



Published in final edited form as:

Vaccine. 2011 August 5; 29(34): 5611–5622. doi:10.1016/j.vaccine.2011.06.017.

Prime-boost vaccination with heterologous live vectors encoding SIV *gag* and multimeric HIV-1 gp160 protein: efficacy against repeated mucosal R5 clade C SHIV challenges

Samir K. Lakhsha^{a,b}, Vijayakumar Velu^{c,d}, Gaia Sciaranghella^{a,b}, Nagadenahalli B. Siddappa^{a,b}, Janet M. DiPasquale^e, Girish Hemashettar^a, John K. Yoon^a, Robert A. Rasmussen^{a,b}, Feng Yang^{a,b}, Sandra J. Lee^{a,b}, David C. Montefiori^f, Francis J. Novembre^{c,d}, François Villinger^{c,d}, Rama Rao Amara^{c,d}, Maria Kahn^g, Shiu-Lok Hu^g, Sufen Li^h, Zhongxia Li^h, Fred R. Frankel^h, Marjorie Robert-Guroff^e, Welkin E. Johnsonⁱ, Judy Lieberman^j, and Ruth M. Ruprecht^{a,b}

^aDepartment of Cancer Immunology and AIDS, Dana-Farber Cancer Institute, Boston, MA 02215, USA

^bDepartment of Medicine, Harvard Medical School, Boston, MA 02215, USA

^cDivision of Microbiology and Immunology, Yerkes National Primate Research Center, Emory University, Atlanta, GA 30329, USA

^dEmory Vaccine Center, Emory University School of Medicine, Atlanta, GA 30329, USA

^eVaccine Branch, National Cancer Institute, National Institute of Health, Bethesda, MD 20892, USA

^fDepartment of Surgery, Duke University Medical Center, Durham, NC 27710, USA

^gUniversity of Washington, Seattle, WA 98195, USA

^hDepartment of Microbiology, University of Pennsylvania School of Medicine, 3610 Hamilton Walk, Philadelphia, PA 19104, USA

ⁱNew England Primate Research Center, Southborough, MA 01772, USA

^jImmune Disease Institute and Department of Pediatrics, Harvard Medical School, Boston, MA, 02115, USA

Abstract

We sought to induce primate immunodeficiency virus-specific cellular and neutralizing antibody (nAb) responses in rhesus macaques (RM) through a bimodal vaccine approach. RM were immunized intragastrically (i.g.) with the live-attenuated *Listeria monocytogenes* (Lm) vector *Lmdd-BdopSIVgag* encoding SIV_{mac239} *gag*. SIV Gag-specific cellular responses were boosted by intranasal and intratracheal administration of replication-competent adenovirus (*Ad5hr-SIVgag*)

© 2011 Elsevier Ltd. All rights reserved.

Corresponding author: Ruth M. Ruprecht, MD, PhD, Dana-Farber Cancer Institute, 450 Brookline Ave, Boston, MA 02215 USA, Phone: 617-632-3719; Fax: 617-632-3112; ruth_ruprecht@dfci.harvard.edu.

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.

Current addresses for GS: Ragon Institute of MGH, MIT and Harvard, Boston, MA 02129, USA; and for ZL: Walter Reed Army Institute, Silver Spring, MD 20910, USA

encoding the same *gag*. To broaden antiviral immunity, the RM were immunized with multimeric HIV clade C (HIV-C) gp160 and HIV Tat. SIV Gag-specific cellular immune responses and HIV-1 nAb developed in some RM. The animals were challenged intrarectally with five low doses of R5 SHIV-1157ipEL-p, encoding a heterologous HIV-C Env (22.1% divergent to the Env immunogen). All five controls became viremic. One out of ten vaccinees was completely protected and another had low peak viremia. Sera from the completely and partially protected RM neutralized the challenge virus >90%; these RM also had strong SIV Gag-specific proliferation of CD8⁺ T cells. Peak and area under the curve of plasma viremia (during acute phase) among vaccinees was lower than for controls, but did not attain significance. The completely protected RM showed persistently low numbers of the α 4 β 7-expressing CD4⁺ T cells; the latter have been implicated as preferential virus targets in-vivo. Thus, vaccine-induced immune responses and relatively lower numbers of potential target cells were associated with protection.

Keywords

Live attenuated *Listeria monocytogenes*; Replication-competent adenovirus; Live oral vaccine; HIV/AIDS vaccine; Multimeric HIV clade C gp160; Prime-boost vaccination; α 4 β 7 integrin

1. Introduction

A safe and efficacious prophylactic vaccine should reduce the number of new HIV-1 acquisitions that affect two million individuals each year. To accomplish this goal, a number of groups are attempting to develop a vaccine that can induce virus neutralizing antibodies (nAbs) as well as virus-specific cellular immunity. Since HIV-1 acquisition mostly occurs mucosally, efforts are being made to induce antiviral responses at the mucosa via vaccination at mucosal sites (reviewed in [1-3]). Viral and bacterial vectors engineered to express lentivirus proteins (reviewed in [4, 5]) are being tested for efficacy using simian immunodeficiency virus (SIV) or chimeric simian-human immunodeficiency virus (SHIV) challenge in nonhuman primates [6]. Some of the vaccine vectors are used as either replication-defective or replication-competent forms [7]. Since many successful vaccines against viral diseases, such as measles, mumps, rubella, polio and yellow fever, employ attenuated but replicating forms of the respective pathogen, replication-competent vectors deserve more attention. Immunization with a live, replicating rhesus cytomegalovirus (RhCMV) vector expressing SIV proteins induced and maintained SIV-specific effector memory T cells [8]. After mucosal low-dose challenges with SIVmac239, the vaccinated animals showed increased resistance to acquisition of infection and some of the vaccinees controlled systemic spread of the challenge virus. Protection was associated with SIV-specific effector memory T cells that were maintained due to continuous antigenic exposure by replicating RhCMV [8].

We used a combination of two live vectors (*Listeria monocytogenes* (Lm) and replication-competent adenovirus type 5 host range mutant (*Ad5hr*) [9]) to prime and boost T-cell responses against SIV Gag. The RM were first immunized with a new, live-attenuated strain of Lm (*Lmdd-BdopSIVgag*) expressing SIV Gag [10-12] and the Gag-specific responses were boosted using *Ad5hr* that encoded the same *gag* (*Ad5hr-SIVgag*) [13]. The common features of Lm and adenovirus, such as mucosal route of infection, preferential targeting of antigen-presenting cells (including dendritic cells), infection of epithelial cells, stimulation of innate immune responses and high levels of transgene expression, make them attractive tools to induce transgene-specific immune responses (reviewed in [7, 14, 15]). The adenovirus also shows another safety feature, i.e., lack of integration in the host cell genome.

Lm has been studied in animal models as a vector for candidate cancer vaccines [16-21] and was recently used in a Phase I clinical trial among cervical carcinoma patients [22]. Oral immunization of mice with Lm expressing HIV-1 Gag induced strong mucosal Gag-specific T-cell responses and protected the vaccinees against vaginal challenge with recombinant vaccinia virus expressing HIV-1 *gag* [11, 23]. Oral immunization of cats with Lm expressing feline immunodeficiency virus (FIV) Gag was also partially effective against vaginal FIV challenge by allowing the vaccinated cats to suppress viral replication although infection was not prevented [24]. Different serotypes of adenovirus (Ad) such as Ad4, Ad5, Ad7, Ad26 and Ad35 are being explored as vaccine vectors. Live, non-attenuated Ad4 and Ad7-based vaccine were found to be safe and effective against acute respiratory syndrome and have been administered orally to more than 10 million military recruits [25, 26]. Replication-defective Ad35 and Ad26 carrying HIV-1 genes are being tested in Phase I clinical trials, whereas Ad5 is being tested in a Phase II trial that has enrolled Ad5 nAb-negative and circumcised male volunteers (<http://clinicaltrials.gov>). The Ad5-based HIV-1 vaccine constructs are under extensive investigation for human use [27-30].

The *Lmdd-BdopSIVgag* prime and *Ad5hr-SIVgag* boost was designed to induce strong cellular responses against SIV Gag in RM. However, the encouraging data of the recent RV144 trial [31] suggest that a combination of immunogens that induce humoral as well as cellular responses may provide protection from HIV-1 acquisition. Hence, we boosted the RM with trimeric HIV-1 gp160, an important target for humoral responses. Along with gp160, the HIV-1 Tat protein was also administered to increase breadth of immune responses [32]. Subsequently, the vaccinated RM were challenged intrarectally (i.r.) with five low doses of the newly constructed SHIV-1157ipEL-p [33] that encodes an R5 HIV clade C *env* (22.1% divergent to the vaccine Env). Here, we present the efficacy data of *Lmdd-BdopSIVgag* prime, *Ad5hr-SIVgag* boost followed by HIV-1 gp160 immunization against heterologous SHIV-1157ipEL-p (SHIV-C) mucosal challenges.

2. Methods

2.1. Immunogens

Construction of *Lmdd-BdopSIVgag* and control vector *Lmdd-Bdop* was described earlier [11, 12], as was that of *Ad5hr-SIVgag* and the *Ad5hr* empty vector [13, 34, 35]. Administration of *Lmdd-BdopSIVgag* is also described [10]. The first dose of *Ad5hr-SIVgag* or *Ad5hr* was given intranasally and intragastrically (i.g.), whereas the second dose was given by the intratracheal route. Fifteen min before oral vector administration, the RM were anesthetized and pretreated with a saturated sodium bicarbonate solution via nasogastric tube to neutralize stomach acid. Each dose consisted of 5×10^8 plaque forming units (pfu) of adenovirus suspended in 500 μ l of phosphate-buffered saline (PBS).

HIV1084i was isolated from a Zambian infant [36]. Multimeric HIV1084i gp160 was produced by recombinant vaccinia virus technology as described [37], whereas HIV IIIB Tat was purchased from Advanced Bioscience Laboratories, Inc. (Kensington, MD). For each protein immunization, 100 μ g of protein in incomplete Freund's adjuvant (IFA) was administered i.m.

2.2. Animals

Indian-origin RM (*Macaca mulatta*) were housed at the Yerkes National Primate Research Center (YNPRC), Atlanta, Georgia, USA. YNPRC is fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC). Approval for all procedures was received from the Institutional Animal and Care and Use

Committees of Dana-Farber Cancer Institute and Emory University. All animals were MHC typed for Mamu-A*001, B*008 and B*17 alleles (Fig. 1).

Three groups, each consisting of 5 RM, (Groups 1A, 1B and 2), were enrolled. Group 1A and 1B were vaccinees that differed in the number of Lm doses, whereas Group 2 were control animals (Fig. 1). The safety and immunogenicity data after vaccinations with *Lmdd-BdopSIVgag* have been described earlier [10], where Group 1A, 1B and 2 are referred as Group C, B and D, respectively.

2.3. Challenge virus

The challenge virus, SHIV-1157ipEL-p, was generated as described earlier [33]. The virus encodes SHIV-1157ip [38] *env* (originally derived from an HIV-C infected Zambian infant) in the backbone of SHIV-1157ipd3N4 [39] (a virus that contains additional NF- κ B sites in the long terminal repeats to boost viral replicative capacity). An animal-titrated stock of SHIV-1157ipEL-p was prepared using concanavalin A (con A)-stimulated RM PBMC cultured in the presence of interleukin (IL)-2 and tumor necrosis factor (TNF)- α (10 ng/ml). For low-dose SHIV-1157ipEL-p challenges, inocula of 8,000 50% tissue culture infectious doses (TCID₅₀; titrated on TZM-bl cells) was used.

2.4. Measurement of plasma vRNA

Plasma viral RNA (vRNA) was isolated by QiaAmp Viral RNA Mini-Kit (Qiagen, Germantown, MD, USA); vRNA levels were measured by quantitative reverse-transcriptase polymerase chain reaction (RT-PCR) for SIV *gag* sequences [40]. Additionally, primers/probes according to Lifson were used [41]. Assay sensitivity was 50 vRNA copies/ml [40].

2.5. Interferon (IFN)- γ ELISPOT assay

The assay was performed as described earlier [42]. Multiscreen-IP plates (Millipore, Billerica, MA) were coated with anti-human IFN- γ antibody (clone B27, BD Pharmingen, San Jose, CA), blocked with 10% heat inactivated fetal bovine serum (FBS) in RPMI-1640 (R-10). 1×10^5 cells were incubated overnight with SIVmac239 Gag or HIV-1 consensus B Tat peptides (each peptide, 2 μ g/ml) obtained through NIH AIDS Research and Reference Reagent Program (ARRRP). Each peptide set consisted of 15-mer peptides with 11-amino acid overlaps between sequential peptides and represented the complete protein sequence. For SIVmac239 Gag peptides, three pools were prepared (pools #1, #2 and #3 consisting of peptides 1–42, 43–84 and 85–125, respectively), whereas a single pool was prepared for the set of 23 HIV-1 Tat peptides. The IFN- γ -secreting cells were detected by using biotinylated anti-human IFN- γ antibody (clone 7-B6-1, Mabtech, Cincinnati, OH), horseradish peroxidase-conjugated streptavidin (BD Biosciences, San Jose, CA) and AEC chromogen substrate (BD Biosciences). The spots were enumerated using an Immunospot ELISPOT reader (CTL, Cleveland, OH). Assays were done in duplicate and background counts with no peptide stimulation were subtracted.

2.6. Intracellular cytokine staining

Cryopreserved PBMC were thawed, washed and rested in R-10 medium overnight at 37°C. Next, PBMC were suspended in R-10 containing brefeldin A (10 μ g/ml; Sigma-Aldrich, St. Louis, MO), monensin (GolgiStop, BD Biosciences), anti-CD49d antibody (clone 9F10, BD Biosciences) and anti-CD28-PECy7 antibody (clone CD28.2, eBioscience, San Diego, CA). The cells were stimulated with SIVmac239 Gag and HIV-1 Tat peptides at 37°C for 6 h. Cells were stained with anti-CD3-AlexaFluor700 (clone SP34-2, BD-Pharmingen), anti-CD4-PE (clone M-T477, BD-Pharmingen), anti-CD8-AmCyan (clone SK1, BD-Biosciences) and anti-CD95-PE-Cy5 (clone DX2, BioLegend, San Diego, CA) antibodies.

The cells were then fixed/permeabilized with cytofix/cytoperm (BD Pharmingen) and stained intracellularly with anti-IL-2-APC (clone MQ1-17H12, BD Pharmingen), anti-IFN- γ -PerCP/Cy5.5 (clone 4S.B3, BioLegend) and anti-TNF- α -PacificBlue (clone MAb11, eBioscience) antibodies. At least 50,000 lymphocytes were acquired on an LSR-II (BD Immunocytometry Systems) and data were analyzed using FlowJo 6.0 (Tree Star, Inc., Ashland, OR) software. Percentages of CD3⁺CD4⁺ and CD3⁺CD8⁺ cells producing each of seven different possible combinations of IL-2, IFN- γ and TNF- α were determined. Background numbers of cells producing cytokines without peptide stimulation were subtracted. Cytokine-producing cells were characterized for their memory phenotype based on cell-surface expression of CD28 and CD95 (central memory (CM): CD28⁺CD95⁺; effector memory (EM): CD28⁻CD95⁺; naïve: CD28⁺CD95⁻).

2.7. Lymphocyte proliferation assay

PBMC were stained with CFSE (CellTrace™ CFSE Cell Proliferation Kit, Invitrogen, Camarillo, CA) and incubated with or without antigen (SIV Gag, HIV-1 Tat; 2 μ g/ml; ARRRP) for 5 days at 37°C. Positive controls were stimulated with con-A (5 μ g/ml); cells without any stimuli were used to determine background proliferation. After incubation, cells were stained with anti-CD3-Alexa Fluor 700 (clone SP34-2), anti-CD4-PerCP (clone L200) and anti-CD8-PE (clone RPA-T8) antibodies (all from BD Pharmingen). After fixation, at least 10,000 lymphocytes (based on forward and side scatter) were acquired by flow cytometry and data were analyzed using FACSDiva (BD Biosciences) software. The percentages of proliferating CD3⁺CD4⁺ and CD3⁺CD8⁺ cells were determined by CFSE dilution; background proliferation (no stimulation) was subtracted.

2.8. Tetramer staining

Indian rhesus macaques display a high frequency of MHC class I allele Mamu-A*001 which binds SIV Gag peptide p11C, the immunodominant epitope for this allele. Using this peptide-MHC tetramer complex, we determined the frequency of p11C-specific, CD8⁺ T cells among PBMC or rectal mononuclear cells as described earlier [10]. Cells were considered to be tetramer positive if they represented at least 0.03% of the total CD3⁺CD8⁺ T lymphocytes, and if the cell cluster was clearly separated from the tetramer-negative cell population.

2.9. Serum antibody binding titers

ELISA plates were coated with SIVmac251 p27 (Immunodiagnosics, Inc., Woburn, MA), HIV-1 Tat (ARRRP) and HIV_{CN54} gp120 (ARRRP), diluted to 2 μ g/ml. After blocking with 3% BSA, serial serum dilutions were added in duplicate wells. Antibody binding was detected by horseradish peroxidase conjugated rabbit anti-monkey IgG (Sigma-Aldrich, St. Louis, MO) and O-Phenylenediamine dihydrochloride (OPD) + hydrogen peroxide substrate. Titers were calculated as the greatest reciprocal serum dilution giving OD readings >mean + 5 standard deviations above background as measured using preimmune serum from the same RM at the same dilution.

HIV-C_{CN54} gp120 produced in insect cells was used to measure anti-Env binding antibody titers. This ruled out the possibility of detection of anti-vaccinia antibodies that might have been induced due to vaccinia proteins possibly associated with our immunogens that were produced by recombinant vaccinia technology.

2.10. PBMC-based nAb assay

Sera were tested against SHIV-1157ipEL-p in the PBMC-based neutralization assay [43]. Predetermined virus inocula were incubated with diluted sera in triplicate wells at 37°C for 1

h. Virus was also incubated with medium alone (to determine 100% virus production). After incubation, 0.5×10^6 phytohemagglutinin (PHA)-stimulated human PBMC were added to all wells in the presence of IL-2 (40 U/ml) and polymyxin B (15 $\mu\text{g/ml}$). From days 3 to 8, 100 μl /well of supernatant was collected and replaced with fresh medium. Supernatants were tested for SIV p27 by ELISA (Advanced Bioscience Laboratories, Kensington, MD). The % neutralization was determined for each serum sample as: %neutralization = $100 - [(\text{avg. p27 in wells containing virus} + \text{plasma mixture} \times 100) / \text{avg p27 in wells assigned for virus production}]$. Pooled sera collected from naïve RM were used as negative control, and a mixture of four broadly reactive nAbs (b12, 2F5, 4E10 and 2G12) served as positive control. Serum dilutions that showed 50 and 90% inhibition of virus replication (IC_{50} and IC_{90} respectively) were determined by logarithmic regression analysis.

2.11. TZM-bl cell-based neutralization assay

Sera collected from all monkeys on the day of the first low-dose virus challenge were tested to determine nAb titers against the SHIV-1157ipEL-p (challenge virus), SHIV-2873Nip (clade C, tier 2 virus [44]), and SHIV_{SF162P4} (clade B, tier 1 virus [45]). Human PBMC-grown virus was incubated with different serum dilutions and virus neutralization was determined by using the TZM-bl cell-based assay as described [46].

2.12. Determination of CD4⁺ α 4 β 7⁺ T cells

Peripheral blood CD4⁺ T cells were analyzed for expression of α 4 and β 7 markers as described [47]. In brief, the PBMC were stained with anti-CD3 – AlexaFluor 700 (clone: SP34-2), anti-CD4-FITC (clone: m-T477), anti-CD49d (clone: 9F10) and anti- β 7-APC (clone: FIB504) antibodies (all from BD Pharmingen). After fixation, at least 10,000 CD3⁺CD4⁺ cells were acquired on LSR-II and analyzed using FACSDiva (BD Biosciences) software. The percentage (%) of CD3⁺CD4⁺ cells expressing α 4 and β 7 markers was determined.

2.12. Measurement of nAb titer against Ad5

Ad5 nAb titers were determined as described [48]. Briefly, 50 μl aliquots of 4-fold dilutions of heat-inactivated plasma were incubated for 1 h at 37°C in 5% CO₂ in 96-well flat bottom plates with 50 μl of a titrated Ad5-luciferase stock (Ad5-Apt-Luc, Crucell Holland B.V., Leiden, The Netherlands) giving approximately 500 viral particles/cell. A549 cells ($1 \times 10^4/100 \mu\text{l}$) were added to each well, and the plates were incubated 24 h at 37°C, 5% CO₂. The culture medium for all steps was F-12 Kaighn's containing 10% FBS, 50 $\mu\text{g/ml}$ gentamicin and 2 mM L-glutamine. Subsequently, 100 μl of medium was removed from each well and replaced with 100 μl reconstituted britelite solution (Britelite plus 100 kit, Perkin Elmer), mixed and incubated 2 min. Luciferase activity was detected by transferring 200 μl of each cell/substrate mixture to 96 well black flat-bottom plates, and measuring luminescence within 15 min on a Victor² 1420 multilabel counter. Neutralizing titers are defined as the plasma dilutions resulting in 50% (or 90%) reduction in luciferase activity relative to that of pre-bleed samples diluted 1:20.

2.13. Statistical analysis

Log-rank tests were used to compare time to infection among vaccinees and controls. Time to infection was defined as the time from entry into the virus challenge phase to the first evidence of viremia (event), or censored at the last known uninfected date. Kaplan-Meier estimates of time to infection were calculated. The Wilcoxon rank-sum test was used to compare peak viremia and the area-under-the-curve (AUC) of the vaccinees and controls. For all the comparisons of treatments, a two-sided significance level of 5% was assumed. A *P*-value <0.05 was considered statistically significant.

3. Results

3.1. Priming of SIV Gag-specific cellular immune responses by *Lmdd-BdopSIVgag* immunization

The safety and immunogenicity of the new vector, *Lmdd-BdopSIVgag*, in RM was reported earlier [10]. *Lmdd-BdopSIVgag* was safe in macaques via i.g. route and induced higher SIV Gag-specific cellular responses than the earlier Lm strain [10]. Here, RM in Group 1A received two sets of *Lmdd-BdopSIVgag* i.g. immunizations at an interval of six weeks (Fig. 1). Two weeks after the first set of Lm administrations, SIV Gag-specific cellular responses were detected among four of five RM. The responses were low (<300 spot-forming units (SFU)/10⁶ PBMC), but above the pre-immunization responses (0 - 20 SFU/10⁶ PBMC). After the second set of Lm administrations, four out of five RM showed significantly higher responses. One RM showed a peak response of 2,920 SFU/10⁶ PBMC within one week, whereas three animals reached peaks (280 to 610 SFU/10⁶ PBMC) after 5 weeks (Fig. 2A).

Group 1B received four sets of *Lmdd-BdopSIVgag* immunizations via the i.g. route (Fig. 1). After the first set, four of five RM showed SIV Gag-specific cellular responses similar to Group 1A. After administration of the second set of *Lmdd-BdopSIVgag*, two RM showed strong responses (1,200 and 2,200 SFU/10⁶ PBMC) within two weeks (Fig. 2A). The responses declined after reaching a peak, but reemerged at low levels after each subsequent dose of *Lmdd-BdopSIVgag* (Fig. 2A).

As described earlier [10], the vaccination induced anti-Gag cellular responses despite pre-existing anti-Lm cellular immune responses. Although the vaccination induced cellular responses, anti-Gag antibodies were not detected in any vaccinee (data not shown). These results are in accordance with our earlier study [49]. Although these vaccinations were performed by the i.g. route, we did not see p11C-specific T cells in rectal mucosal tissues after staining of mononuclear cells from biopsies (data not shown).

3.2. SIV Gag-specific immune responses after administration of *Ad5hr-SIVgag*

In an attempt to strengthen antiviral cellular immune responses in mucosal tissues, we boosted the RM with *Ad5hr-SIVgag*. Eighteen weeks after the administration of the last dose of *Lmdd-BdopSIVgag*, Groups 1A and 1B received the first dose of *Ad5hr-SIVgag* intranasally and i.g. (Fig. 1). On the day of adenovirus administration, all RM had nearly undetectable SIV Gag-specific cellular responses (0 to 55 SFU/10⁶ PBMC). After the administration of *Ad5hr-SIVgag*, one RM from Group 1A showed a strong Gag-specific cellular response (3,200 SFU/10⁶ PBMC) within two weeks, whereas the other four RM showed peak responses of 140 to 625 SFU/10⁶ PBMC between two to six weeks (Fig. 2A). All Group 1B animals showed Gag-specific cellular responses (range: 260 to 4510; mean \pm SD: 1631 \pm 1677 SFU/10⁶ PBMC) within one week (Fig. 2A). After twelve weeks, both Groups received a second dose of *Ad5hr-SIVgag* intratracheally. On the day of the second adenovirus administration, Groups 1A and 1B had low Gag-specific responses (range: 55 to 690 and 70 to 220 SFU/10⁶ PBMC, respectively). After adenovirus administration, boosting of Gag-specific cellular responses was not detected for Group 1A animals by IFN- γ ELISPOT assay, but all Group 1B RM again showed strong cellular responses (range: 1,130 to 5,165 SFU/10⁶ PBMC) within one week. The responses significantly declined in one to two weeks after the peak but reappeared among Group 1B RM intermittently (~ 15 weeks after the last adenovirus dose), which may be due to the replication-competence of *Ad5hr-SIVgag* [13] (Fig. 2A).

After administration of the first dose of *Ad5hr-SIVgag*, anti-Gag antibodies became detectable. The binding antibodies reached titers of >1/1000 in all RM from Group 1A but in only one RM from Group 1B (Fig. 2B). Thus, either cellular and/or humoral anti-Gag

responses were generated. The Mamu-A*001⁺ RM preferentially generated cellular responses. We also measured the titers of anti-*Ad5hr* vector nAbs before and after adenovirus boosting (Fig. 2C). Before vaccination, nAbs were not detected, but after *Ad5hr* administration, three RM from Group 1A and all RM from Group 1B developed anti-*Ad5hr* nAbs (Fig. 2C). All RM from Group 2 (controls) that were immunized with empty vector, i.e. *Ad5hr*, developed anti-*Ad5hr* nAbs (Fig. 2C). Anti-Gag cellular or humoral responses were not detected among these control RM. Higher *Ad5hr* nAb titers among Group 2 RM compared to Group 1A and 1B are likely to be due to better replication of *Ad5hr* compared to *Ad5hr-SIVgag*.

We also measured the frequency of CD3⁺CD8⁺ Gag p11C tetramer⁺ cells for Mamu-A*001⁺ RM (three from Group 1A and all five from Group 1B) in PBMC (Fig. 3A) and the mononuclear cells from rectal mucosa (Fig. 3B) collected two weeks after the second dose of *Ad5hr-SIVgag*. PBMC of two out of three Group 1A RM were positive (~0.5% of CD3⁺CD8⁺ lymphocytes), whereas all RM of Group 1B showed 1 to 3 % CD3⁺CD8⁺ Gag p11C tetramer⁺ cells (Fig. 3A). This is in agreement with the ELISPOT data that showed higher frequencies of SIV Gag-specific cells among Group 1B compared to Group 1A animals. Mononuclear cells from rectal mucosa of one out of three Group 1A RM were positive (0.3% of CD3⁺CD8⁺ lymphocytes), whereas all RM of Group 1B showed 0.1 to 0.3 % CD3⁺CD8⁺ Gag p11C tetramer⁺ cells (Fig. 3B). Results were considered positive if they were >0.03%, a cut-off that was determined from analysis of naïve monkeys. No positive cells were detected among Mamu-A*001 negative vaccinees.

3.3. Polyfunctionality and phenotype of SIV Gag-specific T cells

We analyzed cellular immune response by intracellular cytokine staining (ICS) for Group 1B animals at one week after administration of each dose of adenovirus and fifteen weeks after the last dose (when peaks of anti-Gag cellular responses were detected by ELISPOT assay, Fig. 2A). After the first adenovirus dose (peak 1), the two animals that had shown >1500 ELISPOTs/10⁶ PBMC (RCg-10 and RWy-9) showed relatively high SIV Gag-specific cytokine⁺ T cells (Supplementary Fig. 1A). The majority of cytokine⁺ cells were CD8⁺ T cells with a central memory phenotype that produced IFN- γ + TNF- α (0.3 to 0.6 %); a relatively small fraction showed production of IFN- γ + TNF- α + IL-2 (~0.1%). Another RM, RFa-10, also showed 0.3% CD8⁺ T cells with central memory phenotype that produced IFN- γ + TNF- α (ELISPOT data: 835 SFU/10⁶ PBMC). Most RM also showed Gag-specific, TNF- α -producing CD4⁺ as well as CD8⁺ T cells (Supplementary Fig. 1A). After the second boost with *Ad5hr-SIVgag*, three RM (RFa-10, RKs-9 and RWy-9) showed higher numbers of IFN- γ - and TNF- α -producing CD8⁺ T cells compared to results after the first adenovirus boost (Supplementary Fig. 1B). RM RKs-9 also showed >1% Gag-specific CD4⁺ T cells (Supplementary Fig. 1B). After both adenovirus administrations, most Gag-specific cytokine⁺ CD8⁺ T cells were of the central memory phenotype, but 15 weeks after the last adenovirus administration (when another peak cellular response was detected by ELISPOT assay), the number of effector memory CD8⁺ T cells was higher than their central memory counterpart in four of five RM (Supplementary Fig. 1C). However, the total frequency of Gag-specific T cells was lower than that observed during the second peak of cellular responses (Supplementary Fig. 1B).

3.4. Immune responses at the time of first SHIV-C challenge

To increase the breadth of cellular responses and to induce HIV-1 nAb, the RM were also immunized with three i.m. doses of HIV-1 Tat and multimeric HIV-C gp160 proteins in IFA. The first dose was given 11 weeks after the last adenovirus administration; the second dose was given six weeks after the first protein dose and the last dose was given two weeks before the first virus challenge (Fig. 1).

We measured SIV Gag- and HIV Tat-specific cellular responses and Gag-, Tat-, Env-specific humoral responses at the time of the first SHIV-1157ipEL-p challenge. The frequency of Gag-specific cells had declined to <500 SFU/ 10^6 PBMC except in RM RWy-9 (which showed 1,730 SFU/ 10^6 PBMC) (Fig. 4A). The HIV-1 Tat-specific cellular responses were also modest (<200 SFU/ 10^6 PBMC) (Fig. 4A). ICS data also supported the low frequency of SIV Gag-specific IFN- γ -producing cells (Supplementary Fig. 2). Most vaccinees showed a high fraction of TNF- α -producing T cells; some also showed IL-2 + TNF- α -producing T cells with an effector memory phenotype (Supplementary Fig. 2). The majority of the vaccinees showed proliferation of CD4⁺ and CD8⁺ T cells after stimulation with SIV Gag protein. The highest proliferation of CD8⁺ T cells was shown by RWy-9 followed by RQe-10 (Fig. 4B). All vaccinees had binding antibodies against all immunogens, i.e., SIV Gag, HIV-1 Tat and Env (Fig. 4C).

NAb titers measured by PBMC-based assays against the heterologous challenge virus (SHIV-1157ipEL-p) are shown in Fig. 4D. Two RM from Group 1B (RQe-10 and RWy-9) showed $>90\%$ neutralization of the challenge virus (although titers were low with IC₉₀ $< 1:50$ and IC₅₀ $\sim 1:1,200$). The virus-neutralizing activity was also measured by the TZM-bl-based assay (Table 1) against SHIV-1157ipEL-p, SHIV-2873Nip (clade C, tier 2 virus) and SHIV_{SF162P4} (clade B, tier 1 virus). The RM RWy-9 which showed the highest IC₉₀ in the PBMC-based assay also had the highest IC₅₀ value in the TZM-bl-based assay (Table 1), and a relatively high neutralizing activity of RQe-10 was detected by both assays.

Replicating vectors such as RhCMV are known to induce and maintain antigen-specific effector memory T cells [8]. We also noted a trend towards the emergence of SIV Gag-specific effector memory T cells after the administration of replication competent *Ad5hr* vector. We determined the memory phenotype of SIV Gag-specific CD4⁺ and CD8⁺ T cells (producing any combination of IL-2, IFN- γ and TNF- α). The analysis was done for Group 1B RM one week after each dose of *Ad5hr-SIVgag* (Supplementary Fig. 1A,B) and 15 weeks after the last dose (when another peak of cellular responses was detected by ELISPOT assay (Supplementary Fig. 1C), and on the day of the first virus challenge (Supplementary Fig. 1D). At the first two time points (Supplementary Fig. 1A,B), the numbers of antigen-specific T cells were higher in the central memory (CD28⁺CD95⁺) compartment than the effector memory (CD28⁻CD95⁺) compartment. At the third time point (15 weeks after last *Ad5hr-SIVgag* dose), four out of five Group 1B animals showed higher numbers of SIV Gag-specific CD8⁺ T cells in the effector memory compartment. On the day of the first virus challenge (week 0), the majority of antigen-specific T cells were found in the effector memory compartment for Group 1B RM (Supplementary Fig. 2). As measured by ICS assay, a significant fraction of antigen-specific T cells produced IFN- γ and TNF- α immediately after *Ad5hr-SIVgag* administration (Supplementary Fig. 1A,B) but at week 0, most antigen-specific T cells produced only TNF- α (Supplementary Fig. 1D). To summarize, our data suggest emergence of effector memory T cells after administration of replication-competent *Ad5hr-SIVgag*.

3.5. Vaccine efficacy against multiple, low-dose challenges with heterologous clade C SHIV

Primate challenge models should recapitulate HIV-1 transmission as closely as possible. Therefore, the challenge virus should be inoculated by the mucosal route, should show CCR5 tropism (since almost all sexually transmitted HIV-1 strains are CCR5 tropic) and gradual disease progression, and should represent virus clades with high worldwide prevalence. Importantly, the challenge virus should reflect the relative low virus inoculum typical of human exposures and be heterologous to the strain of virus used for vaccine preparation since no human vaccinee is likely to be exposed to an HIV-1 strain exactly matching his/her vaccine strain. To fulfill these requirements, animals were challenged i.r.

with low-doses of SHIV-1157ipEL-p that encodes a heterologous, recently transmitted R5 HIV-C Env.

All RM were given five SHIV-1157ipEL-p challenges (one i.r. dose/week) and were monitored for plasma vRNA levels (Fig. 5A, B and C). There were an additional twelve control RM that were challenged by the same protocol with the same stock of SHIV-1157ipEL-p in other studies (submitted) (Fig. 5D). We used data of these additional control animals to increase the statistical power. All five control RM from Group 2 became viremic and showed peak plasma viremia ranging from 0.2×10^6 to 11×10^6 vRNA copies/ml (Fig. 5C). Eleven out of 12 additional control RM were infected and showed similar peak plasma viremia levels (0.2×10^6 to 12×10^6 copies/ml) (Fig. 5D). Among vaccinees, all five Group 1A RM became viremic, but in Group 1B, one RM (RQe-10) remained aviremic, whereas another RM (RWy-9) had a low peak viremia of only 32,000 vRNA copies/ml. Peak plasma viremia among the remaining vaccinees ranged between 0.2×10^6 to 2.0×10^6 vRNA copies/ml, and thus trended toward lower values than those for controls. Next, we assessed time to first detection of viremia by Kaplan-Meier analysis. Although onset of viremia in control animals briefly preceded that in the vaccinees, this parameter did not reach statistical significance compared to either Group 2 controls (Fig. 5E) or all 17 controls (Fig. 5F). Similarly, the difference in peak plasma viremia and AUC between vaccinees and controls were not statistically significant (Fig. 5G-J). However, peak plasma viremia among all vaccinees (Groups 1A + 1B) tended to be lower compared to all 17 control RM ($P = 0.067$).

To confirm complete protection of RQe-10, RT-PCR analysis was performed to detect vRNA in a peripheral lymph node as well as ultracentrifuged plasma taken 4 weeks after the last virus challenge; no vRNA copies were detected (data not shown). Importantly, PBMC of RQe-10 supported SHIV-1157ipEL-p replication in vitro (Fig. 6), suggesting that the cells of RQe-10 were not intrinsically resistant to SHIV-C replication.

3.6. The protected RM showed low numbers of CD4⁺α4β7⁺ T cells

Despite robust anti-Gag cellular responses after *Ad5hr-SIVgag* administration (Fig. 2A), peak viremia and AUC were not significantly lower for Group 1B RM compared to Group 2 (control) RM (Fig. 5G,I). Recently, it was reported that adenovirus vaccination leads to expansion of CD4⁺ T cells that express the α4β7 marker [50] and that such cells are potential targets for HIV-1 replication in vivo [51, 52]. To test whether adenovirus vaccination induced an expansion of α4β7-expressing CD4⁺ T cells, PBMC of Group 1B vaccinees were analyzed at various time points after adenovirus administration (Fig. 7A). Although no significant changes were seen, the protected animal (RQe-10) showed a low number of α4β7-expressing CD4⁺ T cells at all time points (Fig. 7A). It would have been interesting to determine α4β7 expression on CD4⁺ T cells of Group 1B RM from other tissue compartments, such as rectal mucosa, but such specimens were not available. Because of the repeated low-dose SHIV-C i.r. challenges, rectal tissue specimens from the Group 1B RM could not be collected during the course of the viral challenges. Rectal biopsies were only collected later from RQe-10 and five other unrelated, naïve animals. Although there was considerable variation in the number of α4β7-expressing CD4⁺ T cells from rectal mucosa (from 4.8% to 24.5%), RM RQe-10 again showed the lowest number of CD4⁺α4β7⁺ T cells (Fig. 7B).

4. Discussion

Here, we showed that 1) the novel *Listeria prime/rAd5* boost strategy was safe and immunogenic; 2) the anti-Gag cellular responses, measured by IFN-γ ELISPOT assay, were high but short-lived; 3) additional boosting with recombinant HIV-1 gp160 protein

generated nAb responses; 4) the bimodal vaccine strategy prevented SHIV-C acquisition in one vaccinee and led to low peak viremia in another; and 5) when all vaccinees were compared to the expanded control group of 17 RM, there was a trend toward lower peak viremia in the vaccinees compared to controls.

In an earlier study performed in a murine model, a prime-boost strategy employing recombinant live Lm and replication-defective Ad5 vector by various routes produced strong SIV Gag-specific cellular responses and protected the mice against mucosal challenge with recombinant vaccinia virus (rVV) expressing HIV-1 *gag* [11]. We tested this strategy in RM using the newly developed Lm vector, *Lmdd-BdopSIVgag*, and replication-competent *Ad5hr-SIVgag*. Compared to the mouse study, our findings in the RM model showed only partial protection against SHIV-1157ipEL-p challenge and were less impressive than those in the mouse study. However, the rVV-based challenge system in mice does not involve a lentivirus. Our experience with lower immunogenicity and efficacy in primates compared to the mouse model does not stand alone since a number of vaccine approaches, most notably DNA vaccination, have faced similar discrepancies in immunogenicity/efficacy when translating a mouse-tested concept to primates/humans (reviewed in [53]).

Despite setbacks, Ad remains an attractive vector for vaccine development [54]. Replicating Ad vectors encoding HIV-1 genes were more immunogenic than their non-replicating counterparts in chimpanzees [55]. Priming with replicating Ad expressing HIV-1 *env/rev* followed by boosting with HIV-1 Env protein protected chimpanzees against a nonpathogenic HIV-1 strain (reviewed in [15]). However, the replicating Ad strategy could not be tested against a virulent strain of HIV-1 since chimpanzees are endangered. Testing replicating Ad as vaccine vectors in RM became possible after identification of a host range mutant of Ad5 (Ad5hr) that replicated in monkey cells [9]. Subsequently, replication-competent Ad5hr constructs encoding SIV *env*, *gag* or *nef* genes were tested in the RM/SIV model and were found to control SIV replication (reviewed in [15]). Similarly, priming with replication-competent Ad5hr encoding HIV_{89.6P} *env* or HIV_{III B} *tat* followed by boosting with the respective proteins significantly reduced peak as well as set point viremia in RM after homologous SHIV_{89.6p} challenge [56]. Although Ad5hr vectors encoding lentiviral genes have been employed to prime antiviral immune responses in several studies, we used *Ad5hr-SIVgag* to boost Gag-specific responses in RM that were primed with *Lmdd-BdopSIVgag*. Our strategy provided strong boosting of Gag-specific IFN- γ secreting cells but the responses were short-lived. This is in agreement with the observation that replication of Ad5hr in RM is of relatively short duration [57].

Oligomeric HIV-1 gp140 or gp160 has been used as immunogens by many investigators (reviewed in [58]). Immunization of macaques with V2-deleted HIV_{SF162} gp140 trimers induced nAbs and protected 12 of 12 monkeys against mucosal challenge with the tier 1 SHIV_{SF162P4} [59] however, the challenge virus exactly matched the immunogen and thus is not reflective of a real-life situation, in which human AIDS vaccine recipients will be exposed to HIV-1 strains that do not match their vaccine. Emerging data indicate that the configuration of the HIV-C envelope from recently transmitted viruses differs significantly from late-stage HIV-C Envs [46, 60]. In the context of HIV-1 infection, clade C Envs are also more immunogenic and more likely to induce cross-neutralizing Abs than HIV-1 clade B Envs [61]. In this current study, we therefore immunized RM with multimeric gp160 protein derived from a recently transmitted HIV-C strain and challenged the animals with a SHIV-C encoding a heterologous HIV clade C *env*. Our strategy induced nAbs in most RM and >90% neutralization of the challenge virus was achieved by sera from two vaccinees.

After SHIV-C challenge, all five controls of Group 2 became viremic, whereas one out of ten vaccinees (RQe-10) remained aviremic and another one (RWy-9) controlled virus

replication with peak viremia <35,000 vRNA copies/ml (partial protection). Out of an additional 12 control RM given the identical challenge virus in the same dose-regimen, 11 became viremic. The control animal that remained aviremic can be considered as representative of uneventful mucosal virus exposures. Whether the same explanation applies to the aviremic status of RQe-10, rather than protection resulting from vaccine-induced immune responses, is unclear. Both RQe-10 and RWy-9 were the only two animals that showed >90% neutralization of the challenge virus and also showed strong SIV Gag-specific proliferation of CD8⁺ T cells on the day of first virus challenge. RQe-10 showed a high number of SIV Gag-specific TNF- α -producing effector memory CD4⁺ T cells, whereas RWy-9 showed the highest numbers of IFN- γ -secreting cells on the day of first virus exposure. Thus, relatively strong vaccine-induced immune responses were present only in the two protected RM. Of note, in the persistently aviremic vaccinee RQe-10, no boosting of IFN- γ ELISPOT reactivity was seen during and after the five low-dose SHIV-C challenges (data not shown). Since both RM had challenge virus-specific nAb responses, we believe that nAbs may have contributed to their protection.

Although our functional assays of humoral immunity focused on neutralizing activity, other antibody effector functions, such as antibody-dependent cellular cytotoxicity (ADCC) and antibody-dependent cell-mediated viral inhibition (ADCVI), can also contribute to protection, as described by other investigators [62-65]. Analysis of such additional antibody effector functions in our study is complicated by the challenge virus-specific neutralizing activity shown by both protected vaccinees. Recently, it has been reported that mucosal IgAs that inhibit HIV-1 transcytosis and mucosal IgGs with neutralizing and/or ADCC activities (in the absence of serum nAb) protected RM against vaginal SHIV challenge [66]. Unfortunately, mucosal secretions were not available after the gp160 boosts for assay of functional anti-Env activities.

Genetic factors can also contribute to protection against viral infections. Individuals homozygous for deletions in CCR5 show resistance to HIV-1 acquisition [67]. Among our RM vaccinees, the protected animal RQe-10 was positive for the Mamu A*001 and B08 alleles, both of which have been associated with slower SIV disease progression and a tendency for strong cellular immune responses to lentiviral antigens [68]. The RM also displayed a moderately resistant TRIM5 α genotype (TRIM TFP/TRIM Q). Of note, four of five control RM of Group 2 had the same, whereas the fifth control RM had a more resistant TRIM5 α genotype.

In addition, integrin $\alpha 4\beta 7$ plays an important role in the initial stages of HIV-1 acquisition. Integrin $\alpha 4\beta 7$ is closely associated with CD4 and CCR5 and interacts specifically with a conserved tripeptide in the V2-loop of HIV-1 Env ([51, 52] reviewed in [69]); since $\alpha 4\beta 7$ is ~3 times longer than CD4, it may efficiently capture virions. Blocking $\alpha 4\beta 7$ during acute infection led to significantly lower viremia in SIV-challenged RM [70]. These data imply that $\alpha 4\beta 7$ likely facilitates lentiviral infection in vivo, and one may postulate that the relative frequency of CD4⁺ $\alpha 4\beta 7$ ⁺ T cells affects the susceptibility of animals to lentiviral infection. Despite comparable levels of virus-specific immune responses in vaccinees RQe-10 and RWy-9, RQe-10 with persistently low numbers of CD4⁺ $\alpha 4\beta 7$ ⁺ T cells remained aviremic, whereas RWy-9 was only partially protected. It should be noted that mitogen-stimulated cultured PBMC of RQe-10 supported SHIV-1157ipEL-p replication. This is in agreement with the earlier notion that integrin $\alpha 4\beta 7$ is not required for viral replication in cultured cells (reviewed in [69]). Challenge virus-specific nAbs and low numbers of potential CD4⁺ $\alpha 4\beta 7$ ⁺ target cells likely provided the basis for the apparent uneventful mucosal virus exposures to RQe-10.

When peak plasma viremia levels of all 10 vaccinees (Groups 1A and 1B) were compared to the expanded group of control animals (Group 2 + 12 additional controls), the vaccinees tended to show lower peak plasma viremic ($P = 0.067$). However, peak plasma viremia levels of the vaccinees (Groups 1A and 1B) were not significantly different from those of the Group 2 controls. An expanded group size would have increased the statistical power of this study.

To summarize, mucosal priming with live attenuated Lm followed by mucosal boosting with an Ad5Hr vector and trimeric gp160 was safe and immunogenic and showed some efficacy against repeated mucosal challenges with SHIV-C encoding heterologous *env*. Persistently low levels of CD4⁺α4β7⁺ T cells may also have contributed to the complete protection seen in one vaccinee. A vaccination strategy that induces virus-specific cellular as well as nAb responses warrants further investigation. Genetic factors that can influence host susceptibility should also be taken into account while determining vaccine efficacy.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank Julie Johnson-McGrath for assistance with the preparation of this manuscript, Stephanie Ehnert for the coordination of the primate studies, and Dr. Richard Raybourne (formerly of the Food and Drug Administration) for assistance and protocols to assess anti-Lm immune responses. We thank Stefan Kostense, Crucell Holland B.V., for providing the Ad5-luciferase construct. We thank the NIH AIDS Research and Reference Reagent Program for reagents. This study was supported by NIH grants P01 AI054558 to FF, FJN, RMR and JL and P01 AI048240 to RMR, RR-00165 to the YNPRC, RR00168 to the NEPRC, and by Center for AIDS Research Immunology Core grants, P30 AI060354 and P30 AI 050409, and in part by the Intramural Research Program of the NIH, National Cancer Institute.

References

- [1]. Demberg T, Robert-Guroff M. Mucosal immunity and protection against HIV/SIV infection: strategies and challenges for vaccine design. *Int Rev Immunol.* 2009; 28(1):20–48. [PubMed: 19241252]
- [2]. Yu M, Vajdy M. Mucosal HIV transmission and vaccination strategies through oral compared with vaginal and rectal routes. *Expert Opin Biol Ther.* Aug; 2010 10(8):1181–95. [PubMed: 20624114]
- [3]. Duerr A. Update on mucosal HIV vaccine vectors. *Curr Opin HIV AIDS.* Sep; 2010 5(5):397–403. [PubMed: 20978380]
- [4]. McElrath MJ, Haynes BF. Induction of immunity to human immunodeficiency virus type-1 by vaccination. *Immunity.* Oct 29; 2010 33(4):542–54. [PubMed: 21029964]
- [5]. Liu MA. Immunologic basis of vaccine vectors. *Immunity.* Oct 29; 2010 33(4):504–15. [PubMed: 21029961]
- [6]. Morgan C, Marthas M, Miller C, Duerr A, Cheng-Mayer C, Desrosiers R, et al. The use of nonhuman primate models in HIV vaccine development. *PLoS Med.* Aug 12.2008 5(8):e173. [PubMed: 18700814]
- [7]. Robert-Guroff M. Replicating and non-replicating viral vectors for vaccine development. *Curr Opin Biotechnol.* Dec; 2007 18(6):546–56. [PubMed: 18063357]
- [8]. Hansen SG, Vieville C, Whizin N, Coyne-Johnson L, Siess DC, Drummond DD, et al. Effector memory T cell responses are associated with protection of rhesus monkeys from mucosal simian immunodeficiency virus challenge. *Nat Med.* Mar; 2009 15(3):293–9. [PubMed: 19219024]
- [9]. Cheng SM, Lee SG, Ronchetti-Blume M, Virk KP, Mizutani S, Eichberg JW, et al. Coexpression of the simian immunodeficiency virus Env and Rev proteins by a recombinant human adenovirus host range mutant. *J Virol.* Nov; 1992 66(11):6721–7. [PubMed: 1404612]

- [10]. Sciaranghella G, Lakhashe SK, Ayash-Rashkovsky M, Mirshahidi S, Siddappa NB, Novembre FJ, et al. A live attenuated *Listeria monocytogenes* vaccine vector expressing SIV Gag is safe and immunogenic in macaques and can be administered repeatedly. *Vaccine*. Nov 9.2010
- [11]. Li Z, Zhang M, Zhou C, Zhao X, Iijima N, Frankel FR. Novel vaccination protocol with two live mucosal vectors elicits strong cell-mediated immunity in the vagina and protects against vaginal virus challenge. *J Immunol*. Feb 15; 2008 180(4):2504–13. [PubMed: 18250460]
- [12]. Li Z, Zhao X, Zhou C, Gu B, Frankel FR. A truncated *Bacillus subtilis* dal gene with a 3' *ssrA* gene tag regulates the growth and virulence of racemase-deficient *Listeria monocytogenes*. *Microbiology*. Oct; 2006 152(Pt 10):3091–102. [PubMed: 17005988]
- [13]. Zhao J, Lou Y, Pinczewski J, Malkevitch N, Aldrich K, Kalyanaraman VS, et al. Boosting of SIV-specific immune responses in rhesus macaques by repeated administration of Ad5hr-SIVenv/rev and Ad5hr-SIVgag recombinants. *Vaccine*. Sep 8; 2003 21(25-26):4022–35. [PubMed: 12922139]
- [14]. Lieberman J, Frankel FR. Engineered *Listeria monocytogenes* as an AIDS vaccine. *Vaccine*. May 6; 2002 20(15):2007–10. [PubMed: 11983264]
- [15]. Patterson LJ, Robert-Guroff M. Replicating adenovirus vector prime/protein boost strategies for HIV vaccine development. *Expert Opin Biol Ther*. Sep; 2008 8(9):1347–63. [PubMed: 18694354]
- [16]. Singh R, Paterson Y. *Listeria monocytogenes* as a vector for tumor-associated antigens for cancer immunotherapy. *Expert Rev Vaccines*. Aug; 2006 5(4):541–52. [PubMed: 16989634]
- [17]. Wallecha A, Maciag PC, Rivera S, Paterson Y, Shahabi V. Construction and characterization of an attenuated *Listeria monocytogenes* strain for clinical use in cancer immunotherapy. *Clin Vaccine Immunol*. Jan; 2009 16(1):96–103. [PubMed: 19020110]
- [18]. Shahabi V, Reyes-Reyes M, Wallecha A, Rivera S, Paterson Y, Maciag P. Development of a *Listeria monocytogenes* based vaccine against prostate cancer. *Cancer Immunol Immunother*. Sep; 2008 57(9):1301–13. [PubMed: 18273616]
- [19]. Peng X, Hussain SF, Paterson Y. The ability of two *Listeria monocytogenes* vaccines targeting human papillomavirus-16 E7 to induce an antitumor response correlates with myeloid dendritic cell function. *J Immunol*. May 15; 2004 172(10):6030–8. [PubMed: 15128786]
- [20]. Sewell DA, Pan ZK, Paterson Y. *Listeria*-based HPV-16 E7 vaccines limit autochthonous tumor growth in a transgenic mouse model for HPV-16 transformed tumors. *Vaccine*. Sep 26; 2008 26(41):5315–20. [PubMed: 18680778]
- [21]. Kim SH, Castro F, Paterson Y, Gravekamp C. High efficacy of a *Listeria*-based vaccine against metastatic breast cancer reveals a dual mode of action. *Cancer Res*. Jul 15; 2009 69(14):5860–6. [PubMed: 19584282]
- [22]. Maciag PC, Radulovic S, Rothman J. The first clinical use of a live-attenuated *Listeria monocytogenes* vaccine: a Phase I safety study of Lm-LLO-E7 in patients with advanced carcinoma of the cervix. *Vaccine*. Jun 19; 2009 27(30):3975–83. [PubMed: 19389451]
- [23]. Zhao X, Zhang M, Li Z, Frankel FR. Vaginal protection and immunity after oral immunization of mice with a novel vaccine strain of *Listeria monocytogenes* expressing human immunodeficiency virus type 1 gag. *J Virol*. Sep; 2006 80(18):8880–90. [PubMed: 16940500]
- [24]. Stevens R, Howard KE, Nordone S, Burkhard M, Dean GA. Oral immunization with recombinant *Listeria monocytogenes* controls virus load after vaginal challenge with feline immunodeficiency virus. *J Virol*. Aug; 2004 78(15):8210–8. [PubMed: 15254192]
- [25]. Top FH Jr, Grossman RA, Bartelloni PJ, Segal HE, Dudding BA, Russell PK, et al. Immunization with live types 7 and 4 adenovirus vaccines. I. Safety, infectivity, antigenicity, and potency of adenovirus type 7 vaccine in humans. *J Infect Dis*. Aug; 1971 124(2):148–54. [PubMed: 4330997]
- [26]. Top FH Jr, Buescher EL, Bancroft WH, Russell PK. Immunization with live types 7 and 4 adenovirus vaccines. II. Antibody response and protective effect against acute respiratory disease due to adenovirus type 7. *J Infect Dis*. Aug; 1971 124(2):155–60. [PubMed: 4330998]
- [27]. Peiperl L, Morgan C, Moodie Z, Li H, Russell N, Graham BS, et al. Safety and immunogenicity of a replication-defective adenovirus type 5 HIV vaccine in Ad5-seronegative persons: a randomized clinical trial (HVTN 054). *PLoS One*. 2010; 5(10):e13579. [PubMed: 21048953]

- [28]. Migueles SA, Rood JE, Berkley AM, Guo T, Mendoza D, Patamawenu A, et al. Trivalent Adenovirus Type 5 HIV Recombinant Vaccine Primes for Modest Cytotoxic Capacity That Is Greatest in Humans with Protective HLA Class I Alleles. *PLoS Pathog.* Feb.2011 7(2):e1002002. [PubMed: 21383976]
- [29]. Hutnick NA, Carnathan DG, Dubey SA, Cox KS, Kierstead L, Makadonas G, et al. Vaccination with Ad5 vectors expands Ad5-specific CD8 T cells without altering memory phenotype or functionality. *PLoS One.* 2010; 5(12):e14385. [PubMed: 21203546]
- [30]. Fitzgerald DW, Janes H, Robertson M, Coombs R, Frank I, Gilbert P, et al. An Ad5-vectored HIV-1 vaccine elicits cell-mediated immunity but does not affect disease progression in HIV-1-infected male subjects: results from a randomized placebo-controlled trial (the Step study). *J Infect Dis.* Mar 15; 2011 203(6):765–72. [PubMed: 21343146]
- [31]. Rerks-Ngarm S, Pitisuttithum P, Nitayaphan S, Kaewkungwal J, Chiu J, Paris R, et al. Vaccination with ALVAC and AIDSVAX to prevent HIV-1 infection in Thailand. *N Engl J Med.* Dec 3; 2009 361(23):2209–20. [PubMed: 19843557]
- [32]. Gavioli R, Cellini S, Castaldello A, Voltan R, Gallerani E, Gagliardini F, et al. The Tat protein broadens T cell responses directed to the HIV-1 antigens Gag and Env: implications for the design of new vaccination strategies against AIDS. *Vaccine.* Jan 30; 2008 26(5):727–37. [PubMed: 18096278]
- [33]. Siddappa NB, Watkins JD, Wassermann KJ, Song R, Wang W, Kramer VG, et al. R5 clade C SHIV strains with tier 1 or 2 neutralization sensitivity: tools to dissect env evolution and to develop AIDS vaccines in primate models. *PLoS One.* 2010; 5(7):e11689. [PubMed: 20657739]
- [34]. Schwartz S, Campbell M, Nasioulas G, Harrison J, Felber BK, Pavlakis GN. Mutational inactivation of an inhibitory sequence in human immunodeficiency virus type 1 results in Rev-independent gag expression. *J Virol.* Dec; 1992 66(12):7176–82. [PubMed: 1433510]
- [35]. Schneider R, Campbell M, Nasioulas G, Felber BK, Pavlakis GN. Inactivation of the human immunodeficiency virus type 1 inhibitory elements allows Rev-independent expression of Gag and Gag/protease and particle formation. *J Virol.* Jul; 1997 71(7):4892–903. [PubMed: 9188551]
- [36]. Grisson RD, Chenine AL, Yeh LY, He J, Wood C, Bhat GJ, et al. Infectious molecular clone of a recently transmitted pediatric human immunodeficiency virus clade C isolate from Africa: evidence of intraclade recombination. *J Virol.* Dec; 2004 78(24):14066–9. [PubMed: 15564517]
- [37]. Rasmussen RA, Ong H, Kittel C, Ruprecht CR, Ferrantelli F, Hu SL, et al. DNA prime/protein boost immunization against HIV clade C: safety and immunogenicity in mice. *Vaccine.* Mar 20; 2006 24(13):2324–32. [PubMed: 16406147]
- [38]. Humbert M, Rasmussen RA, Song R, Ong H, Sharma P, Chenine AL, et al. SHIV-1157i and passaged progeny viruses encoding R5 HIV-1 clade C env cause AIDS in rhesus monkeys. *Retrovirology.* 2008; 5:94. [PubMed: 18928523]
- [39]. Song RJ, Chenine AL, Rasmussen RA, Ruprecht CR, Mirshahidi S, Grisson RD, et al. Molecularly cloned SHIV-1157ipd3N4: a highly replication-competent, mucosally transmissible R5 simian-human immunodeficiency virus encoding HIV clade C Env. *J Virol.* Sep; 2006 80(17):8729–38. [PubMed: 16912320]
- [40]. Hofmann-Lehmann R, Swenerton RK, Liska V, Leutenegger CM, Lutz H, McClure HM, et al. Sensitive and robust one-tube real-time reverse transcriptase-polymerase chain reaction to quantify SIV RNA load: comparison of one-versus two-enzyme systems. *AIDS Res Hum Retroviruses.* Sep 1; 2000 16(13):1247–57. [PubMed: 10957722]
- [41]. Cline AN, Bess JW, Piatak M Jr, Lifson JD. Highly sensitive SIV plasma viral load assay: practical considerations, realistic performance expectations, and application to reverse engineering of vaccines for AIDS. *J Med Primatol.* Oct; 2005 34(5-6):303–12. [PubMed: 16128925]
- [42]. Rasmussen RA, Ong H, Song R, Chenine AL, Ayash-Rashkovsky M, Hu SL, et al. Efficacy of a multigenic protein vaccine containing multimeric HIV gp160 against heterologous SHIV clade C challenges. *Aids.* Sep 12; 2007 21(14):1841–8. [PubMed: 17721091]
- [43]. Polonis VR, Brown BK, Rosa Borges A, Zolla-Pazner S, Dimitrov DS, Zhang MY, et al. Recent advances in the characterization of HIV-1 neutralization assays for standardized evaluation of the antibody response to infection and vaccination. *Virology.* Jun 5; 2008 375(2):315–20. [PubMed: 18367229]

- [44]. Siddappa NB, Song R, Kramer VG, Chenine AL, Velu V, Ong H, et al. Neutralization-sensitive R5-tropic simian-human immunodeficiency virus SHIV-2873Nip, which carries env isolated from an infant with a recent HIV clade C infection. *J Virol.* Feb; 2009 83(3):1422–32. [PubMed: 19019970]
- [45]. Balfe P, Shapiro S, Hsu M, Buckner C, Harouse JM, Cheng-Mayer C. Expansion of quasispecies diversity but no evidence for adaptive evolution of SHIV during rapid serial transfers among seronegative macaques. *Virology.* Jan 5; 2004 318(1):267–79. [PubMed: 14972553]
- [46]. Li M, Salazar-Gonzalez JF, Derdeyn CA, Morris L, Williamson C, Robinson JE, et al. Genetic and neutralization properties of subtype C human immunodeficiency virus type 1 molecular env clones from acute and early heterosexually acquired infections in Southern Africa. *J Virol.* Dec; 2006 80(23):11776–90. [PubMed: 16971434]
- [47]. Wang X, Xu H, Gill AF, Pahar B, Kempf D, Rasmussen T, et al. Monitoring alpha4beta7 integrin expression on circulating CD4+ T cells as a surrogate marker for tracking intestinal CD4+ T-cell loss in SIV infection. *Mucosal Immunol.* Nov; 2009 2(6):518–26. [PubMed: 19710637]
- [48]. Sprangers MC, Lakhai W, Koudstaal W, Verhoeven M, Koel BF, Vogels R, et al. Quantifying adenovirus-neutralizing antibodies by luciferase transgene detection: addressing preexisting immunity to vaccine and gene therapy vectors. *J Clin Microbiol.* Nov; 2003 41(11):5046–52. [PubMed: 14605137]
- [49]. Jiang S, Rasmussen RA, Nolan KM, Frankel FR, Lieberman J, McClure HM, et al. Live attenuated *Listeria monocytogenes* expressing HIV Gag: immunogenicity in rhesus monkeys. *Vaccine.* Oct 16; 2007 25(42):7470–9. [PubMed: 17854955]
- [50]. Benlahrech A, Harris J, Meiser A, Papagatsias T, Hornig J, Hayes P, et al. Adenovirus vector vaccination induces expansion of memory CD4 T cells with a mucosal homing phenotype that are readily susceptible to HIV-1. *Proc Natl Acad Sci U S A.* Nov 24; 2009 106(47):19940–5. [PubMed: 19918060]
- [51]. Cicala C, Martinelli E, McNally JP, Goode DJ, Gopaul R, Hiatt J, et al. The integrin alpha4beta7 forms a complex with cell-surface CD4 and defines a T-cell subset that is highly susceptible to infection by HIV-1. *Proc Natl Acad Sci U S A.* Dec 8; 2009 106(49):20877–82. [PubMed: 19933330]
- [52]. Arthos J, Cicala C, Martinelli E, Macleod K, Van Ryk D, Wei D, et al. HIV-1 envelope protein binds to and signals through integrin alpha4beta7, the gut mucosal homing receptor for peripheral T cells. *Nat Immunol.* Mar; 2008 9(3):301–9. [PubMed: 18264102]
- [53]. Kutzler MA, Weiner DB. DNA vaccines: ready for prime time? *Nat Rev Genet.* Oct; 2008 9(10):776–88. [PubMed: 18781156]
- [54]. Lasaro MO, Ertl HC. New insights on adenovirus as vaccine vectors. *Mol Ther.* Aug; 2009 17(8):1333–9. [PubMed: 19513019]
- [55]. Peng B, Wang LR, Gomez-Roman VR, Davis-Warren A, Montefiori DC, Kalyanaraman VS, et al. Replicating rather than nonreplicating adenovirus-human immunodeficiency virus recombinant vaccines are better at eliciting potent cellular immunity and priming high-titer antibodies. *J Virol.* Aug; 2005 79(16):10200–9. [PubMed: 16051813]
- [56]. Demberg T, Florese RH, Heath MJ, Larsen K, Kalisz I, Kalyanaraman VS, et al. A replication-competent adenovirus-human immunodeficiency virus (Ad-HIV) tat and Ad-HIV env priming/Tat and envelope protein boosting regimen elicits enhanced protective efficacy against simian/human immunodeficiency virus SHIV89.6P challenge in rhesus macaques. *J Virol.* Apr; 2007 81(7):3414–27. [PubMed: 17229693]
- [57]. Buge SL, Richardson E, Alipanah S, Markham P, Cheng S, Kalyan N, et al. An adenovirus-simian immunodeficiency virus env vaccine elicits humoral, cellular, and mucosal immune responses in rhesus macaques and decreases viral burden following vaginal challenge. *J Virol.* Nov; 1997 71(11):8531–41. [PubMed: 9343211]
- [58]. Forsell MN, Schief WR, Wyatt RT. Immunogenicity of HIV-1 envelope glycoprotein oligomers. *Curr Opin HIV AIDS.* Sep; 2009 4(5):380–7. [PubMed: 20048701]
- [59]. Barnett SW, Srivastava IK, Kan E, Zhou F, Goodsell A, Cristillo AD, et al. Protection of macaques against vaginal SHIV challenge by systemic or mucosal and systemic vaccinations with HIV-envelope. *AIDS.* Jan 30; 2008 22(3):339–48. [PubMed: 18195560]

- [60]. Derdeyn CA, Decker JM, Bibollet-Ruche F, Mokili JL, Muldoon M, Denham SA, et al. Envelope-constrained neutralization-sensitive HIV-1 after heterosexual transmission. *Science*. Mar 26; 2004 303(5666):2019–22. [PubMed: 15044802]
- [61]. Dreja H, O'Sullivan E, Pade C, Greene KM, Gao H, Aubin K, et al. Neutralization activity in a geographically diverse East London cohort of human immunodeficiency virus type 1-infected patients: clade C infection results in a stronger and broader humoral immune response than clade B infection. *J Gen Virol*. Nov; 2010 91(Pt 11):2794–803. [PubMed: 20685933]
- [62]. Xiao P, Zhao J, Patterson LJ, Brocca-Cofano E, Venzon D, Kozlowski PA, et al. Multiple vaccine-elicited nonneutralizing anti-envelope antibody activities contribute to protective efficacy by reducing both acute and chronic viremia following simian/human immunodeficiency virus SHIV89.6P challenge in rhesus macaques. *J Virol*. Jul; 2010 84(14):7161–73. [PubMed: 20444898]
- [63]. Florese RH, Demberg T, Xiao P, Kuller L, Larsen K, Summers LE, et al. Contribution of nonneutralizing vaccine-elicited antibody activities to improved protective efficacy in rhesus macaques immunized with Tat/Env compared with multigenic vaccines. *J Immunol*. Mar 15; 2009 182(6):3718–27. [PubMed: 19265150]
- [64]. Hidajat R, Xiao P, Zhou Q, Venzon D, Summers LE, Kalyanaraman VS, et al. Correlation of vaccine-elicited systemic and mucosal nonneutralizing antibody activities with reduced acute viremia following intrarectal simian immunodeficiency virus SIVmac251 challenge of rhesus macaques. *J Virol*. Jan; 2009 83(2):791–801. [PubMed: 18971271]
- [65]. Forthal DN, Moog C. Fc receptor-mediated antiviral antibodies. *Curr Opin HIV AIDS*. Sep; 2009 4(5):388–93. [PubMed: 20048702]
- [66]. Bomsel M, Tudor D, Drillet AS, Alfsen A, Ganor Y, Roger MG, et al. Immunization with HIV-1 gp41 subunit virosomes induces mucosal antibodies protecting nonhuman primates against vaginal SHIV challenges. *Immunity*. Feb 25; 2011 34(2):269–80. [PubMed: 21315623]
- [67]. Samson M, Libert F, Doranz BJ, Rucker J, Liesnard C, Farber CM, et al. Resistance to HIV-1 infection in caucasian individuals bearing mutant alleles of the CCR-5 chemokine receptor gene. *Nature*. Aug 22; 1996 382(6593):722–5. [PubMed: 8751444]
- [68]. Loffredo JT, Maxwell J, Qi Y, Glidden CE, Borchardt GJ, Soma T, et al. Mamu-B*08-positive macaques control simian immunodeficiency virus replication. *J Virol*. Aug; 2007 81(16):8827–32. [PubMed: 17537848]
- [69]. Cicala C, Arthos J, Fauci AS. HIV-1 envelope, integrins and co-receptor use in mucosal transmission of HIV. *J Transl Med*. 2011; 9(Suppl 1):S2. [PubMed: 21284901]
- [70]. Ansari AA, Reimann KA, Mayne AE, Takahashi Y, Stephenson ST, Wang R, et al. Blocking of alpha4beta7 gut-homing integrin during acute infection leads to decreased plasma and gastrointestinal tissue viral loads in simian immunodeficiency virus-infected rhesus macaques. *J Immunol*. Jan 15; 2011 186(2):1044–59. [PubMed: 21149598]

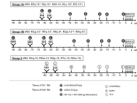


Fig. 1.

Vaccination schedule and SHIV-1157ipEL-p challenges. Group 1A RM were immunized with *Lmdd-BdopSIVgag* (L) at weeks -68 and -62, and Group 1B RM received *Lmdd-BdopSIVgag* at weeks -90, -77, -66 and -60. At weeks -90 and -77, 3×10^{12} CFU of Lm was given intragastrically (i.g.) on days 0, 2, 4 and 6. At the remaining time points, the same dose of Lm was given on days 0, 1 and 2 i.g. Both groups received two doses of *Ad5hr-SIVgag* (A) at the time points indicated. The first adenovirus dose (A) was given intranasally as well as intragastrically; the second dose was given intratracheally. These animals were also vaccinated with HIV Tat and HIV1084i gp160 proteins (P) (100 μ g each given intramuscularly (i.m.)) in IFA at the time points shown. Group 2 (control) RM were given empty vectors (Lm, adenovirus) and IFA. All animals were challenged 5x with a low dose (8,000 50% tissue culture infectious doses (TCID₅₀)) of SHIV-1157ipEL-p (SHIV-C for short) intrarectally (i.r.). The virus challenges were given at weeks 0, 1, 2, 3 and 4.

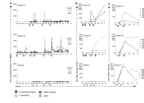


Fig. 2.

Frequency of circulating SIV Gag-specific cells, binding antibody titers against SIV Gag and neutralizing antibody titers against *Ad5hr* during vaccination. (A): The frequency of SIV Gag-specific cells was measured by IFN- γ ELISPOT assay. (B): Serial plasma dilutions were tested by ELISA to determine binding antibody titers against SIV Gag. (C): Serial plasma dilutions were tested to determine neutralizing antibody titers against *Ad5hr*. The time points of administration of *Lmdd-BdopSIVgag* or *Lmdd-Bdop* and *Ad5hr-SIVgag* or *Ad5hr* are indicated (see also Fig. 1 and legend). SFU, spot-forming units.

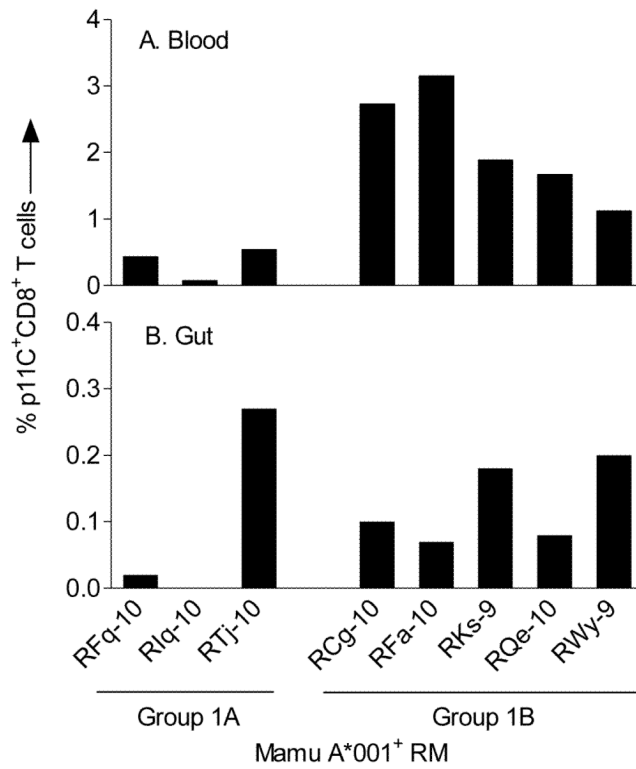


Fig. 3.

The frequency of CD3⁺CD8⁺ Gag p11C tetramer⁺ cells among Mamu A*001⁺ RM. PBMC (A) and mononuclear cells from rectal mucosa (B) obtained two weeks after administration of the second dose of *Ad5hr-SIVgag* were analyzed. Results were considered positive if they were >0.03%, a cut-off that was determined from analysis of naïve monkeys. No positive cells were detected among Mamu-A*001 negative vaccinees.

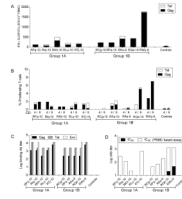


Fig. 4.

Immune responses on the day of first virus challenge. (A): Frequency of circulating SIV Gag-specific, IFN- γ -secreting cells, measured by ELISPOT assay. (B): SIV Gag- and HIV-1 Tat-specific proliferation of CD4⁺ and CD8⁺ T cells. (C): Binding antibody titers against SIVmac251 Gag, HIV Tat (IIIB) and Env (HIV-1_{CN54} gp120). The latter was used because it had been produced by the baculovirus expression system in insect cells. HIV1084i gp160, which had been used as immunogen, could not be used due to potential binding of antibodies to vaccinia virus components that may have been present in the immunogen preparation. (D): Neutralizing antibody titer against SHIV-1157ipEL-p, the challenge virus.

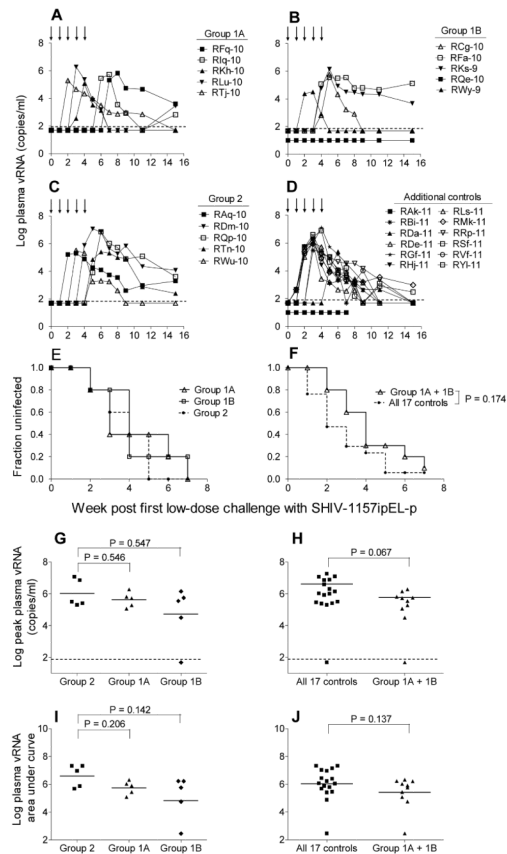


Fig. 5. Plasma viral RNA (vRNA) loads after SHIV-1157ipEL-p challenges (A to D), Kaplan-Meier plots depicting the fraction of RM remaining aviremic (E-F), comparison of peak plasma vRNA (G-H), and AUC (I-J) of vaccinees vs. controls. Five low-dose i.r. challenges (once a week, indicated by arrows) of SHIV-1157ipEL-p were given and plasma vRNA levels were monitored. Additional controls consisted of animals challenged i.r. with the same virus stock and dose as part of another study. Horizontal dashed line in A-D, the lower limit of plasma vRNA detection (50 RNA copies/ml); horizontal solid lines in G-J, mean peak plasma vRNA or mean AUC.

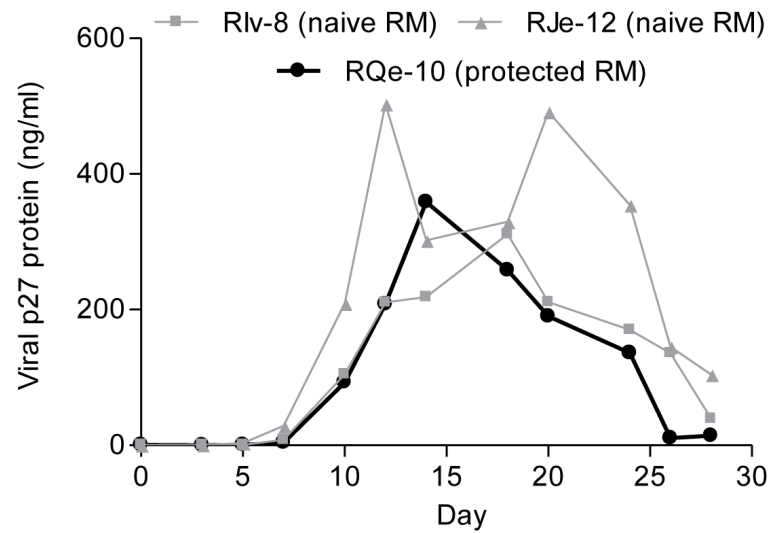


Fig. 6. In vitro SHIV-1157ipEL-p replication in the PBMC of the protected animal, RQe-10. PBMC were stimulated with concanavalin A (5 μ g/ml) in the presence of IL-2 (10 U/ml) and were exposed to SHIV-1157ipEL-p (1×10^4 TCID₅₀). Virus replication was monitored by p27 ELISA of culture supernatants. As control, virus replication in PBMC of two naïve RM (Rlv-8 and RJe-12) was also measured.

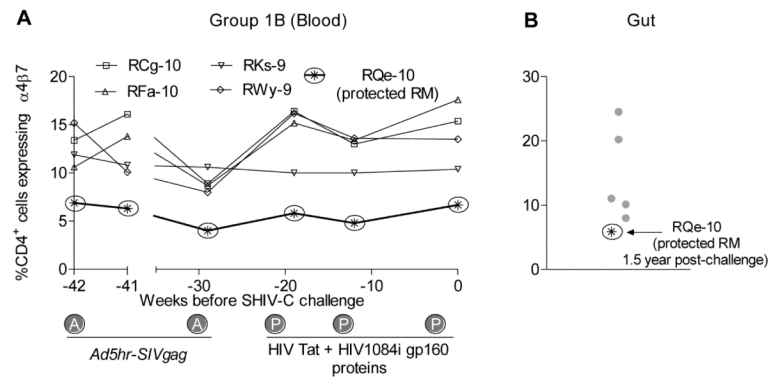


Fig. 7. The frequency of CD4⁺α4β7⁺ T cells. (A): Frequency of CD4⁺α4β7⁺ in peripheral blood of Group 1B animals during vaccination. The protected animal, RQe-10, showed a low frequency at all time points. (B): Frequency of CD4⁺α4β7⁺ T cells derived from rectal biopsies of RQe-10 and five naïve, randomly selected RM.

Table 1

Neutralizing activity of plasma samples (IC₅₀ titers) collected on the day of first virus challenge as measured by TZM-bl assay.

Group	RM	SHIV-1157ipEL-p* (tier 1, clade C)	SHIV-2873Nip (tier 2, clade C)	SHIV _{sf162p4} (tier 1, clade B)
1A	RFq-10	37	37	29
	RIq-10	31	29	21
	RKh-10	<20	<20	26
	RLu-10	22	35	29
	RTj-10	30	<20	<20
1B	RCg-10	<20	22	<20
	RFa-10	<20	21	<20
	RKs-9	<20	22	<20
	RQe-10	48	46	54
	RWy-9	62	42	22
2	RAq-10	<20	<20	<20
	RDm-10	<20	<20	<20
	RQp-10	<20	<20	<20
	RTn-10	<20	<20	<20
	RWu-10	<20	<20	<20

* Challenge virus encoding an *env* gene that was heterologous to the HIV1084i gp160 immunogen. In general, IC₅₀ titers obtained by TZM-bl assays were lower than those obtained by PBMC assays (see Fig. 4D).