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A Live Attenuated *Listeria Monocytogenes* Vaccine Vector Expressing SIV Gag Is Safe and Immunogenic in Macaques and Can Be Administered Repeatedly

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Abstract

Listeria monocytogenes (Lm) is known to induce strong cellular immune responses. We constructed a live-attenuated Lm vector, *Lmdd-BdopSIVgag*, which encodes SIVmac239 *gag*. Intragastric (i.g.) administration of 3×10^{12} bacteria to rhesus macaques was safe and induced anti-Gag cellular but no humoral immune responses. Boosting of Gag-specific cellular responses was observed after i.g. administration of *Lmdd-BdopSIVgag* to previously vaccinated RM despite preexisting anti-Lm immunity shown by lymphoproliferative responses. Surprisingly, anti-Lm cellular responses were also detected in non-vaccinated controls, which may reflect the fact that Lm is a ubiquitous bacterium. The novel, live-attenuated *Lmdd-BdopSIVgag* may be an attractive platform for oral vaccine delivery.

Keywords

Live attenuated *Listeria monocytogenes*; live oral vaccine; HIV/AIDS vaccine; cellular immune responses; anti-vector immunity

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1. Introduction

According to recent UNAIDS (www.UNAIDS.org) global estimates, 2.7 million new HIV-1 infections occur each year, yet a safe and effective HIV-1/AIDS vaccine has remained elusive. The encouraging results of the recent RV144 Phase III Thai AIDS vaccine trial suggest that a vaccine strategy seeking to generate cellular immune responses may need to be combined with one designed to induce humoral responses in order to gain substantial vaccine protection against HIV-1. Although neutralizing human monoclonal antibodies provided complete protection from acquisition of neutralization-sensitive simian-human immunodeficiency virus (SHIV) strains by passive immunization [1–4], induction of antibodies capable of neutralizing diverse HIV-1 strains has not been achieved with the current experimental immunogens. Primate model studies [5,6] suggest that vaccine-induced cellular responses may limit lentiviral replication during the acute phase of infection; lowering of peak and steady-state viremia is associated with better clinical outcome. In a recent SIV/rhesus macaque model study, vaccine-induced cellular responses offered protection against persistent systemic infection [7]. Development of immunogens that can induce potent cellular responses is ongoing in several laboratories.

Among vaccination strategies to stimulate adaptive immunity, an important niche is occupied by live bacterial vectors. Live intracellular bacteria carrying foreign genes [8–11] are attractive candidates for the induction of cellular immune responses. Among the intracellular bacteria, the ubiquitous gram positive bacterium *Listeria monocytogenes* (Lm) has been studied in animal models as a vector for candidate cancer vaccines [12–20]; and was recently used in a Phase I human clinical trial among cervical carcinoma patients [21]. Several features of Lm, such as infection of antigen-presenting cells and the mucosal route of infection, make it an attractive vector for AIDS vaccine development (reviewed in [22]). Since most HIV-1 infections occur at mucosal surfaces, induction of immune responses at mucosal sites is crucial and Lm is a potential tool to achieve this goal. Lm has been used to induce HIV-1 Gag-specific immunity in murine [23–25] and non-human primate models [26]. Oral immunization of mice with Lm expressing HIV-1 Gag induced strong mucosal Gag-specific T-cell responses and protected against vaginal challenge with recombinant vaccinia virus expressing HIV-1 *gag* [25,27,28]. Lm induces multiple effector mechanisms, including antigen presentation via MHC class I and II pathways as well as induction of innate immune responses (reviewed in [29,30]). Lm is able to survive in macrophages [31], in antigen presenting cells (APC), and also in dendritic cells (DC) [32], by escaping from the phagosomic compartment into the cytosol of target cells [33]. Lm can replicate in mammalian cells that are normally non-phagocytic, such as epithelial gastrointestinal cells, by actively translocating across the intestinal barrier and entering organs via the lymphatic system (reviewed in [34–36]). Of particular interest is the fact that Lm is naturally transmitted by the oral route. Thus, Lm-based vaccine vectors may be potential oral vaccines, which may be attractive for use in developing countries.

The use of Lm also presents major concerns. Lm is a food-borne pathogen that causes listeriosis, which can be manifest clinically by signs ranging from gastroenteritis to encephalitis and meningitis, which could be fatal (reviewed in [37]). Listeriosis mostly affects vulnerable individuals, such as immune compromised hosts, neonates, and pregnant women [37–39]. Thus, a number of strategies have been considered to attenuate this bacterium [11,40,41].

Another concern for using Lm vectors as vaccine platform is pre-existing immunity against *Listeria*, a ubiquitous bacterium. Although few studies have shown that pre-existing immunity against Lm did not preclude the generation of immunity to foreign antigens

expressed by the *Listeria* vector in feline [42] and murine [43,44] models, pre-existing immunity against the vector is a widely discussed concern.

Earlier, we reported the hyperattenuated strain of *Listeria*, *Lmdd*, which lacked alanine racemase (*dal*) and D-amino acid aminotransferase (*dat*), the genes responsible for the synthesis of D-alanine, an essential component of the bacterial cell wall. Consequently, *Lmdd* critically depended on an exogenous supply of this amino acid [45]. While *Lmdd* encoding HIV *gag* was safe in rhesus monkeys (RM), the immunogenicity was not as potent as in mouse models [26], a difference we ascribed in part to the difficulties in co-administering the live-attenuated bacteria with D-alanine. Therefore, we further modified *Lmdd* to eliminate the strict D-alanine dependence. The new strain, *Lmdd-Bdop*, was constructed by allelic recombination between the *Lmdd* and the shuttle vector pKSV-7 carrying a *Bacillus subtilis dal* gene [27,46].

Here, we present safety and immunogenicity data on the recombinant *Lmdd-BdopSIVgag*, which encodes Lm-codon-optimized SIV *gag*, in Indian-origin rhesus macaques. In parallel, we tested the parental vector, *Lmdd-Bdop*. Furthermore, we addressed the issue of boosting cellular immune responses to the vector transgene in monkeys with known prior Lm exposure.

2. Methods

2.1. Construction of recombinant *Lmdd-BdopSIVgag*

The hyperattenuated, double-deletion Lm mutant, *Lmdd*, lacks alanine racemase (*dal*) and D-amino acid aminotransferase (*dat*), the genes responsible for the synthesis of D-alanine (an essential component of the bacterial cell wall) and hence required exogenous D-alanine for its replication [45]. *Lmdd-BdopSIVgag* was constructed by allelic recombination between *Lmdd* and the shuttle vector pKSV-7 carrying a *B. subtilis dal* gene [27,46] and a Lm-codon-optimized SIV *gag*; the *Lmdd-Bdop* control vector did not contain any SIV insert. Both *Lmdd-Bdop* and *Lmdd-BdopSIVgag* can grow transiently in infected mammalian cells without exogenous D-alanine. The final recombinant bacteria carry neither plasmids nor plasmid-encoded drug-resistance genes (Fig. 1A), but retain a significant degree of attenuation, with a 50% lethal dose (LD₅₀) for the intravenous (i.v.) route in BALB/c mice of about 10⁸ colony-forming units (CFU) versus 10⁴ CFU for wild-type Lm.

Lmdd-BdopSIVgag and the corresponding empty vector were grown in Bacto Brain Heart Infusion broth (DIFCO, BD Diagnostic, Sparks, MD) overnight at 30°C and stored at -80°C. The expression of SIV Gag by *Lmdd-BdopSIVgag* was confirmed by the Western blot analysis of bacterial culture supernatants (Fig. 1B).

2.2. Animals

Indian-origin rhesus monkeys (*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 Emory University and the Dana-Farber Cancer Institute. All animals were MHC typed for the Mamu-A*001 allele (Table 1).

2.3. Immunization regimen

Immunizations were performed in two phases (Table 1). During Phase I, five RM were given the empty vector *Lmdd-Bdop* (Group A, Phase I controls) and five RM received *Lmdd-BdopSIVgag*. In Phase II, two new groups of five RM (Groups C and D) were

enrolled. Group C RM were given *Lmdd-BdopSIVgag*, whereas Group D received the empty vector *Lmdd-Bdop* (Phase II controls). Group B (re-enrolled from Phase I) was the only group to be continued for the Phase II and again received two sets of *Lmdd-BdopSIVgag* immunizations. Fifteen min before the vector administration, each animal was anesthetized with ketamine and pretreated with a saturated sodium bicarbonate solution given by nasogastric (NG) tube to neutralize gastric acid. During Phases I and II, the immunizations differed in the number of doses and schedules of administration as described in Table 1.

2.4. Sample collection

Peripheral blood was collected in CPT tubes (Becton Dickinson, Franklin Lakes, NJ) containing sodium heparin as an anticoagulant. Peripheral blood mononuclear cells (PBMC) and plasma were separated and used for various assays. Remaining cells were cryopreserved and plasma aliquots were stored at -80°C .

In Phases I and II, rectal pinch biopsies were collected after 3 and 2 weeks following each immunization, respectively. Mononuclear cells were obtained from the biopsies as described earlier [47]. Briefly, the tissue was washed with Hanks balanced salt solution (HBSS), digested by mixture of DNase (Roche, Indianapolis, IN, USA) and collagenase IV (Sigma, St. Louis, MO, USA). The digest was passed through needles of different sizes and filtered to obtain single cell suspension.

2.5. Monitoring of fecal shedding of Lm-based vaccine vectors

To monitor shedding of live organisms, rectal swabs or fecal samples were collected following each immunization. These specimens were plated directly on selective media and incubated at 35°C for 24 h. Biochemical tests were performed to confirm colonies of Lm and the colony counts were documented (Tables 2 and 3).

2.6. ELISPOT assay

The assay was performed as described earlier [48]. Multiscreen-IP plates (Millipore, Billerica, MA, USA) were coated with mouse monoclonal anti-human interferon (IFN)- γ antibody (clone B27, BD Pharmingen, San Jose, CA, USA) overnight at 4°C , then blocked with 10% heat-inactivated fetal bovine serum (FBS) in RPMI-1640 (R-10) for 2 h at room temperature. PBMC were isolated from whole blood by centrifugation in heparinized CPT tubes (BD Pharmingen) and washed three times. For antigen-specific T-cell stimulation, SIVmac239 Gag peptides (obtained through the AIDS Research & Reference Reagent Program (ARRRP), Division of AIDS, NIAID, NIH) were used; the set consisted 15-mers with 11-amino acid overlaps between sequential peptides and represented the complete protein sequence. Three pools of peptides were prepared (pools #1, #2 and #3 consisting of peptides 1–42, 43–84 and 85–125, respectively). Cells were incubated overnight at 1×10^5 cells/well in R-10 medium containing 100 U/ml penicillin, 100 $\mu\text{g/ml}$ streptomycin and 0.02 mM β -mercaptoethanol along with a pool of peptides (each peptide at 2 $\mu\text{g/ml}$). After washing the plate, biotinylated mouse anti-human IFN- γ antibody (clone 7-B6-1, Mabtech, Cincinnati, OH, USA) was added. Horseradish peroxidase-conjugated streptavidin (BD Biosciences, San Jose, CA, USA) and AEC chromogen substrate (BD Biosciences) was used to develop the spots. IFN- γ -secreting cells were enumerated using an Immunospot ELISPOT reader (CTL, Cleveland, OH, USA). Assays were done in duplicates and background counts with no peptide stimulation were subtracted. The results were expressed as the average number of spot-forming unit (SFU) per million PBMC. Cells of each sample were also stimulated with a mixture of phorbol myristic acid (PMA) and ionomycin (0.01 and 0.5 $\mu\text{g/ml}$, respectively) as positive control. The assay also included cells obtained from a naïve animal as well as from a RM with known strong SIV Gag-specific cellular responses (from a different study [48,49]) as negative and positive controls, respectively.

2.7. Tetramer staining

Indian rhesus macaques display a high frequency of MHC class I allele Mamu-A*001 [50], which binds SIV Gag peptide p11C, the immunodominant epitope for this allele. Using this tetramer-peptide complex, we monitored frequency of p11C-specific, CD8⁺ T cells for all Mamu-A*001⁺ RM. Fresh PBMC or rectal mononuclear cells (1×10^6 cells) were stained at room temperature in the dark for 30 min with a mixture of anti-human CD3-Alexa Fluor 700 (SP34-2; BD Pharmingen), anti-human CD8-APC-Cy7 (RPA-T8; BD Pharmingen) antibodies and Gag p11C–Mamu-A*001 tetrameric complex-APC (kindly provided by Dr. Norman Letvin, Beth Israel Deaconess Medical Center, Boston, MA). Cells were washed twice with FACS buffer (PBS with 2% FBS) and fixed in 2% paraformaldehyde. PBMC from a Mamu-A*001-positive macaque with known SIV Gag-specific cellular response and a Mamu-A*001-negative macaque were used as positive and negative controls, respectively. Cells were analyzed using flow cytometer (LSR II; BD Biosciences) and FlowJo 6.0 (Tree Star) software. 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.

2.8. Intracellular Cytokine staining (ICS)

PBMC collected at weeks 0, 2, 4, 6, 8 and 10 during Phase II of immunization were used for ICS. Cells (1×10^6) were suspended in 100 μ l R-10 containing anti-CD49d (1 μ g/ml, clone 9F10, BD Biosciences) and anti-CD28 (20 μ l, clone CD28.2, eBioscience, San Diego, CA, USA) antibodies. The cells were stimulated with a mixture of all three Gag SIVmac239 peptide pools. To allow cytokine accumulation in the Golgi complex, GolgiPlug (BD Pharmingen) was added according to the manufacturer's instructions after 1.5 h. Cells without peptide stimulation were used to measure background cytokine production. Cells stimulated with a mixture of PMA and ionomycin (0.01 and 0.5 μ g/ml, respectively) served as positive controls. After overnight incubation, cells were washed with PBS containing 2% FBS and surface-stained with anti-human CD3-FITC (SP34, BD Pharmingen), anti-human CD4PerCP-Cy5.5 (L200, BD Pharmingen), anti-human CD8-PE (RPA-T8, BD Pharmingen). After surface staining, cells were fixed/permeabilized with BD Cytotfix/Cytoperm solution (BD Cytotfix/Cytoperm Plus Fixation/Permeabilization kit, BD Bioscience). The cells were then intracellularly stained with the following monoclonal antibodies (mAbs): anti-human IFN- γ -APC Cy7 (4S.B3, eBioscience, San Diego, CA), anti-human IL-2-APC (MQ1-17H12, BD Pharmingen) and anti-human TNF- α -Alexa Fluor 700 (mAb11, BD Pharmingen). The cells were then washed and fixed in 2% paraformaldehyde (PFA). Lymphocytes (based on forward and side scatter) were acquired by flow cytometry (LSR-II; BD Immunocytometry Systems) and analyzed using FACSDiva 6.0 software. The percentage of CD3⁺CD4⁺ or CD3⁺CD8⁺ T cells producing IFN- γ , IL-2 or TNF- α was determined after subtracting the corresponding percentages from unstimulated cells (cytokine production background).

2.9. Anti-Lm and anti-SIV Gag antibody ELISA

Antibodies against SIV Gag or against whole Lm in the plasma of immunized animals were measured by ELISA. To determine the presence of IgG against whole Lm, a suspension of heat-inactivated bacteria was prepared as follows. An inoculum of Lm (monkey clinical isolate 12443, bacterial strain RM3102, serotype 1/2a) [51] was grown overnight in Bacto Brain Heart Infusion broth (DIFCO); the concentration of bacteria was evaluated spectrophotometrically at 550 nm. After three washes in PBS, the bacterial suspension was heat-inactivated for 1 h at 65°C and resuspended in PBS to a concentration of 10^8 CFU/ml. Nunc-Immuno Maxi-Sorp 96-well ELISA plates (Nalgene, Nunc International, Rochester, NY, USA) were coated overnight at 4°C with 50 μ l/well of Lm suspension; plates were washed with PBS/0.05% Tween 20 (Sigma), and blocked for 2 h at 37°C with the same

buffer containing also 2% FBS. The plates were washed and incubated 2 h at 37°C with 100 µl/well of plasma samples diluted 1/100 in washing buffer. After additional washing, plates were incubated for 1.5 h at 37°C with goat HRP-anti-monkey IgG Fc (1:4000) (Accurate/Nordic, Westbury, NY, USA), washed, and finally developed by ABTS substrate solution (2,2'-azinobis (3-ethylbenzthiazoline-6-sulfonic acid-diammonium salt dissolved in stable peroxide substrate buffer 1X) (Pierce, Rockford, IL, USA) for 30 min at room temperature. The absorbance was measured at 410 nm. Negative controls consisted of six plasma samples obtained from naïve monkeys; positive control was plasma from a monkey previously vaccinated with *Lmdd-HIVgag*, known to have high anti-Lm antibody titers [26]. A cut-off value for the OD was obtained from plasma of six naïve animals, and was expressed as the mean OD₄₁₀ + 3 SD. Antibody titers are expressed as the dilution at which the sample OD₄₁₀ value was equal or above the cut-off OD₄₁₀ value.

Anti-SIV Gag antibodies were assessed in the same samples. Plates were coated with 3 µg/ml SIVmac251 p55 Gag (ARRRP) in Coating Buffer B (Invitrogen, Carlsbad, CA, USA) and incubated overnight at 4°C. The same protocol as described above was followed, but samples were diluted 1/50, and plasma from an animal with high antibody titers against SIV Gag [48] was used as a positive control for the assay.

2.10. Lymphocyte proliferation assay

PBMC (5×10^6) were suspended in PBS (1×10^6 /ml) containing 0.1% BSA and 5 µM CFSE (CellTrace™ CFSE Cell Proliferation Kit, Invitrogen, Camarillo, CA, USA) and incubated at 37°C for 10 min. To quench the staining, ice-cold R-10 media was added and the cell suspension was incubated on ice for 5 min. The cells were washed twice, resuspended in R-10, seeded at 1×10^6 cells/well in a 48-well plate and stimulated with either SIVmac251 Gag proteins (2 µg/ml; ARRRP) or 10 µl/well of heat-inactivated Lm suspension. Positive controls were stimulated with Con-A (5 µg/ml); cells without any stimuli were used to determine background proliferation. The cells were incubated at 37°C for 5 d then stained with anti-human CD3-Alexa Fluor 700 (SP34-2; BD Pharmingen), anti-human CD4PerCP-Cy5.5 (L200, BD Pharmingen) and anti-human CD8-APC-Cy 7 (RPA-T8; BD Pharmingen). After fixation (2% formaldehyde in PBS), at least 10,000 lymphocytes (based on forward and side scatter) were acquired by flow cytometry and data analyzed using FACSDiva (BD Biosciences) software. The percentages of antigen-specific CD3⁺CD4⁺ and CD3⁺CD8⁺ proliferating cells were determined by measuring CFSE fluorescence intensity for each subset; background proliferation (no stimulation) was subtracted.

2.11. Statistical analysis

Statistical analysis of data was performed by GraphPad Prism 4. The difference in frequency of SIV Gag-specific, IFN-γ-producing cells (SFU / 10^6 PBMC; measured by ELISPOT assay) between Groups B and C (during Phase II of immunization) was assessed by the Mann-Whitney U test. A *P* value < 0.05 was considered statistically significant.

3. Results

3.1. Safety of the new vector, *Lmdd-BdopSIVgag*

A major objective of this study was to test the safety of the new, live attenuated Lm vector in primates. In Phase I, five RM received 1×10^{12} of *Lmdd-Bdop* organisms (Group A) at weeks 0 and 13 (Table 1); the four doses were given on alternate days (days 0, 2, 4 and 6). An equivalent dose of *Lmdd-BdopSIVgag* (3×10^{12} organisms) was given to another five RM (Group B) using the same schedule of i.g. vaccination. The higher dose of *Lmdd-BdopSIVgag* compared to that of the parental vector accounted for the higher degree of attenuation of the former as tested in mice by LD₅₀ (chromosomal integration of the

transgene further attenuates Lm organisms). The animals were monitored for adverse clinical reactions and shedding of live Lm organisms in feces, which was detected within 24 h of inoculation. Twenty-two of 40 samples tested 48 h after each vaccine administration during the first set of four immunizations and 32 of 40 samples tested after the same time interval during second set of four immunizations were fecal culture negative for Lm (Table 2).

In Phase II, ten additional monkeys were enrolled (Groups C and D) that received three doses on consecutive days of either *Lmdd-Bdop* or *Lmdd-BdopSIVgag* at weeks 0 and 6 (Table 1). Group B was re-enrolled from Phase I and received additional *Lmdd-BdopSIVgag* according to the administration schedule followed for Groups C and D. These animals also cleared shedding of *Listeria*; 6 of 15 fecal samples tested 48 h after last dose of the first set of three immunizations and 11 of 15 samples tested at same time interval after last dose of the second set of immunizations were culture negative for Lm (Table 3).

Administration of a single dose of 1×10^9 wild-type Lm in monkeys caused illness that included septicemia, irritability, loss of appetite and occasional diarrhea; the animals shed *Listeria* in feces for about 21 days [52]. These animals also showed lymphopenia and neutrophilia within 48 h after Lm administration. We monitored all animals for 4 months after the last dose of Lm administration. Routine physical exams, complete blood count (CBC) and liver function tests were performed periodically. After administration of high doses of the live attenuated *Lmdd-Bdop* (1×10^{12} CFU) and *Lmdd-BdopSIVgag* (3×10^{12} CFU), none of the macaques showed any of the above mentioned clinical signs and cleared fecal shedding of Lm within 48 to 72 h (Tables 2 and 3). Fig. 2 shows lymphocyte and neutrophil counts after Lm administration. Our data indicate that the new, live attenuated *Lmdd-Bdop* and *Lmdd-BdopSIVgag* vectors were safe and well tolerated by the rhesus monkeys.

3.2. *Lmdd-BdopSIVgag* induced anti-SIV Gag cellular but not humoral immune responses after i.g. immunization

Next, we evaluated the immunogenicity of *Lmdd-BdopSIVgag* after i.g. administration of the vaccine vector. During Phase I, RM were exposed to *Lmdd-bdop* (Group A) or *Lmdd-BdopSIVgag* (Group B) on weeks 0 and 13 on alternate days for a total of four doses (two sets of immunization). The frequency of SIV Gag-specific, IFN- γ secreting cells was monitored by ELISPOT assay. Low-level cellular responses were first detected among Group B animals between weeks 2 to 6 (Fig. 3A and 3B) after the initial priming. p11C (SIV Gag epitope)-specific CD8⁺ T cells were also detected in PBMC among 3 out of 5 macaques 3 weeks after first set of *Lmdd-BdopSIVgag* administration (Fig. 4A). Two weeks after boosting with the second set of exposures to *Lmdd-BdopSIVgag*, two RM showed strong SIV Gag-specific responses (>1000 SFU/ 10^6 PBMC; Fig. 3B). Tetramer+, p11C-specific CD8⁺ T cells were detected in the peripheral blood of two RM (Fig. 4B). These data indicate that i.g. priming / boosting induced strong anti-SIV Gag cellular immune responses in two of the five vaccinated RM. No anti-Gag antibody responses were observed in any vaccinees (data not shown).

3.3. Pre-existing anti-Lm immunity was detected among all RM

Next, we sought to test whether the known exposure of Group B RM during the Phase I immunogenicity testing had induced anti-Lm immune responses, and whether they would preclude recall responses to the transgene. To do this, we re-enrolled Group B monkeys into Phase II immunogenicity testing. We recruited two more groups of five RM each. Group C consisted of naïve monkeys that would receive the same vaccine, *Lmdd-BdopSIVgag*, as the animals in Group B. The difference between Groups B and C was prior exposure of Group B

monkeys to the vaccine. Group D animals were enrolled as recipients of the empty vector, *Lmdd-Bdop*. Eleven weeks after the last immunization during Phase I, we tested Group B animals as well as the newly enrolled Groups C and D for anti-Lm humoral and cellular immune responses. None of the animals had measurable anti-Lm antibodies (data not shown). However, all animals of all three groups had proliferative responses among the CD4⁺ T cells, and the fraction of CD8⁺ T cells proliferating in response to whole Lm stimulation was surprisingly high for all three groups (Fig. 5A). It should be noted that all RM had been pre-screened prior to enrollment for antibody responses to Lm (all had been negative). We ascribe the proliferative responses of all monkeys to whole bacteria – with or without the additional vaccination with *Lmdd-BdopSIVgag* – to pre-existing Lm antigen-specific memory T cells, given that *Listeria* is a ubiquitous bacterium present in soil.

3.4. Prior immunization with *Lmdd-BdopSIVgag* did not prevent anamnestic responses to SIV Gag

Next, we sought to test whether the previously vaccinated Group B monkeys would show increases in anti-SIV Gag ELISPOT responses upon further exposure to the vaccine vector. Phase II re-vaccination was performed in two sets of three immunizations given on three consecutive days (Fig. 3, panels on right). At week 6, prior to the second set of immunization in Phase II, four out of the five RM of Group B had measurable ELISPOT responses (Fig. 3B, right panel); intracellular cytokine staining showed low and variable positivity (data not shown), and p11C-specific CD8⁺ T cells were detected in the peripheral blood of one out of five RM (monkey RCg-10, Fig. 4C). Overall, four out of five monkeys of Group B showed positive anti-SIV Gag responses by ELISPOT assay after only one set of immunization during Phase II, which is consistent with an anamnestic anti-Gag response.

Next, we compared the anti-Gag cellular responses of the re-exposed vaccine recipients of Group B with those of Group C animals that had never seen the vaccine vector before. As expected, the single set of immunization (priming) given at week 0 did not induce high ELISPOT responses (Fig. 3C), and at week 6, the latter were similar to those of monkeys of control Group D. When compared to Group B (re-exposed) monkeys, ELISPOT responses at week 6 in Group C were significantly lower ($P = 0.031$). This indicates that the re-exposed Group B animals mounted significant anamnestic response, whereas Group C monkeys needed boosting with antigen to develop anti-Gag ELISPOT responses, which peaked 2 to 4 weeks after the boost given at week 6 (Fig. 3C).

After second set of immunizations during Phase II, we also observed proliferation of CD4 as well as CD8 T cells in response to SIV Gag protein (Fig. 4B), suggesting induction of SIV Gag-specific memory T cells. Although this vaccination was performed i.g., we did not see p11C-specific T cells in rectal mucosal tissues after staining of mononuclear cells from biopsies (data not shown).

3.5 *Lmdd-BdopSIVgag* induced equivalent anti-Gag immune responses among animals immunized according to schedules yielding persistent versus non-persistent antigen exposure

Next, we compared the immunogenicity of the alternate day dose-schedule with daily vaccine administration schedule for three consecutive days. In this pilot study, we compared the anti-Gag ELISPOT responses of Group B during Phase I with that of the animals in Group C. Phase I data of *Listeria* shedding in feces suggest that the bacteria did not persist in the animals' gut until the administration of next dose given at 48 h later. Therefore in Phase II, the dose schedule was modified and the oral dose of *Listeria* vector was given on 3 consecutive days (days 0, 1, 2). After this modification, all animals showed persistent shedding of *Listeria* from day 1 to 3 (Table 3), implying that continuous antigen exposure

was achieved during this set of immunization, with which we sought to mimic the course of natural infection. In this pilot study, we compared the anti-Gag ELISPOT responses of Group B during Phase I with those of the RM in Group C. Anti-Gag ELISPOT responses did not differ much following vaccination according to the two different dose-schedules. After the initial prime/boost immunizations at weeks 0 and 13, two out of five RM in Group B (Phase I) versus three out of five RM in Group C (Phase II) were clearly positive. Given the small group size, this difference was not significant.

3.6. *Lmdd-BdopSIVgag* did not induce anti-Lm or anti-SIV Gag humoral responses

Although our vaccination induced SIV Gag-specific T-cell responses, we did not detect humoral responses to Gag or whole Lm. ELISA results were negative even after multiple i.g. Lm exposures of Group B animals (data not shown). The exclusive activation of cellular immunity without humoral counterpart is characteristic of the intracellular survival and proliferation of *Listeria* and these results are in accordance with our earlier study [26].

4. Discussion

The intracellular bacterium Lm has been considered as live vaccine vector to deliver foreign antigens to elicit antigen-specific cellular immune responses. However, Lm is a food-borne pathogen that causes listeriosis and hence, attempts are directed toward attenuating bacterial pathogenicity while retaining immunogenicity. We developed new live attenuated strain of Lm, *Lmdd-BdopSIVgag*, to induce SIV Gag-specific cellular immune responses.

We found that our new *Listeria* vector was safe and well tolerated by all RM. No adverse reaction or illnesses were induced even after administration of multiple high doses (3×10^{12} organisms). In contrast, a single oral dose of 1×10^9 wild-type Lm caused severe listeriosis in non-human primates [52]. Shedding of *Lmdd-BdopSIVgag* was observed only for the limited duration following i.g. administration. Hence, we conclude that our new vaccine vector was safe among non-human primates. The vector also induced SIV Gag-specific cellular immune responses that were higher than those induced by our earlier live attenuated strain, based upon the *Lmdd* vector [26]. Thus, *Lmdd-BdopSIVgag* was more immunogenic than the earlier version and was also easier to administer since it did not require exogenous D-alanine for its replication.

Besides safety, another major concern associated with the use of vectored vaccines is the issue of preexisting anti-vector immunity [53], which has gained more attention after the failed STEP trial. Despite initially encouraging data from preclinical and clinical studies, the efficacy of human adenoviral vector serotype 5 (AdHu5) was hampered by a strong preexisting anti-vector immunity among vaccinated macaques, in which transgene-specific T cells homed to different organs in the presence of anti-vector immunity [54]. Lower vaccine-vector transgene immunogenicity has been reported for the adeno-associated virus (AAV) vector in animal models in the presence of anti-vector nAbs [55]. Furthermore, prior exposure to wild-type *Salmonella* also negatively affected the use of this bacterium as vaccine vector [56,57]. Since Lm is a ubiquitous bacterium, anti-Lm immune responses are likely to be present among the majority of individuals. The issue of preexisting immunity in the context of development of *Listeria* as vaccine vector has been addressed only in murine [43,44] and feline [42] systems. These studies showed that despite previous exposure to Lm, induction of immune responses to the new antigens carried by *Listeria* was not hampered. Our current findings are in agreement with these earlier reports. Although we detected strong lymphocyte proliferation to whole Lm before the administration of the *Lmdd-BdopSIVgag* vaccine vector, SIV Gag-specific cellular responses were induced among the vaccinated monkeys. Moreover, we also found that secondary anamnestic cellular responses

were generated after re-exposure to this vaccine strain, suggesting that tolerance was not induced despite repeated oral administrations of live attenuated *Lmdd-BdopSIVgag*.

We did not detect any anti-Lm antibodies despite multiple Lm exposures in our primate studies, a finding that is in agreement with earlier reports. Infection of mice with sublethal doses of wild-type Lm did not induce anti-Lm antibodies [58]. In a monkey model of listeriosis during pregnancy, highly invasive Lm infection was required to induce high-titer anti-Lm antibodies, which in turn were associated with placental invasion and stillbirths [51]. Similarly, in our earlier study with the live attenuated Lm vector, *Lmdd-HIVgag*, anti-Lm antibodies were not detected after oral priming and boosting but were detected after oral priming followed by intramuscular boosting in macaques [26]. In humans, characterization of Lm-specific cellular and humoral immune responses among healthy individuals showed that while 60% of individuals had Lm-specific cellular responses, only 16% of the individuals showed low-titer anti-Lm antibodies [43].

The present study shows that our newly developed, live attenuated Lm strain, *Lmdd-BdopSIVgag*, was safe and immunogenic in non-human primates despite preexisting anti-Lm immunity shown by proliferative responses. This new strain induced SIV Gag-specific cellular immune responses after i.g. administration, and repeated mucosal administrations did not lead to induction of tolerance.

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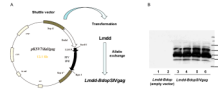


Fig. 1.

Construction of *Lmdd-BdopSIVgag* and analysis of SIV *gag* expression by *Lmdd-BdopSIVgag*. The recombinant *Listeria monocytogenes* (Lm) vector *Lmdd-BdopSIVgag* was obtained by allelic exchange between the Lm *dal dat* vector and pKSV-7 carrying a *Bacillus subtilis dal* gene and Lm-codon-optimized SIV *gag* (A). Culture supernatants from control *Lmdd-Bdop* (lanes 1, 2) and from *gag*-expressing *Lmdd-BdopSIVgag* (lanes 3–6) were analyzed by Western blot. SIV Gag was detected using the anti-SIV Gag monoclonal antibody 2F12 (AIDS Research & Reference Reagent Program, NIH). Cultures contained approximately 7 ng/ml p27 antigen as determined by ELISA. The multiple bands reflect proteolytic processing of Gag within the Lm and following secretion into the medium.

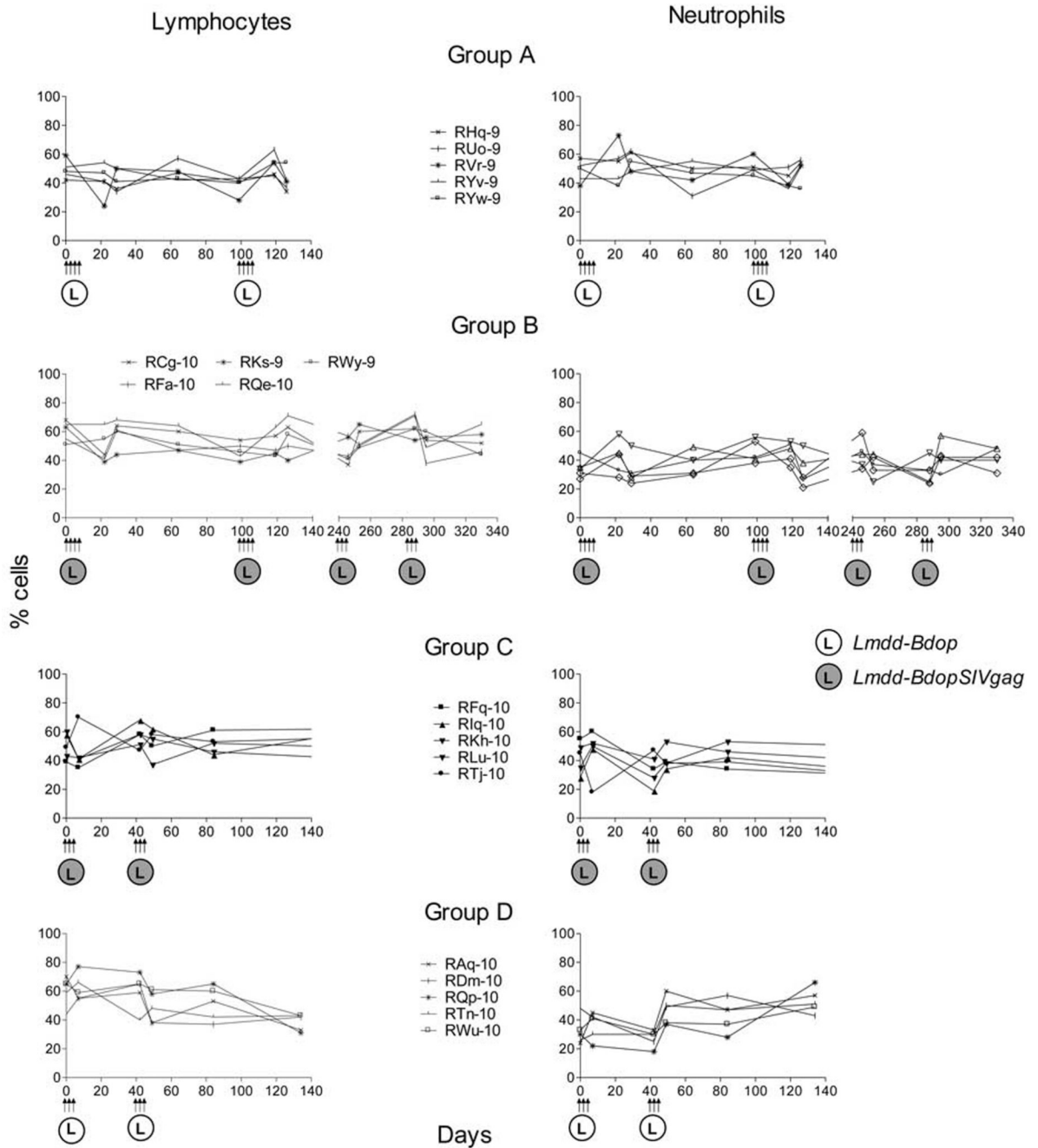


Fig. 2. Hematological profiles of RM following Lm administration. The percent lymphocytes (left panels) and neutrophils (right panels) are shown. No neutrophilia was seen; one Group C RM had an absolute neutrophil count on day 4 after the last dose of *Lmdd-BdopSIVgag* of $14,280 \text{ cells/mm}^3$ (normal range for RM, $0.2\text{--}14.6 \times 10^3 \text{ cells/mm}^3$) without bands or concomitant changes in the percent neutrophils and lymphocytes. This RM also did not show any clinical signs of illness. The normal percentages of lymphocytes and neutrophils range between 8% to 92% and 5% to 88%, respectively, as wide fluctuations have been reported.

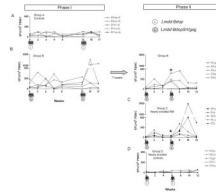


Fig. 3.

The frequency of SIV Gag-specific cells measured by IFN- γ ELISPOT assay at the time points indicated. Arrows, intragastric administration of either *Lmdd-BdopSIVgag* (L in dark circle) or *Lmdd-Bdop* (L in open circle); *, statistically significant difference ($P = 0.031$) between Groups B and C at week 6 in Phase II.

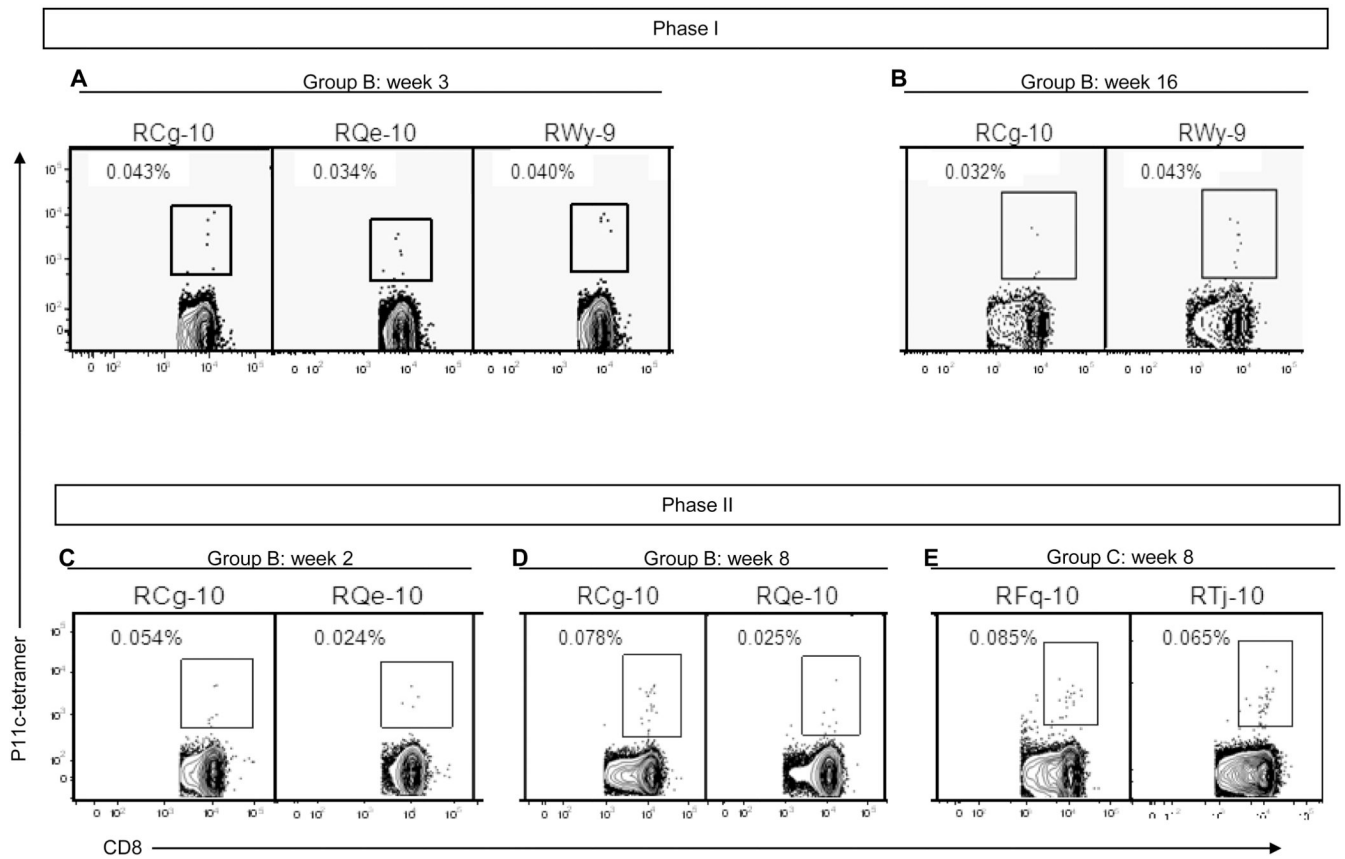


Fig. 4. Frequency of CD3+CD8+ Gag p11C tetramer+ cells among Mamu-A*001+ animals during Phases I and II. The results are shown only for responder RM. Results were considered positive if >0.03%, a cut-off that was determined from analysis of naïve monkeys. No positive cells were detected among Mamu-A*001 negative vaccinated monkeys.

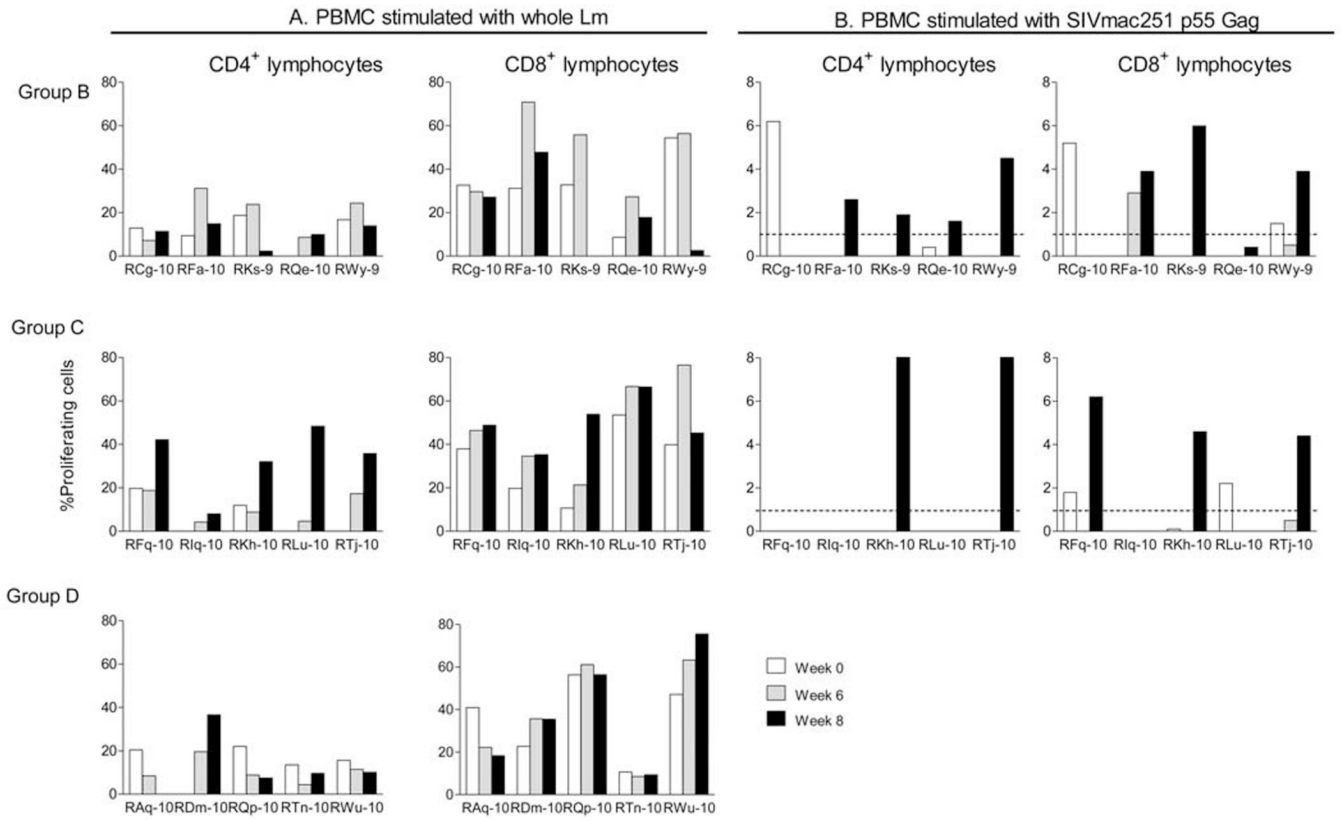


Fig. 5. Proliferation of PBMC after stimulation with whole heat-inactivated Lm (A) or SIV Gag protein (B). Percent CD4⁺ (left panels) and CD8⁺ (right panels) proliferating cells are shown. Dashed line, mean frequency of proliferating cells shown by control (Group D) animals.

Table 1
 Immunization schedule of rhesus monkeys with *Lmdd-BdopSIVgag* and empty vector control

| Vaccination Phase | Groups (status at start) | Monkey name | Immunogen | Dose (CFU) | Schedule | |
|-------------------|------------------------------|------------------|------------------------|-----------------------------------|---------------------------------------|--|
| Phase I | A (naive) | RHq-9 | | | | |
| | | RUo-9 | | | | |
| | | RVr-9 | <i>Lmdd-Bdop</i> | 1×10^{12} | Days 0, 2, 4, and 6 at weeks 0 and 13 | |
| | | RYv-9 | | | | |
| | | RYw-9 | | | | |
| | B (naive) | RCg-10* | | | | |
| | | RFa-10* | | | | |
| | | RKs-9* | <i>Lmdd-BdopSIVgag</i> | 3×10^{12} | Days 0, 2, 4, and 6 at weeks 0 and 13 | |
| | | RQe-10* | | | | |
| | | RWy-9* | | | | |
| Phase II | B (re-enrolled from Phase I) | RCg-10* | | | | |
| | | RFa-10* | | | | |
| | | RKs-9* | <i>Lmdd-BdopSIVgag</i> | 3×10^{12} | Days 0, 1, and 2 at weeks 0 and 6 | |
| | | RQe-10* | | | | |
| | | RWy-9* | | | | |
| | C (naive) | RFq-10* | | | | |
| | | RIq-10* | | | | |
| | | RKh-10 | <i>Lmdd-BdopSIVgag</i> | 3×10^{12} | Days 0, 1, and 2 at weeks 0 and 6 | |
| | | RLu-10 | | | | |
| | | RTj-10* | | | | |
| D (naive) | RAq-10 | | | | | |
| | RDm-10 | | | | | |
| | RQP-10 | <i>Lmdd-Bdop</i> | 1×10^{12} | Days 0, 1, and 2 at weeks 0 and 6 | | |
| | RTn-10 | | | | | |

| Vaccination Phase | Groups (status at start) | Monkey name | Immunogen | Dose (CFU) | Schedule |
|-------------------|-----------------------------|-------------|-----------|------------|----------|
| | | R WU-10 | | | |

* Animals positive for the Mamu-A*001 allele

Table 2

Shedding of live Lm in feces after vaccination during Phase I.

| Groups | RM | Bacterial colonies (CFU) | | | | | | | | | | | | | | | | | | | |
|--------|--------|---|------|----|-----------------|-----|----|-----|----|----|-----|---|-----|----|-----|----|-----|----|-----|----|----|
| | | 1 st set of four immunizations | | | | | | | | | | 2 nd set of four immunizations | | | | | | | | | |
| | | D0 | D1 | D2 | D3 ^a | D4 | D5 | D6 | D7 | D8 | D9 | D0 | D1 | D2 | D3 | D4 | D5 | D6 | D7 | D8 | D9 |
| A | RHq-9 | - | +++ | - | + | + | - | +++ | - | - | +++ | - | +++ | - | +++ | + | +++ | + | +++ | - | |
| | RUo-9 | - | + | - | - | ++ | - | + | + | - | + | - | +++ | - | +++ | - | + | + | +++ | - | |
| | RVr-9 | - | ++ | + | ++ | - | - | ++ | + | - | ++ | + | +++ | - | +++ | - | ++ | ++ | +++ | - | |
| | RYv-9 | - | +++ | - | + | +++ | - | + | - | - | +++ | + | +++ | - | +++ | - | +++ | + | +++ | - | |
| | RYw-9 | - | ++ | - | + | + | - | ++ | - | - | ++ | - | +++ | - | +++ | - | +++ | - | +++ | - | |
| B | RCg-0 | - | ++++ | + | + | + | + | +++ | ++ | - | +++ | - | +++ | - | +++ | + | +++ | + | +++ | - | |
| | RFa-10 | - | ++ | - | ++ | + | - | ++ | ++ | - | +++ | - | - | - | - | + | + | ++ | - | - | |
| | RKs-9 | - | + | ++ | ++ | + | - | ++ | + | - | +++ | - | +++ | - | +++ | - | - | ++ | - | - | |
| | RQe-10 | - | +++ | + | + | + | - | + | - | - | +++ | - | +++ | - | +++ | + | +++ | - | +++ | - | |
| | RWy-9 | - | + | - | - | - | + | ++ | - | - | ++ | - | ++ | - | + | - | - | ++ | - | - | |

++++, > 100 CFU;

+++ , 50 to 100 CFU;

++ , 10 to 50 CFU;

+ , < 10 CFU;

- , no CFU;

^a no samples available

Table 3

Shedding of live Lm in feces after vaccination during Phase II.

| Groups | RM | Bacterial colonies (CFU) | | | | | | | | | | | | | | | | | | | | | |
|--------|--------|--|------|-----|-----|-----|----|----|------|-----|--|-----|-----|-----|----|---|-----|-----|-----|-----|-----|---|---|
| | | 1 st set of three immunizations | | | | | | | | | 2 nd set of three immunizations | | | | | | | | | | | | |
| | | D0 | D1 | D2 | D3 | D4 | D5 | D6 | D0 | D1 | D2 | D3 | D4 | D5 | D6 | | | | | | | | |
| B | RCg-10 | - | +++ | +++ | +++ | + | - | - | +++ | ++ | ++ | ++ | - | - | - | - | +++ | ++ | ++ | ++ | - | - | - |
| | RFa-10 | - | ++ | ++ | +++ | - | - | - | +++ | +++ | +++ | +++ | - | - | - | - | +++ | +++ | +++ | +++ | - | - | - |
| | RKs-9 | - | ++ | + | + | ++ | - | - | +++ | ++ | ++ | + | - | - | - | - | +++ | ++ | ++ | + | - | - | - |
| | RQe-10 | - | +++ | +++ | +++ | + | - | - | +++ | ++ | + | ++ | - | - | - | - | +++ | ++ | ++ | ++ | - | - | - |
| | RWy-9 | - | +++ | ++ | ++ | - | - | - | + | ++ | ++ | ++ | + | - | - | - | +++ | ++ | ++ | ++ | + | - | - |
| C | RFq-10 | - | ++ | ++ | +++ | + | + | - | +++ | +++ | +++ | ++ | - | - | - | - | +++ | +++ | +++ | +++ | - | - | - |
| | RIq-10 | - | ++ | ++ | +++ | + | - | - | +++ | ++ | +++ | +++ | + | - | - | - | +++ | ++ | +++ | +++ | + | - | - |
| | RKh-10 | - | +++ | ++ | +++ | ++ | - | - | ++ | +++ | +++ | +++ | - | - | - | - | +++ | +++ | +++ | +++ | - | - | - |
| | RLu-10 | - | ++ | + | +++ | +++ | - | - | +++ | ++ | ++ | ++ | - | - | - | - | +++ | ++ | ++ | ++ | - | - | - |
| | RTj-10 | - | +++ | + | +++ | + | + | - | +++ | ++ | ++ | +++ | - | - | - | - | +++ | ++ | +++ | +++ | - | - | - |
| D | RAq-10 | - | +++ | +++ | +++ | - | - | - | +++ | +++ | +++ | - | +++ | - | - | - | +++ | +++ | +++ | +++ | - | - | - |
| | RDm-10 | - | ++ | +++ | +++ | - | - | - | +++ | +++ | +++ | ++ | - | - | - | - | +++ | +++ | +++ | +++ | - | - | - |
| | RQp-10 | - | +++ | + | +++ | - | - | - | +++ | +++ | +++ | +++ | - | - | - | - | +++ | +++ | +++ | +++ | - | - | - |
| | RTh-10 | - | + | +++ | +++ | - | - | - | ++++ | ++ | ++ | +++ | +++ | +++ | + | + | +++ | +++ | +++ | +++ | +++ | + | + |
| | RWu-10 | - | ++++ | ++ | +++ | ++ | + | + | +++ | +++ | +++ | +++ | - | - | - | - | +++ | +++ | +++ | +++ | - | - | - |

++++, > 100 CFU;

+++ , 50 to 100 CFU;

++ , 10 to 50 CFU;

+ , < 10 CFU;

- , no CFU;