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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

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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 SIVmac239 *gag*. SIV Gag-specific cellular responses were boosted by intranasal and intratracheal administration of replication-competent adenovirus (*Ad5hr-SIVgag*) 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 α4β7-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.

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

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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 μl 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 μg 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. Groups 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 Groups 1A, 1B and 2 are referred as Groups 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-κB 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 8000 50% tissue culture infectious doses ($TCID_{50}$; 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].

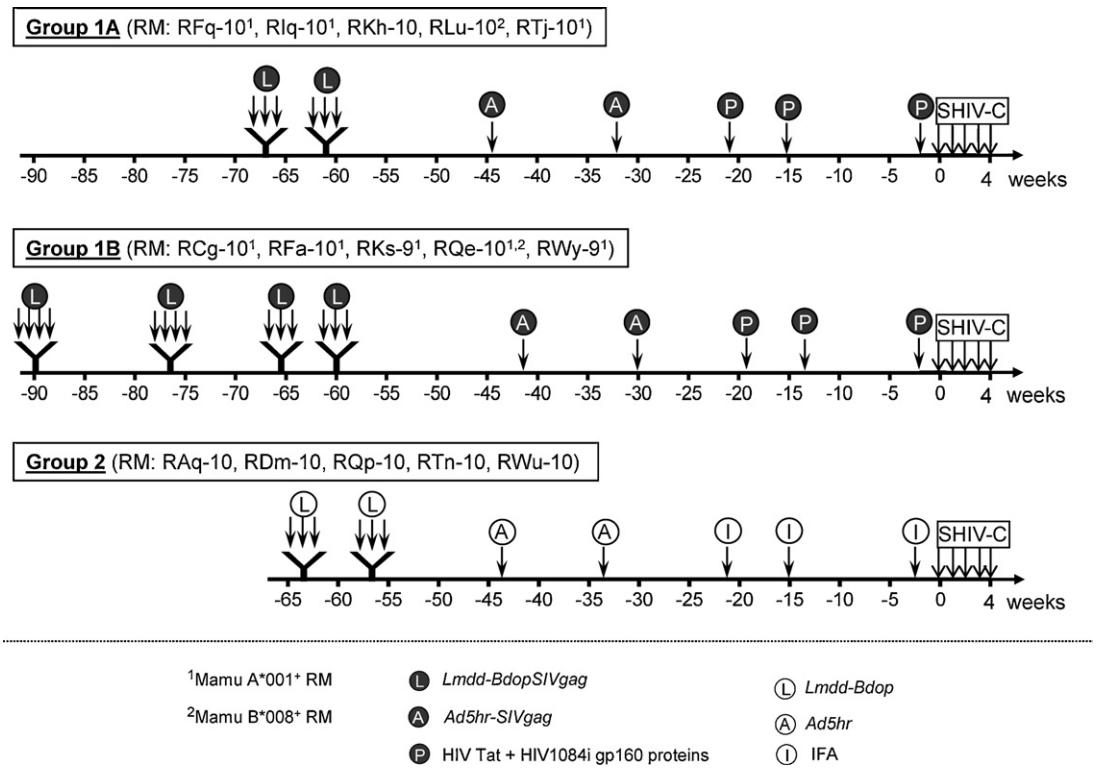

Fig. 1. Vaccination schedule and SHIV-1157ipEL-p challenges. Group 1A RM were immunized with *Lmdd-BdopSIVgag* (L) at weeks −68 and −62, and Group 1B RM received *Lmdd-BdopSIVgag* at weeks −90, −77, −66 and −60. At weeks −90 and −77, 3×10^{12} CFU of Lm was given intragastrically (i.g.) on days 0, 2, 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 μg 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 5× with a low dose (8000 50% tissue culture infectious doses ($TCID_{50}$)) of SHIV-1157ipEL-p (SHIV-C for short) intrarectally (i.r.). The virus challenges were given at weeks 0, 1, 2, 3 and 4.

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 μg/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 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 μg/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 or 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 μg/ml; ARRRP) for 5 days at 37 °C. Positive controls were stimulated with con-A (5 μg/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 (Immunodiagnostics, Inc., Woburn, MA), HIV-1 Tat (ARRRP) and HIV_{CN54} gp120 (ARRRP), diluted to 2 μg/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 phytohemaglutinin (PHA)-stimulated human PBMC were added to all wells in the presence of IL-2 (40 U/ml) and polymyxin B (15 μg/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 − [(avg. p27 in wells containing virus + plasma mixture × 100)/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 using the TZM-bl cell-based assay as described [46].

2.12. Determination of $CD4^+\alpha4\beta7^+$ T cells

Peripheral blood $CD4^+$ T cells were analyzed for expression of α4 and β7 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-β7-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 α4 and β7 markers was determined.

2.13. 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% CO_2 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 (1×10^4/100 μl) were added to each well, and the plates were incubated 24 h at 37 °C, 5% CO_2. The culture medium for all steps was F-12 Kaighn's containing 10% FBS, 50 μg/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 Victor^2 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.

2.14. 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)/10^6 PBMC), but above the pre-immunization responses (0–20 SFU/10^6 PBMC). After the second set of Lm administrations, four out of five RM showed significantly higher responses. One RM showed a peak response of 2920 SFU/10^6 PBMC within one week, whereas three animals reached peaks (280–610 SFU/10^6 PBMC) after 5 weeks (Fig. 2A).

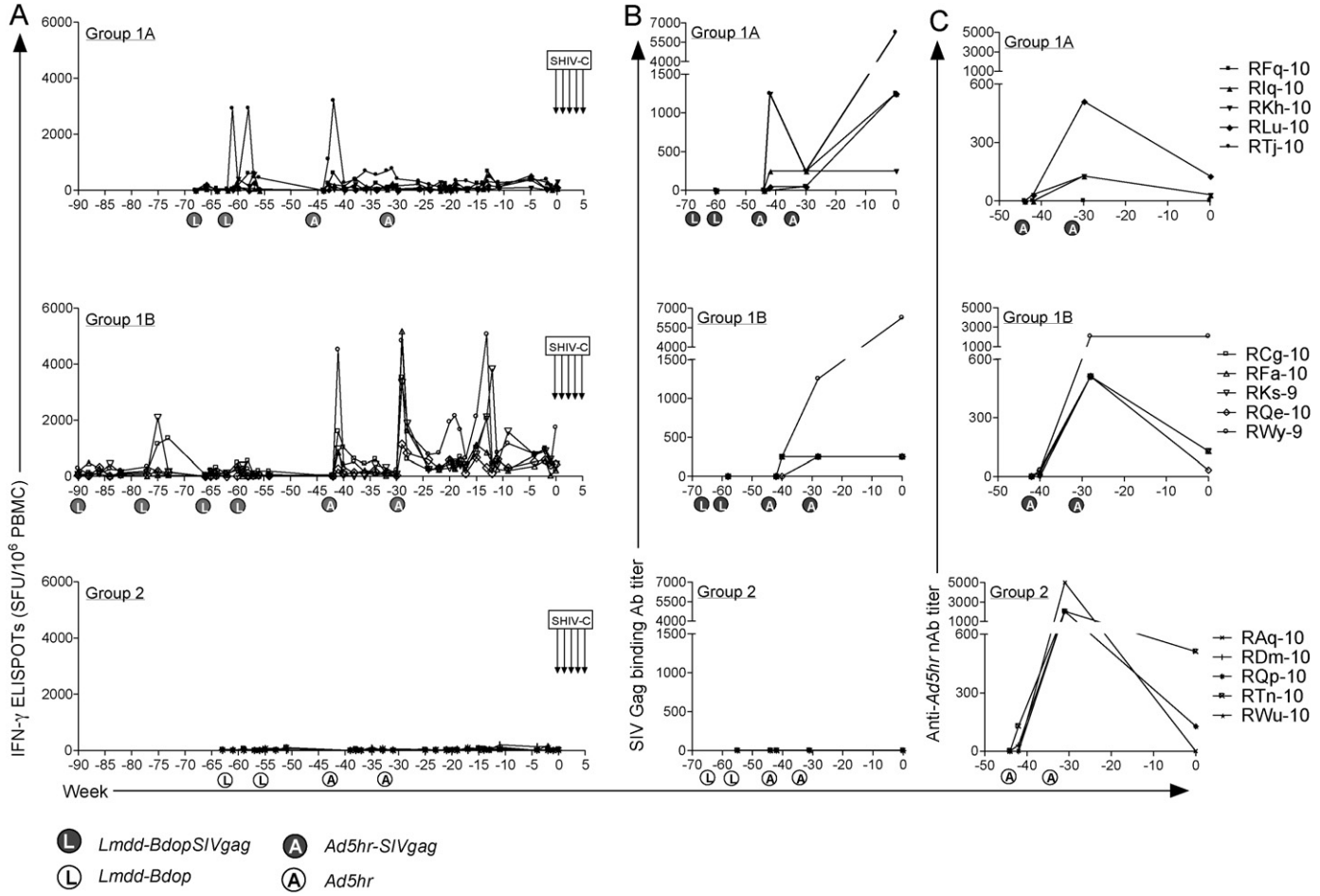

Fig. 2. Frequency of circulating SIV Gag-specific cells, binding antibody titers against SIV Gag and neutralizing antibody titers against *Ad5hr* during vaccination. (A) The frequency of SIV Gag-specific cells was measured by IFN-γ ELISPOT assay. (B) Serial plasma dilutions were tested by ELISA to determine 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.

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 (1200 and 2200 SFU/10^6 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 [49a]. 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 (0–55 SFU/10^6 PBMC). After the administration of *Ad5hr-SIVgag*, one RM from Group 1A showed a strong Gag-specific cellular response (3200 SFU/10^6 PBMC) within two weeks, whereas the other four RM showed peak responses of 140–625 SFU/10^6 PBMC between two and six weeks (Fig. 2A). All Group 1B animals showed Gag-specific cellular responses (range: 260–4510; mean ± SD: 1631 ± 1677 SFU/10^6 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: 55–690 and 70–220 SFU/10^6 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: 1130–5165 SFU/10^6 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).

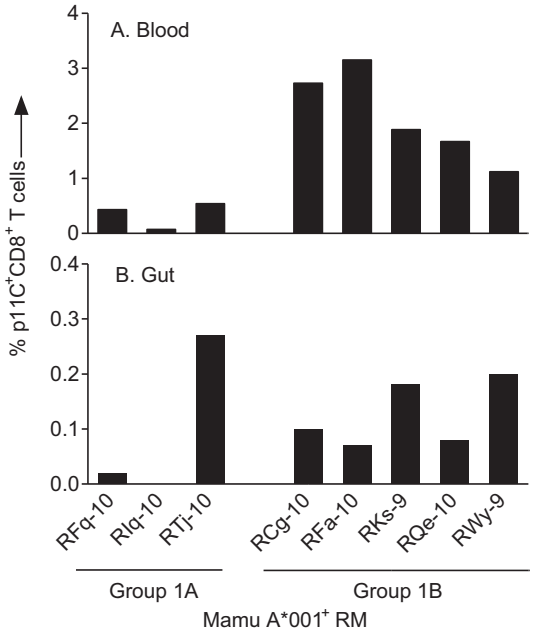

Fig. 3. The frequency of $CD3^+CD8^+$ Gag p11C $tetramer^+$ cells among Mamu $A*001^+$ RM. PBMC (A) and mononuclear cells from rectal mucosa (B) obtained two weeks after administration of the second dose of *Ad5hr-SIVgag* were analyzed. Results were considered positive if they were >0.03%, a cut-off that was determined from analysis of naïve monkeys. No positive cells were detected among Mamu-A*001 negative vaccinees.

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 Groups 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 1–3% $CD3^+CD8^+$ 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/10^6 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 (0.3–0.6%); 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/10^6 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 1730 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 IC_{90} < 1:50 and IC_{50} ~1:1200). 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 IC_{90} in the PBMC-based assay also had the highest IC_{50} 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 toward 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 and 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

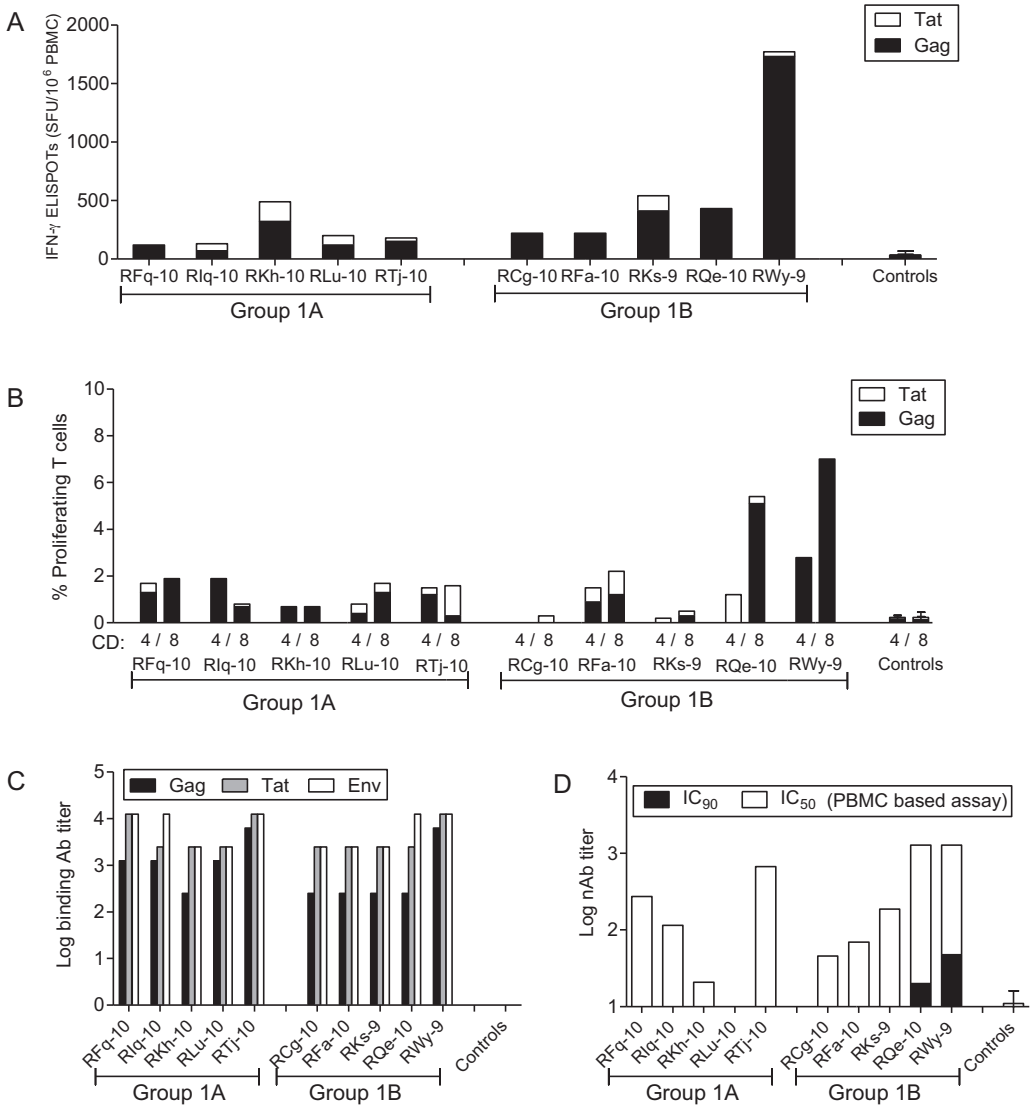

Fig. 4. Immune responses on the day of first virus challenge. (A) Frequency of circulating SIV Gag-specific, IFN-γ-secreting cells, measured by ELISPOT assay. (B) SIV Gag- and HIV-1 Tat-specific proliferation of $CD4^+$ and $CD8^+$ T cells. (C) Binding antibody titers against SIVmac251 Gag, HIV Tat (IIIB) and Env ($HIV\text{-}1_{CN54}$ 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.

Table 1
Neutralizing activity of plasma samples (IC_{50} titers) collected on the day of first virus challenge as measured by TZM-bl assay.

Group	RM	SHIV-1157ipEL-p[a] (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

[a] Challenge virus encoding an *env* gene that was heterologous to the HIV1084i gp160 immunogen. In general, IC_{50} titers obtained by TZM-bl assays were lower than those obtained by PBMC assays (see Fig. 4D).

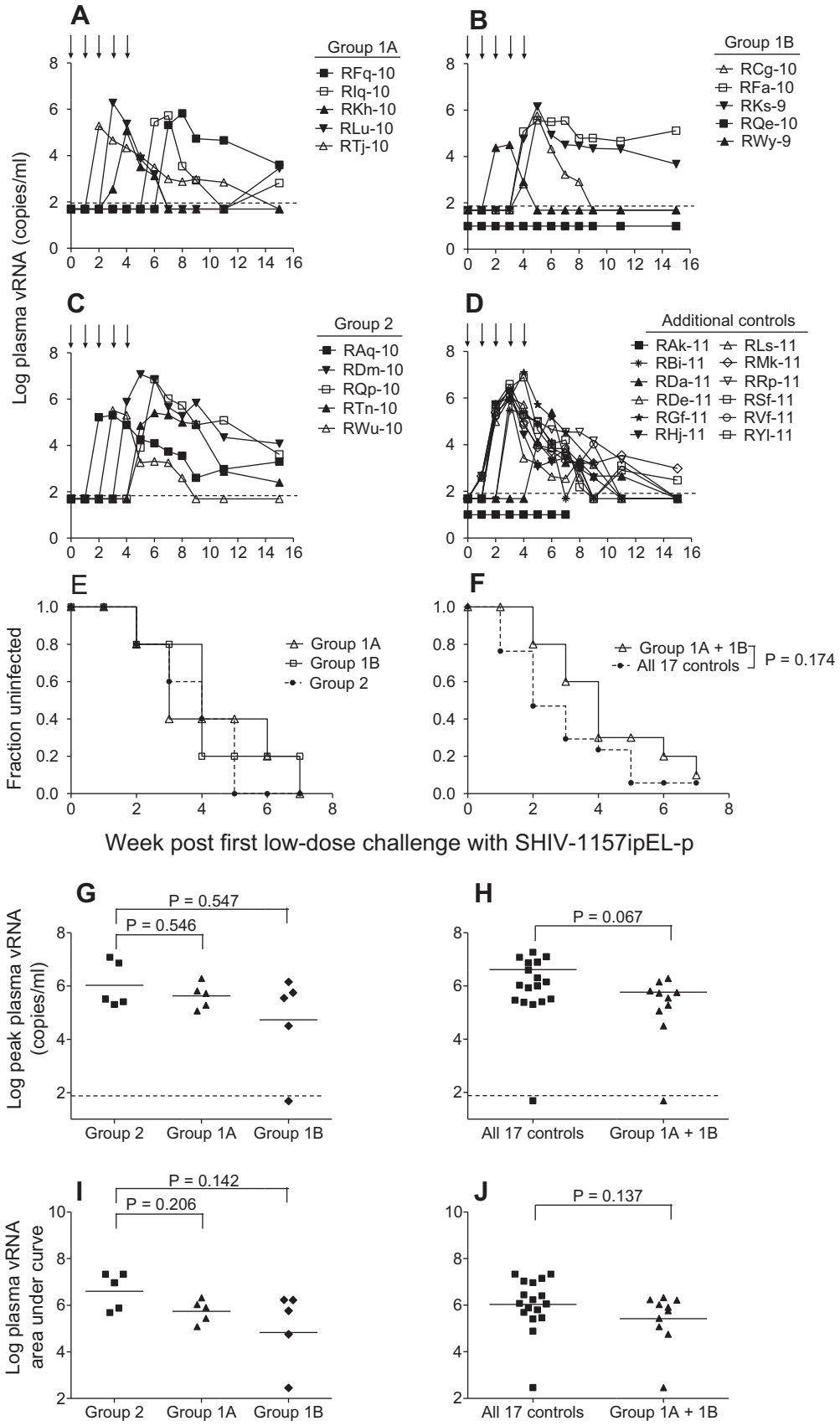

Fig. 5. Plasma viral RNA (vRNA) loads after SHIV-1157ipEL-p challenges (A–D), Kaplan–Meier plots depicting the fraction of RM remaining aviremic (E and F), comparison of peak plasma vRNA (G and H), and AUC (I and J) of vaccinees vs. controls. Five low-dose i.r. challenges (once a week, indicated by arrows) 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. 1D). At the first two time points (Supplementary Fig. 1A and 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 and 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–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 [49b] (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 and 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 ($P = 0.067$).

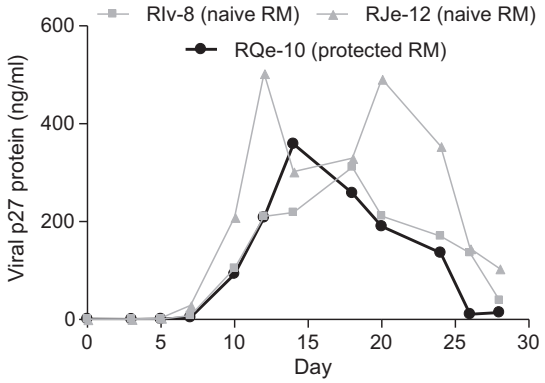

Fig. 6. In vitro SHIV-1157ipEL-p replication in the PBMC of the protected animal, RQe-10. PBMC were stimulated with concanavalin A (5 μg/ml) in the presence of IL-2 (10 U/ml) and were exposed to SHIV-1157ipEL-p (1×10^4 $TCID_{50}$). Virus replication was monitored by p27 ELISA of culture supernatants. As control, virus replication in PBMC of two naïve RM (RIv-8 and RJe-12) was also measured.

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 $CD4^+\alpha4\beta7^+$ 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 and 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

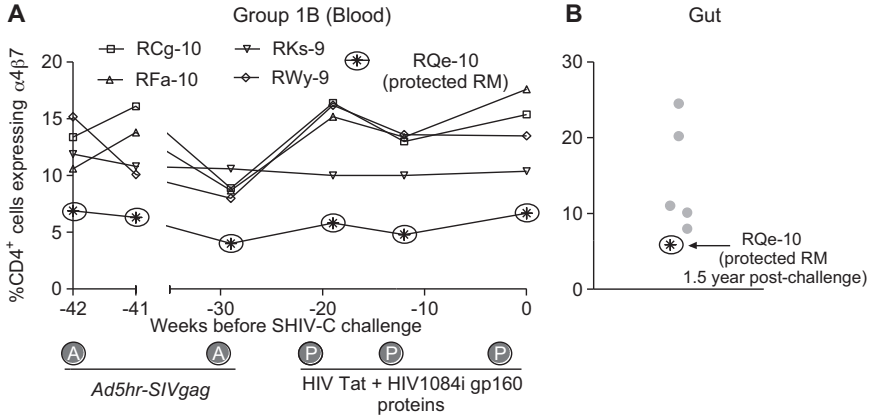

Fig. 7. The frequency of $CD4^+\alpha4\beta7^+$ T cells. (A): Frequency of $CD4^+\alpha4\beta7^+$ in peripheral blood of Group 1B animals during vaccination. The protected animal, RQe-10, showed a low frequency at all time points. (B) Frequency of $CD4^+\alpha4\beta7^+$ T cells derived from rectal biopsies of RQe-10 and five naïve, randomly selected RM.

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_{IIIB} *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 B*008 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 mod-

erately resistant TRIM5α 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 TRIM5α genotype.

In addition, integrin α4β7 plays an important role in the initial stages of HIV-1 acquisition. Integrin α4β7 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 α4β7 is ∼3 times longer than CD4, it may efficiently capture virions. Blocking α4β7 during acute infection led to significantly lower viremia in SIV-challenged RM [70]. These data imply that α4β7 likely facilitates lentiviral infection in vivo, and one may postulate that the relative frequency of $CD4^+\alpha4\beta7^+$ 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 $CD4^+\alpha4\beta7^+$ 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 α4β7 is not required for viral replication in cultured cells (reviewed in [69]). Challenge virus-specific nAbs and low numbers of potential $CD4^+\alpha4\beta7^+$ 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 viremia ($P = 0.067$). 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 $CD4^+\alpha4\beta7^+$ 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.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.vaccine.2011.06.017.

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