adjuvant enhances CD8+ T cell response and mucosal protection from viral challenge. *Journal of virology*. 2011; 85:8316–27. [PubMed: 21653670]
12. Tuyishime S, Haut LH, Zhu C, Ertl HC. Enhancement of recombinant adenovirus vaccine-induced primary but not secondary systemic and mucosal immune responses by all-trans retinoic acid. *Vaccine*. 2014; 32:3386–92. [PubMed: 24780251]
13. Lisulo MM, Kapulu MC, Banda R, Sinkala E, Kayamba V, Sianongo S, et al. Adjuvant potential of low dose all-trans retinoic acid during oral typhoid vaccination in Zambian men. *The Journal of Translational Immunology*. 2013; 175:468–75.
14. Iwata M, Hirakiyama A, Eshima Y, Kagechika H, Kato C, Song SY. Retinoic acid imprints gut-homing specificity on T cells. *Immunity*. 2004; 21:527–38. [PubMed: 15485630]
15. Ganguly S, Manicassamy S, Blackwell J, Pulendran B, Amara RR. Adenovirus type 5 induces vitamin A-metabolizing enzymes in dendritic cells and enhances priming of gut-homing CD8 T cells. *Mucosal immunology*. 2011; 4:528–38. [PubMed: 21289616]
16. Iwata M. Retinoic acid production by intestinal dendritic cells and its role in T-cell trafficking. *Seminars in immunology*. 2009; 21:8–13. [PubMed: 18849172]
17. Hammerschmidt SI, Friedrichsen M, Boelter J, Lyszkiewicz M, Kremmer E, Pabst O, et al. Retinoic acid induces homing of protective T and B cells to the gut after subcutaneous immunization in mice. *The Journal of clinical investigation*. 2011; 121:3051–61. [PubMed: 21737878]
18. Kang SG, Lim HW, Andrisani OM, Broxmeyer HE, Kim CH. Vitamin A metabolites induce gut-homing FoxP3+ regulatory T cells. *J Immunol*. 2007; 179:3724–33. [PubMed: 17785809]
19. Mora JR, Iwata M, Eksteen B, Song SY, Junt T, Senman B, et al. Generation of gut-homing IgA-secreting B cells by intestinal dendritic cells. *Science*. 2006; 314:1157–60. [PubMed: 17110582]
20. Stephensen CB, Rasooly R, Jiang X, Ceddia MA, Weaver CT, Chandraratna RA, et al. Vitamin A enhances in vitro Th2 development via retinoid X receptor pathway. *J Immunol*. 2002; 168:4495–503. [PubMed: 11970994]
21. Guillems M, Crozat K, Henri S, Tamoutounour S, Grenot P, Devilard E, et al. Skin-draining lymph nodes contain dermis-derived CD103(-) dendritic cells that constitutively produce retinoic acid and induce Foxp3(+) regulatory T cells. *Blood*. 2010; 115:1958–68. [PubMed: 20068222]
22. Adams J. The neurobehavioral teratology of retinoids: a 50-year history. *Birth defects research Part A, Clinical and molecular teratology*. 2010; 88:895–905.

23. Egan MA, Megati S, Roopchand V, Garcia-Hand D, Luckay A, Chong SY, et al. Rational design of a plasmid DNA vaccine capable of eliciting cell-mediated immune responses to multiple HIV antigens in mice. *Vaccine*. 2006; 24:4510–23. [PubMed: 16140439]
24. Widera G, Austin M, Rabussay D, Goldbeck C, Barnett SW, Chen M, et al. Increased DNA vaccine delivery and immunogenicity by electroporation in vivo. *J Immunol*. 2000; 164:4635–40. [PubMed: 10779767]
25. Holechek SA, Denzler KL, Heck MC, Schriewer J, Buller RM, Legrand FA, et al. Use of a recombinant vaccinia virus expressing interferon gamma for post-exposure protection against vaccinia and ectromelia viruses. *PloS one*. 2013; 8:e77879. [PubMed: 24147092]
26. Hamann A, Andrew DP, Jablonski-Westrich D, Holzmann B, Butcher EC. Role of alpha 4-integrins in lymphocyte homing to mucosal tissues in vivo. *J Immunol*. 1994; 152:3282–93. [PubMed: 7511642]
27. Evans TI, Reeves RK. All-trans-retinoic acid imprints expression of the gut-homing marker alpha4beta7 while suppressing lymph node homing of dendritic cells. *Clinical and vaccine immunology : CVI*. 2013; 20:1642–6. [PubMed: 23966557]
28. Mora JR, Bono MR, Manjunath N, Weninger W, Cavanagh LL, Roseblatt M, et al. Selective imprinting of gut-homing T cells by Peyer's patch dendritic cells. *Nature*. 2003; 424:88–93. [PubMed: 12840763]
29. Zeng R, Oderup C, Yuan R, Lee M, Habtezion A, Hadeiba H, et al. Retinoic acid regulates the development of a gut-homing precursor for intestinal dendritic cells. *Mucosal immunology*. 2013; 6:847–56. [PubMed: 23235743]
30. Suvas S, Kumaraguru U, Pack CD, Lee S, Rouse BT. CD4+CD25+ T cells regulate virus-specific primary and memory CD8+ T cell responses. *The Journal of experimental medicine*. 2003; 198:889–901. [PubMed: 12975455]
31. Duhon T, Duhon R, Lanzavecchia A, Sallusto F, Campbell DJ. Functionally distinct subsets of human FOXP3+ Treg cells that phenotypically mirror effector Th cells. *Blood*. 2012; 119:4430–40. [PubMed: 22438251]
32. Burocchi A, Colombo MP, Piconese S. Convergences and divergences of thymus-and peripherally derived regulatory T cells in cancer. *Frontiers in immunology*. 2013; 4:247. [PubMed: 23986759]
33. Lund JM, Hsing L, Pham TT, Rudensky AY. Coordination of early protective immunity to viral infection by regulatory T cells. *Science*. 2008; 320:1220–4. [PubMed: 18436744]
34. Shattock RJ, Haynes BF, Pulendran B, Flores J, Esparza J. Improving defences at the portal of HIV entry: mucosal and innate immunity. *PLoS medicine*. 2008; 5:e81. [PubMed: 18384232]
35. Yasuda Y, Okamoto M, Konishi H, Matsuo T, Kihara T, Tanimura T. Developmental anomalies induced by all-trans retinoic acid in fetal mice. I. Macroscopic findings. *Clinical and Molecular Teratology*. 1986; 34:37–49.
36. Peters BS, Conway K. Therapy for HIV: past, present, and future. *Advances in dental research*. 2011; 23:23–7. [PubMed: 21441476]
37. Haut LH, Lin SW, Tatsis N, DiMenna LJ, Giles-Davis W, Pinto AR, et al. Robust genital gag-specific CD8+ T-cell responses in mice upon intramuscular immunization with simian adenoviral vectors expressing HIV-1-gag. *European journal of immunology*. 2010; 40:3426–38. [PubMed: 21108465]
38. Roberts AD, Ely KH, Woodland DL. Differential contributions of central and effector memory T cells to recall responses. *The Journal of experimental medicine*. 2005; 202:123–33. [PubMed: 15983064]
39. Wherry EJ, Blattman JN, Murali-Krishna K, van der Most R, Ahmed R. Viral persistence alters CD8 T-cell immunodominance and tissue distribution and results in distinct stages of functional impairment. *Journal of virology*. 2003; 77:4911–27. [PubMed: 12663797]
40. Suvas PK, Dech HM, Sambira F, Zeng J, Onami TM. Systemic and mucosal infection program protective memory CD8 T cells in the vaginal mucosa. *J Immunol*. 2007; 179:8122–7. [PubMed: 18056354]
41. Kalams SA, Parker SD, Elizaga M, Metch B, Edupuganti S, Hural J, et al. Safety and comparative immunogenicity of an HIV-1 DNA vaccine in combination with plasmid interleukin 12 and impact

- of intramuscular electroporation for delivery. The Journal of infectious diseases. 2013; 208:818–29. [PubMed: 23840043]
42. Mpendo J, Mutua G, Nyombayire J, Ingabire R, Nanvubya A, Anzala O, et al. A Phase I Double Blind, Placebo-Controlled, Randomized Study of the Safety and Immunogenicity of Electroporated HIV DNA with or without Interleukin 12 in Prime-Boost Combinations with an Ad35 HIV Vaccine in Healthy HIV-Seronegative African Adults. PloS one. 2015; 10:e0134287. [PubMed: 26252526]

Highlights

RALDH2 can be used as a mucosal homing DNA adjuvant and as an alternative to ATRA.

Enhanced HIVgag-specific CD8⁺ T-cell responses following immunization with RALDH2.

RALDH2 administration enhances both effector and central memory T cells.

DNA vaccination with RALDH2 elicits mucosal protection against viral challenge.

Fig. 1. HIVgag immunization together with RALDH2 increases the frequency of effector CD8⁺ T cells in the spleen and IFN- γ production following *in vitro* restimulation with HIVgag SQV tetramer

Naïve C57BL/6 mice were immunized three times with RALDH2, HIVgag or HIVgag + RALDH2. Two other groups were also immunized with either HIVgag or HIVgag + RALDH2 and received ATRA on day -1 and day +1. Two weeks following the last immunization, spleens were collected and responses were assessed by gating on CD8⁺ T cells for staining of the SQV tetramer versus CD44 expression. (A) Representative flow plot. (B) Cell numbers for effector CD8⁺ T cells were analyzed for the spleen and shown as representative bar graphs. (C) Number of LCMV-specific CD8⁺ T cells in the spleen producing IFN- γ following three immunizations. Data represent means $\pm$ standard errors of the means (SEM) of data from five repeated experiments with 4 to 5 mice/group/experiment for a total of 24 mice per group. Unpaired Student's t test was performed on the data.

Fig. 2. Enhanced central and effector memory CD8⁺ T cells in mice immunized with HIVgag and RALDH2

(A) Central memory (CD44^{hi}CD62L^{hi}) and (B) effector memory (CD44^{hi}CD62L^{lo}) CD8⁺ T cell compartments in spleens of mice immunized three times with HIVgag, HIVgag + RALDH2 or HIVgag with ATRA on day -1 and +1. Samples were collected two weeks following the last immunization and screened for CD62L and CD44 on gated SQV tetramer-positive CD44⁺CD8⁺ T cells from the spleen. Data represent results from a representative experiment (from 2 replicates) with a total of 5 mice per group. Unpaired Student's t test was performed on the data.

Fig. 3. RALDH2 and ATRA provide similar immune responses in mucosal sites

Naïve C57BL/6 mice were immunized three times with RALDH2, HIVgag or HIVgag + RALDH2. Two other groups were also immunized with either HIVgag or HIVgag + RALDH2 and received ATRA on day -1 and day +1. Two weeks following the last immunization, the Peyer's patches or the lamina propria were collected and responses were assessed by gating on CD8⁺ T cells for staining of the SQV tetramer versus CD44 expression. (A) Representative flow plot. (B) Percentages of effector CD8⁺ T cells in the Peyer's patches were analyzed and shown as representative bar graphs. Similarly, percentages of effector CD8⁺ T cells in the lamina propria (C) were analyzed and shown as a representative bar graph. Data represent means $\pm$ standard errors of the means (SEM) of data from five repeated experiments with 4 to 5 mice/group/experiment for a total of 24 mice per group. Unpaired Student's t test was performed on the data.

Fig. 4. RALDH2 or ATRA provision during vaccination leads to protection from vaginal viral challenge

Naïve C57BL/6 mice were immunized three times with HIV_{gag} in the presence of ATRA or RALDH2. Mice were treated 5 weeks and a half later with progesterone to synchronize mucosal surfaces for 5 days, followed by intravaginal infection with VACVgag at 3×10^7 PFU. Tissue viral loads were assessed on ovaries and uteri on day 6 post-infection and are shown as means $\pm$ SEM. Data represent results from a representative experiment (from 2 replicates) with a total of 5 mice per group ($n=4$ in the control group). Unpaired Student's *t* test was performed on the data.