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A novel retinoic acid, catechin hydrate and mustard oil-based emulsion for enhanced cytokine and antibody responses against multiple strains of HIV-1 following mucosal and systemic vaccinations

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Abstract

Non-replicating protein- or DNA-based antigens generally require immune-enhancing adjuvants and delivery systems. It has been particularly difficult to raise antibodies against gp120 of HIV-1, which constitutes an important approach in HIV vaccine design. While almost all effort in adjuvant research has focused on mimicking the pathogens and the danger signals they engender in the host, relatively little effort has been spent on nutritive approaches. In this study, a new nutritive immune-enhancing delivery system (NIDS) composed of vitamin A, a polyphenol-flavonoid catechin hydrate, and mustard oil was tested for its adjuvant effect in immune responses against the gp120 protein of HIV-1_{CN54}. Following a combination of two mucosal and two systemic vaccinations of mice, we found significant enhancement of both local and systemic antibodies as well as cytokine responses. These data have important implications for vaccine and adjuvant design against HIV-1 and other pathogens.

Keywords

Vaccine; HIV gp120; Immune-enhancing; Adjuvant; nutritive

1. Introduction

Transmission of HIV-1 via the heterosexual route is one of the most common routes of infection. Therefore, induction of local mucosal immunity at the female genitourinary tract as well as systemically against HIV infection and transmission is important in HIV vaccine development [1]. Accumulating evidence indicates that regardless of the route of inoculation, i.e. intra-venous, rectal or vaginal, mucosal CD4+ T cells in vaginal, intestinal or respiratory mucosa are targeted and depleted before peripheral CD4+ T cells [2,3,4] and that the intestine remains a reservoir of CD4+ HIV-infected cells late in SIV infection [5]. Induction of protective immune responses in the female genital tract (FGM) through vaccination has proven difficult, and strong adjuvants and delivery systems are required [6].

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Intra-nasal (IN) immunization can induce immune responses in the upper and lower respiratory as well as the female genitourinary tracts. Of the several mucosal routes available for mucosal immunization, i.e. oral, nasal, vaginal and rectal, the nasal (IN) is the most desired for human vaccinations because the nostrils are readily exposed and IN immunizations generally require lower doses of antigen, with important implications for many costly recombinant antigen-based vaccines. Licensed oral or IN vaccines thus far are based on live attenuated organisms, none of which have lead to late stage clinical trials for HIV vaccines. Consequently, potent adjuvants and optimal delivery systems will be required to enable the development of effective mucosal non-replicating vaccines against HIV-1. Although the IN route of vaccination has clear and well-established advantages over systemic injections, a major perceived risk has been the development of Bell's palsy (partial facial paralysis), particularly following use of toxin-based vaccines [7]. Recent results also support the use of the sublingual (SL) route of inoculation which can elicit responses at multiple mucosal surfaces, including the female genital tract [8]. Sublingual immunization with non-replicating antigens induced antibody-forming cells and cytotoxic T cells in the female genital tract mucosa and protected against genital papillomavirus infection [9]. As opposed to the oral route, vaccines delivered through the sublingual route, do not traverse the harshly acidic stomach or the small intestine with highly degradative enzymes. Therefore, similar to the IN route, lower concentrations of antigen and/or adjuvant can be used for the SL route. An important strategy for induction of enhanced mucosal and systemic responses is vaccinations through both mucosal and systemic routes. It has been shown that mucosal (IN) followed by systemic (IM) immunizations induced antibody responses in serum and the FGT of rhesus macaques [10] and mice [11].

Several mucosal adjuvants and delivery systems for protein-, DNA- and RNA-based vaccines have been devised and used in animal and early clinical studies [12]. It has become increasingly clear that a combination of a delivery system with an immune-enhancing adjuvant will have a synergistic effect. In general, particulate delivery systems present multiple copies of antigens to the immune system and promote trapping and retention of antigens in local lymph nodes. An enhancement of protective efficacy of a meningococcal vaccine was observed following formulation of LTK63 with nanoparticles [13], or with chitosan bioadhesive [14]. Furthermore, using the small molecule immune potentiator, CpG adsorbed to PLG microparticles immunogenicity and protective efficacy of an anthrax vaccine was improved [15]. Conventional and marginal zone B cells express various toll like receptors (TLR) and can be activated through engagement of such receptors with various ligands [16,17,18]. The response to TLR ligands, costimulatory molecules (CD40) and B cell receptor cross-linking are heavily dependent on signaling by TGF β -activated kinase 1 (TAK1) [19]. It has been shown that imiquimod and resiquimod, members of a family of small molecule immune potentiators, can directly act on resting and activated B cells to increase their proliferation and immunoglobulin (Ig) secretion via TLR7 [20]. Specifically, resiquimod was shown to increase IgG2a (an indication of TH1 response) and suppress IgE (an indication of TH2 response). These data support the combined use of immune potentiators and delivery systems for the synergistic enhancement of B and T cell responses at mucosal surfaces.

The main focus of research on vaccine adjuvants and delivery systems in the past several decades has been to mimic pathogens' danger signals. This approach, while successful in some cases, has proven unsafe in several other cases. Specifically, two recent failed clinical trials for HIV vaccines underscored the importance of novel and safe approaches in adjuvant and vaccine design for vaccines against HIV and other pathogens. In one trial, transient facial nerve paralysis (Bell's palsy) was found following intranasal delivery of a genetically detoxified mutant of *Escherichia coli* heat labile toxin (21). The other failed trial was the Merck vaccine study based on adenovirus vectors [22]. Several lines of evidence suggest

that nutritive components such as select vitamins and polyphenols given as nutritional supplements before, during and after vaccinations possess immunomodulatory properties. For instance, the combination of the flavonoid epigallocatechin-3-gallate (EGCG), a polyphenol, and DNA vaccination led to enhanced tumor-specific CD8⁺ T-cell immune responses and enhanced antitumor effects [23,24]. Moreover, retinoic acid, a vitamin A derivative, given as a nutritional supplement, was shown to increase neonatal anti-tetanus toxoid antibody response, and selectively increased anti-TT IgG1 and IL-5, resulting in a skewed TH2 response [25].

This study pertains to the rational design of a novel combination of a rubefacient-based delivery system, i.e. mustard oil, for administration through epithelial cells of the mucosal membranes, as well as systemically formulated with immune-enhancing components, comprising a vitamins A derivative, retinoic acid, and a flavonoid, i.e. catechin hydrate, for enhanced induction of local and systemic antibody as well as TH1 and TH2 responses against the HIV-1 envelope glycoprotein, gp120. The use of the rubefacient is based on the novel idea that a rubefacient will enhance antigen-delivery and presentation through the epithelial cells overlying mucosal membranes as well as increase extravasation of antigen-presenting cells (APC) to the site of administration. Because vitamin A has also been shown to increase TH2 type responses, a flavonoid, i.e. catechin hydrate, was further added into the formulation for further enhancement of the immune responses in an additive or synergistic manner and for induction of balanced TH1 and TH2 type responses. Another important rationale behind selection of these components is their low cost and ease of commercial manufacturing. While we used gp120 from a subtype C HIV-1 strain (CN54) for vaccinations, we sought to determine whether immune responses against heterologous subtype B HIV-1 strains could also be generated. For mucosal vaccinations, we administered the vaccines simultaneously through both the IN and SL routes. We then boosted the mucosally primed mice through intra-muscular vaccinations. Importantly, we used the exact same NIDS formulation for both mucosal and systemic vaccinations.

2. Materials and Methods

2.1 Immune-enhancers, vaccine preparations and vaccinations

The HIV-1 gp120_{CN54} protein was obtained from the NIH AIDS Research and Reference Reagents Program. While CN54 is generally considered a B/C HIV-1 subtype, the purified gp120 CN54 obtained from the NIH AIDS Research and Reference Reagents Program is actually a C subtype. Vitamin A (Retinoic acid, Sigma-Aldrich; Cat# R2625) and Catechin Hydrate (Sigma-Aldrich, Cat# C1251) were dissolved in 200 proof absolute ethanol (Sigma-Aldrich, Cat# 459844). The stock concentration was 3 µg/µl for Vitamin A (VA), 50 µg/µl for Catechin Hydrate (Cat). Mustard seed oil (MO) was purchased from Botanic Oil Innovations inc. (Spooner, WI). Allyl isothiocyanate (AIT) was purchased from Sigma Aldrich (Cat# W203408-500G-K). The amount used for each dose of vaccination was 30 µg for VA, 120 µg for Cat, and 1 µl for AIT. Dulbecco's phosphate buffered saline (Cat# 21-030-CV) was used to adjust the final volume for each dose. All components used in the vaccine including vitamin A, Cat, Tween-20, MO, and PBS were tested for endotoxin with a Genscript kit (Piscataway, NJ08854; Cat# L00350) and the endotoxin content in each component was found to be less than 0.005 EU/ml. All vaccines were prepared in endotoxin free 2.0 ml tubes (Eppendorf biopur safe-lock microcentrifuge tubes). Tween®-80 (Sigma) was added to all groups at a final concentration of 0.1%. The emulsification of the oil in water vaccine formulation was performed manually by repeatedly drawing and purging the contents of the vaccine formulation in an eppendorf tube 15–17 times through 0.5 ml tuberculin syringes with 27 gauge needles (beckton-dickenson; Cat# 305620). The resulting emulsion was stable, with no oil and water phase separation, for 2-3 hours until vaccinations.

The estimated microparticle/micelle size of the prepared vaccines (50% v/v mustard oil, 0.1% Tween 80, 50% aqueous phase v/v) was as follows: largest: 5 μm ; smallest: 0.1 μm ; the majority (70–80%) were between 0.5–1 μm .

The dose volume per mouse for each combined IN/SL (sublingual) vaccination was 38.8 μl , i.e. 19.4 μl for IN and 19.4 μl for SL routes. For intramuscular vaccination, the volume per dose was 100 μl (50 μl on each thigh) using the same tuberculin syringes used for emulsification. Hence, for mucosal (IN and SL) NIDS formulations the total water/alcohol phase was 19.4 μl and the total oil phase was 19.4 μl , containing 30 μg VA (0.77 mg/ml), 120 μg for Cat (3.1 mg/ml), 1 μl v/v for AIT (0.025 ml/ml). The dose of the HIVgp120CN54 was a total of 5 g for the combined IN/SL vaccinations and the first IM vaccination, (0.12 mg/ml), diluted in PBS. For IM vaccinations the final volume per dose was 100 μl . Hence, each IM dose contained 30 μg VA (0.3 mg/ml), 120 μg for Cat (1.2 mg/ml), and no AIT. For IM vaccinations the gp120 dose was 5 μg for the first vaccination (0.12 mg/ml) and 2.5 g (0.064 mg/ml) for the second vaccination, each diluted only in PBS.

Female BALB/c mice at the age of 6–8 weeks at the onset of the studies were used. We performed two independent studies which yielded near identical results with 3 (study one) or 4 (study two) mice per each group at where the mice were vaccinated with HIV-1 gp120CN54 in the with or without the NIDS. The mice were maintained according to IACUC guidelines in the vivarium of Murigenics Inc. (Vallejo, CA 94592). Before the first vaccination, the mice were anesthetized with isoflurane, for simultaneous IN and SL vaccinations, whereas for the second vaccination the mice were not anesthetized for the IN administrations. Thus the following two groups of mice received the combined IN/SL vaccinations twice at 2 weeks intervals and then twice intra-muscularly also at 2 weeks intervals: Group 1: HIVenvgp120_{cn54}+NIDS (MO+VA+Cat+AIT); Group 2: HIVenvgp120_{cn54} in PBS only.

2.2 Test of serum antibodies by ELISA

Sera were collected at 2 weeks after the second and third vaccinations and at one week after the fourth vaccination, respectively. Sera were collected from individual mouse and were kept at -20°C until assayed. To measure anti-HIV gp120_{cn54}IgG1, IgG2a and IgA responses, nunc maxisorp 96-well microplates were coated with 2 $\mu\text{g}/\text{ml}$ PBS of the recombinant gp120 protein from the HIV-1_{cn54}, HIV-1_{BaL}, HIV-1_{CM}, HIV-1_{IIIB}, (all from the NIH AIDS Reagent program) and HIV-1_{SF162} (Immune Technology Corporation, NJ) in 100 μl per well at 4°C overnight. After washing 4 times with PBS containing 0.02% Tween 20 (PBS-T), the plates were blocked with 200 μl blocking solution (1% goat serum, 0.02% Tween-20 in PBS) for 1 hour at room temperature. The serum samples were diluted 1/600 in the blocking solution initially and 3-fold serial dilutions were made in each column of 96-well microplates for each sample. Column 1 was used as blank. A positive reference serum and a negative reference serum were diluted in the same way in column 2 and 3, respectively. After addition and dilution of the serum samples, the plates were incubated for 2 hours at room temperature. The plates were then washed 5 times with PBS-T followed by addition of 100 μl goat anti-mouse IgG1-HRP conjugate (Southern Biotech, Cat# 1070-05) diluted 1/8000 in blocking solution. The plates were incubated for 1 hour at room temperature. After washing 5 times with PBS-T, 100 μl TMB substrate was added for color development. The color development was stopped in about 15–30 minutes by addition of 100 μl of 1N sulfuric acid. The plates were read at 450nm using a microplate reader (Molecular Devices). The cutoff value was determined as two and half times of the background value (average value of blank wells). The titer of each serum sample was calculated automatically using the software based on log-logit curve. For IgG2a titration, all the procedures and reagents were the same as for IgG1 ELISA except for the secondary antibody conjugate. A goat anti-mouse IgG2a-HRP conjugate (Southern Biotech, Cat#

1080-05) diluted 1/8000 was used. For IgA titration, the procedures and reagents used were the same as above except that the serum samples were diluted initially at 1/20 followed by 3-fold serial dilutions in each column and a goat anti-mouse IgA-HRP (Southern Biotech, Cat# 1040-05) diluted 1/8000 was used.

2.3 Test of IgG1 and IgA in vaginal washes by ELISA

Vaginal washes (VWs) about 100 μ l were collected from individual anesthetized mice at two weeks post the second IN vaccination and at one week post the last IM vaccination using a micropipettor by aspirating about 100 μ l PBS inside the cervicovaginal vault several times. The VWs were kept on dry ice for later use. VWs were thawed and vortexed followed by centrifugation at 3000 $\times$ g for 5 minutes. The VWs were tested for IgG1 and IgA by ELISA. The ELISA tests were performed in a similar way to that described for serum IgG1 and IgA above except that the initial dilution for both IgG1 and IgA was 1/3 followed by 3-fold serial dilutions. The tests also employed the PerkinElmer ELAST[®] ELISA Amplification System to increase sensitivity as per manufacturer's protocols.

2.4. Virus neutralization assay

Sera collected at one week after two mucosal followed by two IM immunizations were pooled from each group and used for measurement of neutralization activity against several HIV-1 subtypes. Standard neutralization assays were performed in TZM-bl cells using 293T-grown Env-pseudoviruses. The values are the sample dilution at which relative luminescence units (RLUs) were reduced 50% compared to virus control wells (no test sample).

2.5. Cell cultures and tests of cytokines and antibodies in supernatants by ELISA

At one week after the fourth and final vaccination the mice were sacrificed. Spleens, cervical lymph nodes (CLN) and iliac lymph nodes (ILN) were removed aseptically from individual mouse and were immediately placed into 4.5 ml cold RPMI 1640 basic medium (UCSF Cell culture facility). Individual spleens, CLN, or ILN were meshed through Falcon 70 μ m nylon meshes (Cat#353910) followed by rinsing of the mesh with about 4.5 ml complete RPMI media with 5% fetal bovine serum (FBS). The cells were collected into a 50 ml centrifuge tube and were transferred into a 5 ml Nalgene tube (Cat# 5000-0050). After spinning for 5 minutes at 4°C at 600 $\times$ g in a Eppendorf Benchtop centrifuge (5702R), the cells were gently resuspended in 4.5ml complete RPMI1640 with 5% FBS. The cells were pelleted and resuspended one more time. For cell counting, 20 μ l cell suspensions were mixed with 180 μ l PBS to make a 1/10 dilution. 10 μ l of the diluted cell suspension was mixed with 10 μ l trypan blue. 10 μ l of the mixture was used for hematology. After counting, the cell concentration in each spleen sample was diluted to a final concentration of 2 $\times$ 10⁶/ml.

Cells were cultured in corning 24-well cell culture plates (Cat#3526). 0.5 ml of the cells was added to each well. Two wells were used for each sample. One well will be added with 0.5 ml complete RPMI 1640 and the other well will be added with 0.5 ml complete RPMI 1640 with 2 μ g/ml HIV gp120_{cn54} protein. After incubation at 37 °C in a CO₂ incubator for 24 hours, cells and supernatants were harvested and were centrifuged for 5 minutes at 600 $\times$ g. The supernatants were collected in 2 ml microcentrifuge tubes and stored at -20°C.

IFN γ , IL-5, IL-10 cytokines in the supernatants were measured using the ELISA Max Deluxe kits from Biolegend (Cat#430806, Cat#431206, and Cat#431406, respectively) following the manufacturer's instructions. To enhance the sensitivity of the tests, the ELASTR ELISA Amplification System by PerkinElmer was used according to the manufacturer's protocols. IgG1 anti-gp120CN54 ELISA titers were measured in the

supernatants by standard ELISA coupled with the Perkin Elmer ELAST® ELISA Amplification System as described above under 2.3.

2.7. Statistical Analysis

The data from all the tests were analyzed to show significant differences between vaccination groups, i.e. gp120_{cn54} in NIDS vs. gp120_{cn54} alone, with Students *t* test, two-tailed unless indicated, using Microsoft Excel's statistical tools. The *p* values are indicated in the text and in the Figures.

3. Results

3.1 Serum antibody responses following mucosal vaccinations

At two weeks following two combined intra-nasal/sublingual vaccinations, serum anti-gp120_{cn54} IgG1 responses were enhanced an average of 198 fold in mice vaccinated with gp120_{cn54} in NIDS compared to the mice vaccinated with gp120_{cn54} alone (*p*<0.002) (Figure 1). Moreover, while the average of serum IgG2a anti-HIVgp120_{cn54} responses was also enhanced, it did not reach statistical significance (Figure 1A). These data show that two mucosal vaccinations with gp120_{cn54} in NIDS result in relatively high levels of serum IgG1 and IgG2a antibody responses.

3.2 Serum antibody responses following mucosal priming and systemic boosting vaccinations

Based on previous published data [26,27], in order to further enhance mucosal and systemic antibody and cytokine responses, we boosted the mice with 2 IM booster vaccinations. Following the first IM boosting vaccination, the serum IgG1 antibody responses increased an average of 107 folds, reaching an average titer of over 80,000 (*P*<0.0003), when vaccinations with gp120_{cn54} in NIDS was compared to vaccinations with gp120_{cn54} alone (Figure 1B). Moreover, the serum IgG2a antibody responses increased an average of 169 folds, reaching an average titer of over 29000 (*P*<0.004), when vaccinations with gp120_{cn54} in NIDS was compared to vaccinations with gp120_{cn54} alone (Figure 1B). Furthermore, the serum IgA antibody responses increased an average of 3.4 folds, reaching an average titer of over 333 (*P*<0.035; one-tailed), when vaccinations with gp120_{cn54} in NIDS was compared to vaccinations with gp120_{cn54} alone (Figure 1B). These data show that the NIDS significantly enhanced serum IgG1, IgG2a and IgA responses when administered as two mucosal priming and a single systemic boosting vaccinations.

Following an additional IM boosting vaccination with gp120_{cn54} in NIDS vs. gp120_{cn54} alone, the serum IgG1 antibody titers increased 542 folds, reaching an average titer of 120400 (*P*<0.019). Moreover, the serum IgG2a antibody titers increased 62 folds to an average titer of 5092 (*P*<0.005) and serum IgA responses increased 2.3 folds to an average titer of 340 (*P*<0.05, one tailed) (Figure 1C). These data demonstrate that two mucosal priming vaccinations followed by two systemic boosting vaccinations with gp120_{cn54} in NIDS significantly enhanced serum IgG1, IgG2a and IgA antibody responses compared to vaccinations with gp120_{cn54} alone.

3.3 Vaginal antibody responses following mucosal priming and systemic boosting vaccinations

Because the vaginal mucosa is a major portal of HIV-1 entry, we next determined the antibody titers in vaginal washes. There appeared to be an enhancement of both IgG1 and IgA titers in vaginal washes (VW) in mice vaccinated twice mucosally with gp120_{cn54} in NIDS compared to the mice vaccinated with gp120_{cn54} alone (Figure 2A). However, these responses did not reach statistical significance. We next measured the vaginal IgG1 and IgA

responses in vaginal washes collected after the final vaccination, i.e. following 2 mucosal and 2 systemic vaccinations. We found markedly and significantly enhanced vaginal IgG1 (increased 255 folds to an average titer of 1725; $P < 0.025$) and IgA (increased 8 folds to an average titer of 106; $P < 0.039$) titers in vaginal washes of the mice that were vaccinated with gp120_{cn54} in NIDS compared to gp120_{cn54} alone (Figure 2B). These data show that the NIDS not only enhanced the serum antibody responses, but also induced antibody responses measurable in the vaginal vault.

3.4 Augmentation of local and systemic antibody responses against gp120 by NIDS

Because the antibody responses measured in vaginal washes could be serum derived, we also measured antibody responses in overnight culture supernatants of single cell suspensions isolated from spleens, cervical LN and iliac LN activated with gp120_{cn54} or not activated. We found enhancements of anti-gp120_{cn54} IgG1 titers from the mice vaccinated with gp120_{cn54} in NIDS vs. gp120_{cn54} alone in all tissues examined (Figure 3). These data further support the enhancement of serum and vaginal antibody responses following vaccinations with gp120_{cn54} in NIDS and suggest at least a portion of the vaginal IgG1 responses is locally derived.

3.5 Markedly enhanced breadth of antibody responses

Induction of antibody responses that can bind gp120 from several similar strains of HIV-1 is highly desired in HIV vaccine design and development. Hence, we next determined whether vaccinations with gp120_{cn54} in NIDS induced heterologous antibody responses against strains of HIV-1 other than the vaccine strain, i.e. CN54, a B/C clade HIV-1 strain. To this end, we tested the sera collected after the final vaccination with gp120 derived from several heterologous subtype B HIV-1 strains, i.e. BaL, CM, SF162 and IIIB, as well as against the homologous CN54 subtype B/C. We found while vaccinations with gp120_{cn54} alone induced relatively low levels of only CN54 gp120-specific serum IgG1 antibody responses, vaccinations with gp120_{cn54} in NIDS induced relatively high levels of serum IgG1 responses against not only gp120_{cn54}, but also gp120 derived from BaL, CM, SF162 and IIIB (Figure 4). These data show the feasibility of the NIDS adjuvant to induce enhanced responses against homologous as well as against heterologous strains of subtype B HIV-1 strains.

3.6 Antibody Neutralization titers

We next measured serum neutralization titers against homologous and heterologous strains of subtype B HIV-1 strains. Interestingly, we found several folds enhancement of neutralization titers against the subtype C MW965.26 strain (Figure 5), as well as a modest increase against the subtype B SF162 strain. No other enhancements of the serum neutralization titers were evident against the other strains. These data suggest vaccinations with the gp120_{cn54} in the presence of NIDS may enhance serum neutralizations titers against some heterologous HIV-1 strains.

3.7 Cytokine Responses

To determine T cell responses against gp120_{cn54} following vaccinations in the presence or absence of NIDS, we next measured IFN γ (TH1), IL-5 (TH2) and IL-10 (Treg) responses in spleens (SP). We found low but detectable IFN γ , several folds higher IL-5 and similar IL-10 responses in SP of mice vaccinated with gp120_{cn54} in NIDS compared to vaccinations with gp120_{cn54} alone (Figure 6). While antigen-presenting cells and leukocytes other than CD4⁺ T cells can also make these cytokines, these data suggest that TH1 and TH2-type cytokine responses may be enhanced by mixing of gp120_{cn54} in NIDS.

4. Discussion

Recent HIV vaccine trials have clearly underscored the need to safe vaccines. These include the Novartis LTK63 [21] and the failed Merck clinical trial using a live adenovirus-based vector [22]. Considering the safety issues in vaccine development in general and in HIV vaccine development in particular, this study raised hopes that a clinically safe HIV vaccine could be designed and developed. This is the first study demonstrating the ability of a completely nutritive based immune-enhancing adjuvant/delivery system. All of the NIDS components are GRAS, i.e. generally regarded as safe, as defined by the USA Food and Drug Administration. The LD50 value for vitamin A is well established and is 1.1–26 g/kg for oral administration in mice and 96 mg/kg for intra-peritoneal and 53 mg/kg for subcutaneous administrations in rats. The LD50 for catechin hydrate is also high and is 3.8g/kg for oral and over 5 g/kg for subcutaneous administrations in rats. This is a very important finding in light of the fact that most adjuvants that are based on inducing danger signals, e.g. live attenuated and TLR-based, while successful in animal and preclinical studies may prove toxic in clinical or post marketing studies.

While in this study we did not perform any comparison of the NIDS and HIV gp120 with other adjuvants, we performed a separate study with NIDS and a novel polysaccharide antigen derived from *Neisseria meningitidis* (*Nm*). In comparing the *Nm* antigen alone, mixed with NIDS or adsorbed to alum, we found significantly higher serum IgG2a, responses following vaccinations with the *Nm* antigen in NIDS, but not when adsorbed to alum, suggesting that the NIDS formulation induced better TH1 type responses than alum (our unpublished data).

The delivery system used in this study for the emulsification of vitamin A, catechin hydrate and gp120 was mustard oil, which is a rubefacient. A rubefacient traditionally is a substance for external application that produces redness of the skin e.g. by causing dilation of the capillaries and an increase in blood circulation [28]. Mustard oil is an plant-based rubefacient and is extracted from seeds of *Brassica juncea*, *Brassica nigra*, and *Sinapis alba*. The basic composition of mustard oil is monounsaturated fatty acids (erucic acid (18–51%), oleic acid (7–22%)), polyunsaturated fatty acids (linolenic (9–15%) and linoleic (6–24%)), proteins (30%), isothiocyanates, phenolics, phytin and dithiol thiones, and 9–15% omega 3 fatty acids. Allyl isothiocyanate (AIT) is a colorless oil that is responsible for the pungent taste of mustard, horseradish, and wasabi. It is slightly soluble in water, but well soluble in most organic solvents [29]. AIT comes from the seeds of black mustard (*Brassica nigra*) or brown Indian mustard (*Brassica juncea*). When these mustard seeds are broken, the enzyme myrosinase is released and acts on a glucosinolate known as sinigrin to produce AIT. Known as synthetic mustard oil, AIT can be produced commercially by the reaction of allyl chloride and potassium thiocyanate: $\text{CH}_2=\text{CHCH}_2\text{Cl} + \text{KSCN} \rightarrow \text{CH}_2=\text{CHCH}_2\text{NCS} + \text{KCl}$ [29].

AIT has been used in studies of inflammation to mediate neurogenic vasodilatation and oedema via both neurogenic and non-neurogenic mechanisms to mediate inflammation in the mouse ear [30,31,32]. These studies demonstrate the ability of mustard oil to induce plasma extravasation and skin inflammation due to neutrophil, but not mast cell, accumulation at the site of topical application and possibly through the involvement of neurokinin receptors. These data also support the notion that mustard oil may act as a delivery system on mucosal membranes of the respiratory and other mucosal tracts which also contain neurokinin receptors. Mustard oil has been consumed in several countries for centuries and millions of infants receive daily mustard oil massages in Bangladesh and India [33], suggesting it is generally safe. While several plant-based oils have been used as vaccine adjuvants with varying success [34,35,36], the above data justify the use of mustard

oil containing AIT as a better alternative, particularly for epithelial vaccinations through mucosal membranes. Of note, the manual emulsification method used in these pioneering studies yielded micelles that were mostly 0.5–1 μm in diameter. For stable micro-emulsion preparation with micelles with sizes of 200–400 nm in diameter, a high shear homogenizer or a microfluidizer (e.g. LV1, Microfluidics, Newton, MA) can be used.

Flavonoids are a group of polyphenolic compounds found in the human diet, and they include, quercetin, anthocyanidins/anthocyanins, and catechins, each with various immunomodulatory activities. Anthocyanin-rich extracts induced TNF production in activated macrophages [37], and various flavonoids were shown to be inhibitors of IL-4 and IL-13 production by activated human basophils [38]. Also, catechins inhibited the growth of *Legionella pneumophila* in macrophages and selectively upregulated the production of IL-12, IFN γ and TNF α and down-regulated IL-10 production in a dose-dependent manner [39]. Furthermore, the combination of epigallocatechin-3-gallate (EGCG) dietary supplementation and DNA vaccination led to enhanced tumor-specific CD8+ T-cell immune responses and enhanced antitumor effects [24,40]. However, several other reports support an immunosuppressive role for EGCG [41,42,43]. Contrary to this notion, we detected enhanced splenic IFN γ and serum IgG2a responses following vaccinations with NIDS, which may be attributed to the presence of catechin hydrate. Thus, our data demonstrate that catechin hydrate acted as an immune-enhancer and likely acts through a different immunomodulatory mechanism compared to EGCG. Taken together, these data suggest that catechin hydrate, possesses immune-enhancing TH1-type vaccine adjuvant properties and that its inclusion in varying ratios in the NIDS formulation may further enhance TH1 type responses.

Vitamin A, used individually as nutritional supplement given before, during and after vaccinations, and also not in the proprietary combination in NIDS co-administered with the antigen used in this study, has been shown to possess immunomodulating activity and used as vaccine adjuvant previously. Vitamin A is a fat-soluble vitamin that is derived from two sources: preformed retinoids and provitamin carotenoids. Retinoic acid (RA) and its parent compound retinol (ROH, vitamin A) have been used as important immunopotentiating agents since the early 1900s. Several lines of evidence suggest that RA induced a selective TH2 type response. Recently, RA nutritional supplementation in the presence of synthetic double stranded RNA (poly IC) was shown to increase neonatal anti-tetanus toxoid antibody response, and selectively increased anti-TT IgG1 and IL-5, resulting in a skewed TH2 response [44]. Moreover, vitamin A deficiency diminished TH2-mediated Ab responses and providing vitamin A or its active metabolites reversed this defect. All-trans retinoic acid (ATRA), an acid derivation of Vitamin A, regulated the balance of immune response induced by TR421-hCG β DNA vaccine to a TH2 bias [45]. Lower vitamin A levels were associated with a relative TH1 dominance and proportionately more NK cells [46]. These data show that vitamin A induces a predominantly TH2 response. In this regard, it was interesting that the cytokine responses measured in spleens supported the induction of both enhanced IgG1 (TH2) and IgG2a (TH1) responses, in that both IFN γ and IL-5 responses were enhanced following vaccinations with NIDS compared to vaccinations without NIDS. While the TH2 type responses appeared higher than the TH1-type responses in this formulation, the data indicated that the inclusion of the flavonoid, catechin hydrate, enhanced the TH1 while possibly reducing TH2 responses induced by vitamin A, and that changing the ratios of vitamin A and catechin hydrate may change this balance further. It is important to note, that the cytokine responses we detected may not be exclusively derived from T cells, as the non-irradiated antigen presenting cells and other cell types in the splenic cell cultures may have secreted some of these cytokines as well.

Our data in mice demonstrated that the NIDS is effective in enhancing serum and vaginal antibody responses. It is generally accepted that vaginal antibody responses are required in order to limit or block vaginal transmission of HIV-1. It is well-established that the major antibody isotype in vaginal and cervical secretions is IgG, and not IgA [47,48]. We measured increased IgG responses against gp120 in mice vaccinated with gp120 in NIDS vs. gp120 alone. To address whether the increased vaginal IgG responses were locally or serum derived we also measured IgG antibody titers in gp120-activated overnight culture supernatants of SP, cervical LN and ILN. The detection of increased IgG1 antibody responses in all of these tissues, including in ILN which drains the vaginal mucosa, following vaccinations with gp120 in NIDS compared to gp120 alone, suggests that at least a portion of the increased vaginal IgG1 responses may be locally derived. The presence of locally derived IgG antibody responses is of particular importance in mounting robust and rapid responses against sexual transmission of HIV.

While there is no general consensus that neutralizing antibodies or cytotoxic T lymphocytes correlate with protection against HIV-1, there is general agreement that serum neutralizing antibodies against a broad range of primary CCR5-tropic HIV-1 isolates. In this regard, the results obtained on the serum antibody breadth as measured by ELISA (Figure 4) and particularly the serum neutralization activities against heterologous HIV-1 subtypes (Figure 5) are of particular importance. While several reports have demonstrated that sera from mice neutralize HIV-1 virions, other reports indicate that strong background in neutralization activities from unvaccinated mice, or overt lack of neutralization activity in vaccinated mice make interpretations of neutralization data from mice difficult [49]. Hence our future studies will address the neutralization activity of sera from other animals, e.g. rabbits or rhesus macaques vaccinated with gp120 in the presence or absence of NIDS.

The enhanced IgA responses in serum and vaginal lavages were of importance. IgA antibody responses clearly demonstrated that the NIDS induced heavy chain isotype switch to IgA. Induction of isotype switch to IgA usually requires potent and often toxic adjuvants, such as cholera toxin [50]. Protection from disease has been reported to correlate with local IgA in lungs [51] and the intestine [52]. However, the significance of serum IgA antibody in protection is less well-defined, unless it is correlated with mucosal IgA antibody responses. While the correlates of protection against HIV are also not well-defined, it is generally agreed that vaginal and serum neutralizing antibody responses are important for protection against heterosexual transmission [47]. In our study, we detected enhanced vaginal and serum IgA and IgG antibody responses, suggesting that the NIDS formulation provided isotype switch to both IgG and IgA, with important effector functions for virus neutralization and inhibition of virus attachment, respectively.

Vaccinations against HIV-1 must result in neutralization of multiple primary strains of HIV-1 and the breadth of the antibody responses against gp120 proteins derived from multiple HIV-1 strains of a geographically well defined subtype, e.g. subtype B, is of particular importance. Because we performed these studies in mice it was difficult to assess the breadth of the neutralizing antibodies against various strains of HIV-1. Nonetheless, the data on serum antibody responses measured by ELISA which measured neutralizing and non-neutralizing and functional as well as non-functional antibodies were intriguing [53]. This is in light of the fact that significantly enhanced antibody responses against multiple subtype B strains were detected following vaccinations with gp120 in NIDS compared to gp120 alone. This raises the possibility that future studies in rabbits and rhesus macaques may indeed result in neutralizing antibodies against multiple heterologous HIV-1 strains.

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Fig. 1. Serum anti-HIVgp120_{cn54} antibody responses in mice vaccinated with HIV gp120_{cn54} in NIDS or HIV gp120_{cn54} only. Two groups of 4 mice each received two mucosal and two IM vaccinations sequentially. Sera were collected at various time points and tested for IgG1, IgG2a, and IgA by standard ELISA. (A) Sera collected at 2 weeks post the second mucosal vaccination. (B) Sera collected at two weeks post the two mucosal and one IM vaccination. (C) Sera collected at one week after two mucosal followed by two IM vaccinations.

Fig. 2.

Vaginal IgG1 and IgA anti-HIV gp120_{cn54} responses in mice vaccinated with HIV gp120_{cn54} in NIDS or HIV gp120_{cn54} alone. Two groups of 4 mice each received two mucosal and two IM vaccinations sequentially. Vaginal washes were collected at two time points and were tested by ELISA coupled with the Perkin Elmer ELAST® ELISA Amplification System. (A) Vaginal washes collected at two weeks post the second mucosal vaccination. (B) Vaginal washes collected at one week after two mucosal followed by two IM vaccinations.

Fig. 3. Presence of local and systemic anti-HIV gp120_{cn54} IgG1 in cell culture supernatants. Single cell suspensions were prepared from 4 individual mice for spleen, and cervical LNs and were pooled from 4 mice for iliac LN. The cells were cultured in the presence or absence of gp120_{cn54} for 24 hours. Cell culture supernatants were collected and tested for anti-gp120_{cn54} IgG1 by ELISA, enhanced with the Perkin Elmer ELAST® ELISA Amplification System.

Fig. 4.

Enhanced breadth of serum anti-gp120 IgG1 responses against several heterologous subtype B HIV-1 strains (BaL, CM, SF162 and IIIB) after two mucosal followed by two IM vaccinations with gp120_{cn54} in NIDS compared to gp120_{cn54} alone. Sera were collected at one week post the last vaccination and tested with standard ELISA.

Fig. 5.

Enhanced serum neutralization titers against heterologous strain of HIV-1 strains. Sera were collected and pooled from mice in each group at one week after two mucosal followed by two IM vaccinations with gp120_{cn54} in NIDS compared to gp120_{cn54} alone and tested for neutralization activity against various HIV-1 strains. The values are the sample dilution at which relative luminescence units (RLUs) were reduced 50% compared to virus control wells (no test sample).

Fig. 6.

TH1, TH2 and Treg responses in spleens following vaccinations with gp120_{cn54} in NIDS compared to gp120_{cn54} alone. Single cell suspensions isolated from spleens of 4 individual mice and were cultured with or without gp120_{cn54} for 24 hours. Cell culture supernatants were collected and tested individually for IFN (TH1), IL-5 (TH2) and IL-10 (Treg) concentrations with ELISA.