Published in final edited form as:

J Med Primatol. 2018 October ; 47(5): 288–297. doi:10.1111/jmp.12372.

A simultaneous oral and intramuscular prime/sublingual boost with a DNA/Modified Vaccinia Ankara viral vector-based vaccine induces simian immunodeficiency virus-specific systemic and mucosal immune responses in juvenile rhesus macaques

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Abstract

Background: A pediatric vaccine to prevent breast milk transmission of human immunodeficiency virus (HIV) may generate greater immune responses at viral entry sites if given by an oral route.

Methods: We compared immune responses induced in juvenile macaques by prime/boosting with SIV-expressing DNA/Modified Vaccinia Ankara virus (MVA) by the intramuscular route (IM), the oral (O)/ tonsillar routes (T), the O/sublingual (SL) routes, and O+IM/SL routes.

Results: O/T or O/SL immunization generated SIV-specific T cells in mucosal tissues but failed to induce SIV-specific IgA in saliva or stool or IgG in plasma. IM/IM or O+IM/SL generated humoral and cellular responses to SIV. IM/IM generated greater frequencies of T_{FH} in spleen but O+IM/SL animals had higher avidity plasma IgG and more often demonstrated mucosal IgA responses.

Conclusion: These results suggest that co-delivery of HIV DNA/MVA vaccines by the oral and IM routes might be optimal for generating both systemic and mucosal antibodies.

Keywords

HIV; pediatric vaccine; oral route

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Author contributions: K.D.P., K.V.R., and P.A.K. conceptualized the study. A.D.C., K.J., and P.A.K. performed experiments and data analyses. R.A. provided the DNA vaccine constructs. A.D.C., P.A.K., and K.D.P. wrote the manuscript.

Conflict of Interest: none

INTRODUCTION

Breastfeeding is critical for providing nutrition and passive immunity to infants in resource-poor countries, but it poses a considerable risk for post-partum mother-to-child transmission (MTCT) of human immunodeficiency virus type 1 (HIV) ^{1, 2, 3}. While maternal access to antiretroviral therapy in resource-poor areas is increasing, only 60% of pregnant HIV-positive women receive it ^{4, 5}. Pediatric HIV infection remains an important issue in developing countries, with an estimated 150,000 new cases in 2015, approximately half from breastfeeding, in addition to 110,000 AIDS-related pediatric deaths ⁶. Thus, additional means to prevent MTCT of HIV, such as vaccination, are urgently needed.

We previously showed that intramuscular (IM) poxvirus-vectored simian immunodeficiency virus (SIV) vaccines provided infant macaques with partial protection against oral SIV challenge ⁷. To prevent MTCT of HIV, we hypothesized that including an oral delivery route would augment vaccine-induced SIV-specific T and B cell responses at the sites of viral entry in the oral cavity and intestine. The feasibility and efficacy of oral vaccination routes for preventing SIV infection by vaginal or rectal routes has been demonstrated in several studies with adult macaques. For example, topical application of SIV DNA, MVA and inactivated SIV particles in the small intestine or between the cheek and gum in the oral cavity significantly delayed acquisition of vaginal SIVmac251 infection or resulted in control of viremia ⁸. Topical administration of live attenuated SIV *nef* to the palatine and lingual tonsils (T) provided protection against tonsillar or rectal challenge with SIVmac251 ^{9, 10, 11}. There is more limited information regarding the sublingual (SL) vaccine delivery route in macaques. But in one study, SL immunization of juvenile macaques with live attenuated vaccinia viruses expressing HIV envelope (Env) protein was shown to elicit Env-specific systemic IgG responses, although no mucosal IgA responses were detected ¹².

DNA prime/poxviral vector boost vaccine strategies have been studied extensively in adult rhesus macaques using SIV infection models ¹³. Adult rhesus macaques vaccinated two times by the IM route with DNA encoding SIV virus-like particles and granulocyte-macrophage colony stimulating factor (GM-CSF) ¹⁴ or CD40 ligand (CD40L) ¹⁵ adjuvants and boosted with MVA encoding SIV *gag, pol, env* (SIV-MVA) have been protected against rectal challenge with heterologous SIV ^{14, 15}. Additionally, a recent HIV vaccine efficacy trial with human adults in Thailand utilized a canarypox virus prime, which elicited immune responses capable of reducing infection by 31% ¹⁶.

In this proof-of-concept study, we tested the immunogenicity of the oral, T and SL delivery routes using SIV expressing DNA and MVA in juvenile macaques. Juvenile macaques were chosen because their larger size enabled us to test the feasibility of applying small vaccine volumes to specific oral mucosal sites prior to testing this approach in infant macaques. The results suggest that delivery of vaccine using a combination of oral and IM routes may be superior to oral or IM vaccination routes alone for generating both mucosal and systemic immune responses.

MATERIALS AND METHODS

Humane Care Guidelines:

SIV and type D retrovirus-negative juvenile rhesus macaques (*Macaca mulatta*) were housed in pairs at the California National Primate Research Center (Davis, CA) in accordance with the “Guide for Care and Use of Laboratory Animals” outlined by the American Association for Assessment and Accreditation of Laboratory Animal Care. The UC Davis Institutional Animal Care and Use Committee approved all procedures. All procedures were performed under ketamine anesthesia.

Vaccines and study design:

The vaccination schedule is outlined in Table 1. DNA plasmids encoding SIV_{mac239} gag,pol,env alone (D) or with GM-CSF (D_G) or CD40L (D_{40L}) have been detailed elsewhere^{13, 14, 15, 17}. For oral (O) administration, DNA was formulated in cationic 1,2-dioleoyl-3-trimethylammonium (DOTAP) liposomes by Encapsula Nanosciences (Brentwood, TN) and delivered into the cheek pouches in 1.4mL containing 3mg D or 1.5mg D_G + 1.5mg D_{40L}. IM immunizations with DNA were done by injecting 3mg D or 1.5mg D_G + 1.5mg D_{40L} in 0.3mL into the left quadriceps. MVA expressing SIV_{mac239} gag,pol,env (vector DR2) was provided by Dr. Bernie Moss (National Institute of Allergy and Infectious Diseases) and expanded in chick embryo fibroblasts as described¹⁸. For administration of MVA-SIV by the T route, 10⁸ PFU SIV-MVA in 15μL was pipetted on top of each palatine tonsil. For SL immunizations, 2 × 10⁸ SIV-MVA in 30μL was placed under the tongue, respectively. All IM immunizations with SIV-MVA were done by injecting 0.5 × 10⁸ PFU in 0.25mL into both the left and right quadriceps (1 × 10⁸ PFU total). Animals were euthanized 3 weeks after the last immunization for tissue analysis.

Sample processing:

Plasma was harvested from EDTA anti-coagulated whole blood via centrifugation and stored at −80°C. Mononuclear cells were isolated as described^{19, 20}. Saliva and stool were collected, stored, and processed as described^{21, 22}. Lymph nodes (LNs; submandibular, retropharyngeal, submental, and mesenteric), spleen, tonsil, ileum, and colon were collected at euthanasia and processed as previously described^{3, 19}. Mononuclear cells (MNCs) were used fresh or stored in liquid nitrogen.

Antibody quantification in plasma, saliva, and stool:

SIV-specific and total antibodies in plasma, saliva and fecal extracts prepared from stool (reflective of intestinal antibodies) were assessed by ELISA as described²¹. Antibodies were measured to recombinant SIV_{mac239} gp140 (Immune Technology) or Gag+Pol proteins using TritonX-100-lysed aldrithiol-2-inactivated SIV particles (kindly provided by Dr. Jeff Lifson, AIDS and Cancer Virus Program, Frederick, MD) at a 1/400 coating dilution that lacked detectable Env protein. Concentrations of SIV Env or Gag,Pol-specific IgA in secretions were normalized to the total IgA concentration by calculating the specific activity (sp act; ng specific IgA per μg total IgA). Samples were considered antibody positive if the sp act was the mean sp act + 3 SD in naïve (pre-immunization) secretions.

ELISPOT for antibody-secreting cells:

Polyvinylidene fluoride membranes (Millipore) were coated with 1 μ g SIV_{mac239} gp140 (Immune Technology) per well and blocked for 2h with 2% milk²³. Frozen cell preparations were thawed, washed and stimulated (10⁶ cells/mL) with 1 μ g/mL R848 (InvivoGen) and 10ng/mL human IL-2 (Miltenyi Biotec) for 72h in complete medium (cRPMI): RPMI-1640 medium (Gibco) containing penicillin, streptomycin, L-glutamine (Sigma-Aldrich) and 10% heat inactivated FBS (Gibco)²⁴. Stimulated cells (8 $\times$ 10⁴/well) were incubated in triplicate wells of gp140-coated microtiter plates overnight at 37°C, washed with PBS+0.05% Tween-20, incubated 1h with 1 μ g/mL biotinylated affinity-purified goat anti-human IgG (SouthernBiotech) or anti-rhesus IgA monoclonal antibody (clone DF12; Nonhuman Primate Resource Program) at room temperature. Plates were washed with PBS+0.05% Tween-20, incubated with a 1/4000 dilution of avidin-peroxidase (SouthernBiotech) for 1h at room temperature, and developed using the BD AEC kit. Dried membranes were analyzed with an automated ELISpot Reader System (Autoimmun Diagnostika GmbH). Results are reported as the number of antibody-secreting cells (ASC) per 10⁶ MNCs.

T cell subset analysis:

Frozen cells were thawed, washed and cultured in cRPMI (10⁶/mL) with or without 0.5x cell stimulation cocktail (eBiosciences) and stained with viability dye (Invitrogen) and anti-CD3, -CD4, -CD8, -CD45RA, -CCR7, -CD95 (BD), -CXCR5 and -PD-1 (eBiosciences) antibodies. Intracellular staining was performed with anti-FoxP3, -IFN- γ (BD), -IL-21, -IL-4 (eBiosciences) antibodies after permeabilizing fixed cells with the Human FoxP3 Buffer Set (BD) per the manufacturer's recommendations. T follicular cells (T_{FH}) were defined as live CD3⁺CD4⁺CD95⁺CCR7^{lo}CXCR5⁺PD-1^{hi} cells; T follicular regulatory cells (T_{FR}) were FoxP3⁺ T_{FH}. T helper cells (T_H) were live CD3⁺CD4⁺CD95⁺CXCR5⁺PD-1^{lo}; T regulatory cells (T_{REG}) were FoxP3⁺ T_H^{25, 26}. A total of 300,000 events were collected using an LSRII or LSRFortessa using FACSDiva v8.0 (BD) and analyzed with FlowJo Software v10.2 (TreeStar) applying fluorescence-minus-one controls and Boolean gating.

SIV-specific T cell responses:

10⁶ splenic or LN cells were stimulated with a SIV_{mac239} p27 Gag peptide pool (AIDS Reagent Program, #6883), 0.5x Stimulation Cocktail (eBiosciences), or no stimulant in cRPMI at 37°C for 6h²¹. Brefeldin-A (eBiosciences) was added after 1h. Cells were stained with viability dye (Invitrogen) and anti-CD3, -CD4, -CD8, -CD45RA, and -CCR7 antibodies, treated with Cytotfix/Cytoperm, and stained with anti-TNF- α , -IFN- γ , -IL-2, and -IL-17 antibodies (all from BD). Data collection and analysis were performed as above. SIV Gag-specific cytokine responses were normalized to unstimulated controls.

Statistical analyses:

Due to small group sizes, comparisons were performed using the nonparametric two-tailed Mann-Whitney rank sum test and the GraphPad Prism v6.0 program. P values $\leq$ 0.05 were considered significant.

RESULTS

SIV-specific antibody responses

We quantified SIV gp140 Env-specific antibodies in plasma 3 weeks after the first and second vaccine boosts with SIV-MVA (weeks 6 and 9). No differences were observed in animals primed with adjuvanted vs. non-adjuvanted DNA, possibly because only a single priming immunization with these adjuvants was performed. The O/T and O/SL immunizations failed to induce Env-specific IgG or IgA in plasma (Figures 1A-D). The IM/IM and O+IM/SL vaccinees had Env-specific plasma IgG after the first boost, and the second boost increased concentrations to levels that were roughly 1000-fold above preimmune (Figure 1A & B). Env-specific plasma IgA was only increased roughly 10-fold in these groups (Figure 1D). Although the concentrations of Env-specific IgG did not differ in the IM/IM and O+IM/SL groups, the avidity of Env-specific IgG was significantly higher in the O+IM/SL animals (Figure 1C).

Because the goal of mucosal immunization is to generate immune responses within mucosal tissues, we tested saliva and stool for SIV-specific IgA antibodies after the second boost. There were no salivary IgA responses to Env and Gag,Pol in O/T and O/SL animals, and only 1/6 animals in the IM/IM or O+IM/SL groups had salivary IgA antibodies (Figure 1E & F). Intestinal IgA responses were more prevalent, especially in O+IM/SL animals (Figure 1G & H). Less than 50% of the O/T, O/SL, and IM/IM-immunized animals had Env-specific intestinal IgA on week 9, whereas 4/6 O+IM/SL vaccinees developed these antibodies (Figure 1G). Gag,Pol-specific intestinal IgA responses were also observed in 4/6 O+IM/SL animals, but in none of the O/SL and only 1 IM/IM animal (Figure 1H). These results suggest that simultaneous O+IM priming followed by SL boosting may generate IgA responses in the intestinal mucosa more consistently than prime-boosting by the O/SL or IM/IM routes.

Tissue SIV-specific B cells and Induction of follicular T helper (T_{FH}) cells

We used ELISPOT to measure Env-specific ASC in oral LNs (retropharyngeal, submental or submandibular) and spleen collected 3 weeks after the last immunization. Animals in the O/T and O/SL groups lacked SIV-specific IgG ASC in these tissues, whereas IM immunization alone or in combination with the O immunization elicited SIV-specific ASC in oral LNs (Figure 2A-B) and the spleen (Figure 2C-D). These data are consistent with the observation that IM vaccine delivery was required to generate anti-SIV IgG antibodies in plasma. No anti-Env IgA ASC were detected in oral LNs or spleens from animals in any other group (data not shown), commensurate with the weak salivary and plasma IgA responses (Figure 1). Despite modestly higher numbers of ASC in the spleen of IM/IM D-immunized animals compared to IM/IM D_G+D_{40} animals ($p=0.0192$; Figure 2C), the GM-CSF and CD40L adjuvants did not overall appear to influence systemic or mucosal antibody responses (Figure 1).

To further investigate the lack of antibody responses in the O/T and O/SL groups, we analyzed tissues for T_{FH} , required for maturation of antibody responses, and T_{FR} , which regulate T_{FH} responses^{25, 26, 27, 28} (Figure 3). Due to limited cell numbers, intestinal tissue

could not be evaluated. Frequencies of CD4⁺ T helper cells (T_H), regulatory T cells (T_{REG}), T_{FH}, and T_{FR} were similar in oral LNs from all vaccine groups (Figure 3A-D). Consistent with more SIV-specific IgG ASC in spleen (Figure 2), animals in the IM/IM group exhibited higher splenic T_{FH} frequencies relative to O/T, O/SL and O+IM/SL animals (Figure 3E). In contrast, macaques in the O/SL group had higher T_{REG} frequencies compared to other groups (Figure 3H).

Functionally, CXCR5⁺PD-1⁺ T_{FH} have been defined by IL-21^{29,30} and IL-4³¹ expression. In oral LNs, T_{FH} expressed predominantly IL-4, although relatively few T_{FH} cells expressed IL-21 (Figure 3I). While we did not observe differences in the percentage of IL-4 or IL-21 single positive T_{FH} in oral LNs, IM/IM animals had higher frequencies of IL-4⁺IL-21⁺ dual positive T_{FH}^{29,32,33} compared to O+IM/SL animals (Figure 3I). In the spleen, few IL-4 or IL-21 producing T_{FH} were found (Figure 3J).

SIV Gag-specific tissue T cell responses:

We also tested whether SIV-specific CD4⁺ and CD8⁺ T cell responses were induced in tonsils, LNs draining the oral cavity, and intestinal tissues on week 9 (Figure 4, Table 2). Only 4 animals in the O/T group had sufficient cell numbers to test Gag-specific T cell responses in tonsils, and only 1 had detectable Gag-specific T cells. In contrast, Gag-specific T cells were found in tonsils of 67%, 100%, and 80% of the animals vaccinated by the O/SL, IM/IM, or O+IM/SL routes, respectively (Figure 4, Table 2). However, up to 67% of the O/T animals had Gag-specific T cells in other tissues, with 1 animal (#41145) having unusually high CD4⁺ (84.8%) and CD8⁺ (68.3%) TNF-α responses in the retropharyngeal LNs (Figure 4C-D).

Immunization by the O/SL route resulted in somewhat more consistent T cell responses, with every animal exhibiting Gag-specific T cells in at least one tissue. While all IM/IM animals demonstrated T cell responses in tonsils and intestinal tissues, only a few had responses in oral and mesenteric LNs (Figure 4, Table 2). The most Gag-specific T cell responses were induced in animals primed by both the O+IM routes and boosted by the SL route (Figure 4, Table 2). The different routes of immunization appeared to influence the quality of SIV-specific T cell responses. In the O/T group, TNF-α responses dominated, whereas IL-17 production accounted for the majority of Gag-specific responses in IM/IM animals (Figure 4). The O/SL and O+IM/SL-immunized animals developed strong IL-17 responses in intestinal tissues, but SIV-specific T cell responses in LNs were biased towards production of IFN-γ, IL-2, and TNF-α (Figure 4).

DISCUSSION

A pediatric vaccine to prevent breast milk transmission of HIV likely needs to induce both orogastrintestinal and systemic immune responses. This proof-of-concept study investigated whether an oral-only DNA-SIV prime/MVA-SIV boost strategy, administered at specific sites in the oral cavity, could induce oral, gastrointestinal, and systemic SIV-specific immune responses.

In previous infant macaque studies, we have found that vaccine-induced SIV-specific plasma IgG and mucosal IgA antibodies at the time of oral SIV challenge were associated with reduced viremia^{34, 35}. In contrast to these former studies that utilized peroral (PO) prime/IM boost vaccine regimens, the O (cheek pouch) prime/T boost strategy tested here did not elicit detectable plasma or mucosal antibody responses. The O/SL regimen also failed to generate plasma and salivary antibodies, but it did induce Env- specific IgA antibodies in intestinal secretions (stool) of 50% of vaccinees. IM prime/IM boosting infrequently elicited mucosal IgA responses but induced high levels of anti-Env IgG antibodies in plasma, more T_{FH} cells in spleen, and higher frequencies of IgG antibody-promoting IL-4⁺IL-21⁺ T_{FH} cells³¹ in LNs draining the oral cavity. Simultaneous O+IM priming followed by SL boosting induced comparable levels of Env-specific IgG antibodies in plasma but with higher avidity than those in the IM/IM-immunized animals. The O+IM/SL regimen also induced both Env- and Gag-Pol-specific intestinal IgA responses in 67% of animals, but in only 16% of IM/IM immunized animals. These data, combined with our previous findings, suggest that immunization in the oral cavity may be optimal for generating intestinal responses in infants, but IM immunization will additionally be required in oral HIV vaccine regimens to generate strong systemic antibody responses; a conclusion supported by our finding that IgG-secreting Env-specific B cells could only be detected in LNs and spleens of IM-immunized animals (O+IM/SL or IM/IM).

The lack of detectable SIV-specific plasma antibodies in O/T and O/SL animals does not exclude a priming effect and possible development of memory B cells. In an SIV vaccine study with adult macaques, application of single-cycle viral vectors or Ad5-SIV vaccines to the palatine tonsils induced no or low levels of anti-Env plasma IgG during the immunization phase³⁶. However, vaccinated animals demonstrated an anamnestic anti-Env IgG response after SIV infection, indicating that memory B cells had been generated by tonsillar immunization. Here, despite absent or poor SIV-specific antibody responses observed in O/IT and O/SL-immunized macaques, several animals developed SIV Gag-specific T cell responses in multiple tissues, some at unusually high magnitudes, which were confirmed by repeat testing. Unexpectedly, T cell responses in distal mucosal LNs were superior to those in LNs more proximal to the site of vaccine administration. Another study in adult macaques showed that despite low or absent antibody responses following vaccination in the oral cavity or gastrointestinal tract, circulating SIV-specific IFN- γ ⁺ CD4⁺ and CD8⁺ T cells were associated with prevention or control of SIV infection after mucosal SIV challenge^{8, 37}. The latter study did not assess T_H2 and T_H17 responses, however T_H2 and T_H17 responses were better associated with protection than T_H1 responses in the RV144 HIV vaccine trial³⁸. Here, independent of the delivery route, infant intestinal T cell responses were dominated by IL-17, a hallmark cytokine of T_H17 cells. T_H17 cells are irreversibly lost in acute HIV infection³⁹, whereas increased numbers of T_H17 cells in long-term non-progressors are associated with decreased microbial translocation, reduced immune activation, and slower disease progression⁴⁰. Thus, vaccine-induced IL-17⁺ T_H responses in intestinal tissues may promote intestinal integrity, inhibit immune activation and slow disease progression during SIV/HIV infection.

Poor antibody responses in O/IT and O/SL vaccinees were potentially due to inefficient uptake of the priming DNA in the oral cavity. It was not possible to prime with DNA by the

T or SL routes because the DNA-SIV plasmids were incorporated into cationic liposomes to prevent degradation in the lumen, and this dramatically increased the volume required to deliver the established 3mg DNA dose. For this reason, the DNA vaccine component was given by the O route, which likely resulted in dispersion across the entire oral cavity and most likely some ingestion. MVA uptake through tonsillar M cells was anticipated. However, it is possible that there was insufficient MVA contact time and/or drainage from the tonsil into the oral cavity. Although the amount of vaccine directly taken up by the oral mucosa versus intestinal tissue cannot be quantified, SL vaccine delivery resulted in more consistent T cell and intestinal IgA responses when compared to tonsillar delivery.

SL vaccines to protect against infectious diseases have proved successful in mouse models^{41, 42, 43, 44}, and the first immunogenicity studies of a SL papilloma virus vaccine have been conducted in humans⁴⁵. Although poor neutralizing antibody responses were observed in the latter human study, this could be attributed to delivery of a suboptimal amount of vaccine and adjuvant^{44, 45}. Novel vaccine delivery modes (e.g. films), smaller vaccine volumes, and mucosal adjuvants should be tested in future studies to enhance SL vaccine-induced immune responses. The adjuvants used in the current study were selected for their ability to enhance dendritic cell priming and increase mucosal IgA responses in adult macaques given two IM immunizations with adjuvanted DNA^{14, 15}. The lack of observed benefits of GM-CSF or CD40L DNA adjuvants in this study could be due to our having performed only a single DNA immunization, the small population sizes or possible introduction of a lower DNA dose in the host due to topical oral delivery. Although GM-CSF in high doses can suppress immune responses⁴⁶, the doses of GM-CSF DNA given here to juvenile macaques were the same as those given to adult macaques in previous studies in which no immune suppression was observed. A retrospective PCR analysis of tissue samples for IL-10, TGF- β , and FoxP3 mRNA levels also did not reveal differences in juvenile macaques receiving adjuvanted or non-adjuvanted vaccines, or between vaccine routes (data not shown).

Overall, this study suggests that IM immunization is required for induction of strong systemic antibody responses in this DNA prime/MVA boost vaccine model. However, based on plasma IgG antibody avidity, SIV-specific T cell responses and mucosal IgA responses, a combined O+IM immunization appears superior to the IM/IM strategy, and its relevance should be further explored and confirmed in efficacy studies.

Acknowledgements:

We thank Dr. Bernie Moss and Dr. Patricia Earl (National Institute for Allergy and Infectious Diseases) for providing us with the MVA-SIV vaccine vector, Olga Nichols for producing the SIV-MVA immunization stock, Robert L. Wilson (LSUHSC) for technical assistance in processing secretions and performing ELISAs, Neelima Choudhary and R. Henderson Tuck (UNC) for expert technical assistance with tissue processing, Aanini Dwivedi (UNC) for assistance with ELISPOT and RT-PCR assays, and the CNPRC staff for expert technical assistance with all animal procedures. We gratefully acknowledge the receipt of the anti-CXCR5 and anti-rhesus IgA monoclonal antibodies from the NIH Nonhuman Primate Reagent Resource supported by AI126683 and OD010976. The peptide sets for SIVmac239 Gag were obtained through the AIDS Reagent Program Division of AIDS, NIAID, NIH.

Funding: This work was supported by the National Institutes of Health (R01DE022287) to KDP and by the Office of Research Infrastructure Programs/OD (P51OD011107) to CNPRC. Research reported in this publication was supported by the Center for AIDS Research award number P30AI050410. The UNC Flow Cytometry Core Facility is supported in part by P30 CA016086 Cancer Center Core Support Grant to the UNC Lineberger Comprehensive Cancer Center and in part by the North Carolina Biotech Center Institutional Support Grant 2017-IDG-1025 and by

the National Institutes of Health1UM2AI30836–01. The content of this publication is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

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Figure 1. Systemic and mucosal antibody responses.

SIV gp140 Env-specific IgG binding antibodies in plasma were measured on week 6 (A) and week 9 (B) after immunization using ELISA. (C) The avidity index of anti-gp140 plasma IgG in vaccine groups found to have these antibodies. (D) SIV gp140-specific plasma IgA antibodies on week 9. (E) and (F) IgA specific activity (ng IgA antibody per µg of total IgA) measured in saliva to SIV gp140 Env and SIV Gag,Pol, respectively, on week 9. (G) and (H) IgA specific activity measured in fecal extracts to Env and Gag,Pol, respectively, on week 9. Dotted lines denote cut-offs for significance. Animals in the D and D_G+D_{40L}-primed vaccine groups are denoted by open and filled symbols, respectively. Plasma from one D and D_G+D_{40L}-primed animal on week 9 was not available for analysis. Bars denote medians. *p 0.05; **p 0.01.

Oral LNs

Spleen

Figure 2. Antibody secreting cells in tissues.

(A) Cells from oral LNs (retropharyngeal, submental, or submandibular) or (C) splenic mononuclear cells were stimulated as described in the Methods and assessed by ELISPOT for SIV gp140 Env-specific ASC. Symbols represent the average of three independent measurements for each D-primed (open symbols) or D_G + D_{40L}-primed (filled symbols) animal, and are reported as the number of spots per million mononuclear cells. Note that oral LNs were available from only 2 of 3 animals in Groups A1, A2, B1, B2, and C1.

Representative ELISPOT images from each group for oral LN (B) and spleen (D) are shown.

*p 0.05; **p 0.01.

Frequencies

Figure 3. T_{FH} cell populations in DNA-SIV primed/ MVA-boosted infant rhesus macaques. Frequencies of CD4⁺ T cell populations in oral LNs (A-D) or spleen (E-H), reported as the proportion of CD4⁺CD95⁺ T cells. *Panels I-N* show the frequencies of IL-4⁺, IL-21⁺, or IL-4⁺IL-21⁺ T_{FH} cells in oral LNs (I) or spleen (J) after stimulation as outlined in the Methods. D- or D_G + D_{40L}-primed animals are represented by open or filled symbols, respectively. Note the different scales of the y-axes.

Figure 4. SIV p27 Gag-specific T cell responses.

Frequencies of p27-specific cytokine-secreting CD4⁺ and CD8⁺ T cells in various tissues. Each bar represents the sum of T cells positive for IFN- γ (blue), IL-2 (orange), IL-17 (green), or TNF- α (red) in each animal. Animals in each vaccine group are listed on the x-axis with "+" indicating those primed with D_G + D_{40L}. Note that tissues from some animals could not be analyzed due to low cell numbers. Note the different scales of the y-axes.

Table 1.

Experimental Groups and Vaccine Regimens.

Cohort	n	Prime (Week 0) ¹	Route	Boost #1 (Week 3) ²	Route	Boost #2 (Week 6)	Route
A	3	DNA-SIV ³	O ⁴	MVA-SIV	T ⁵	MVA-SIV	T
B	3	DNA-SIV/GMCSF + DNA-SIV/CD40L ⁶	O	MVA-SIV	T	MVA-SIV	T
C	3		O	MVA-SIV	SL ⁷	MVA-SIV	SL
D	3	DNA-SIV/GMCSF + DNA-SIV/CD40L	O	MVA-SIV	SL	MVA-SIV	SL
E	3	DNA-SIV	IM ⁸	MVA-SIV	IM	MVA-SIV	IM
F	3	DNA-SIV/GMCSF + DNA-SIV/CD40L	IM	MVA-SIV	IM	MVA-SIV	IM
G	3	DNA-SIV	O + IM	MVA-SIV	SL	MVA-SIV	SL
H	3	DNA-SIV/GMCSF + DNA-SIV/CD40L	O + IM	MVA-SIV	SL	MVA-SIV	SL

¹ DNA-SIV expressing SIV gag, pol, env;² MVA-SIV expressing SIV gag, pol, env;³ D,⁴ oral;⁵ tonsillar;⁶ DG+40L;⁷ sublingual;⁸ intramuscular

Table 2.

Frequency of SIV p27 Gag-specific T Cell Responders

Number and (%) of animals with CD4 ⁺ or CD8 ⁺ SIV p27-specific T Cell responses						
Regimen	Tonsil	Retro. LN ¹	Sub. LN ²	Mes. LN ³	Ileum	Colon
O/ T	1 of 4 (25)	3 of 5 (60)	4 of 6 (67)	3 of 6 (50)	3 of 6 (50)	3 of 6 (50)
O/ SL	3 of 6 (50)	4 of 6 (67)	2 of 6 (33)	3 of 6 (50)	5 of 6 (83)	5 of 5 (100)
IM/ IM	5 of 5 (100)	2 of 6 (33)	3 of 6 (50)	1 of 6 (17)	6 of 6 (100)	5 of 5 (100)
O+IM/ SL	5 of 5 (100)	4 of 6 (67)	3 of 6 (50)	5 of 6 (83)	6 of 6 (100)	5 of 5 (100)

¹retropharyngeal LNs;²submandibular LNs;³mesenteric LNs