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Live Attenuated *Listeria monocytogenes* Expressing HIV Gag: Immunogenicity in Rhesus Monkeys

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Abstract

Induction of strong cellular immunity will be important for AIDS vaccine candidates. Natural infection with wild-type *Listeria monocytogenes* (*Lm*), an orally transmitted organism, is known to generate strong cellular immunity, thus raising the possibility that live attenuated *Lm* could serve as a vaccine vector. We sought to examine the potential of live attenuated *Lm* to induce cellular immune responses to HIV Gag. Rhesus macaques were immunized with *Lmdd-gag* that expresses HIV *gag* and lacks two genes in the D-alanine (D-ala) synthesis pathway. Without this key component of the bacterial cell wall, vaccine vector replication critically depends on exogenous D-ala. *Lmdd-gag* was given to animals either solely orally or by oral priming followed by intramuscular (i.m.) boosting; D-ala was co-administered with all vaccinations. *Lmdd-gag* and D-ala were well tolerated. Oral priming/oral boosting induced Gag-specific cellular immune responses, whereas oral priming/i.m. boosting induced systemic as well as mucosal anti-Gag antibodies. These results suggest that the route of vaccination may bias anti-Gag immune responses either towards T-helper type 1 (Th1) or Th2 responses; overall, our data show that live attenuated, recombinant *Lmdd-gag* was safe and immunogenic in primates.

Keywords

rhesus monkey; *Listeria monocytogenes*; D-alanine; cellular immunity; vaccine

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Disclosures

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1. Introduction

It is well accepted that the most effective way to control the HIV pandemic will be to develop a safe, effective vaccine. There is consensus that an ideal vaccine will need to generate both humoral and cellular immune responses that can prevent or at least contain the replication of different HIV strains and clades. However, the relative importance of mobilizing each arm of the immune system and the means to induce each type of immunity are unclear. Although several successful passive immunization studies using neutralizing monoclonal antibodies in macaques strongly suggest that inducing broadly reactive neutralizing antibodies as part of any vaccine regimen would be beneficial [1–5], the levels and breadth of neutralizing antibodies induced by active vaccination have thus far proved insufficient to protect against infection [6–8]. It appears necessary that a vaccine must activate both cellular and humoral immune mechanisms.

The importance of cellular immunity to control human and primate immunodeficiency virus infection has been shown previously [9,10]. In vivo depletion studies with an anti-CD8 monoclonal antibody demonstrated that CD8⁺ cells are directly involved in containing simian immunodeficiency virus (SIV) infection in primates [11,12]. As such, the most promising and effective vaccine candidates to date against simian-human immunodeficiency virus (SHIV) challenge of macaques have aimed at inducing strong cellular immune responses [13–16]. Many vaccine candidates currently in clinical trials are focused on inducing cellular immune responses, especially CD8⁺ cytotoxic T lymphocyte (CTL) responses [17].

One of the most effective ways to induce CD8⁺ CTL responses against a given antigen is to insert the corresponding gene into live, intracellular vectors. Infection of antigen presenting cells (APC) by such recombinant vectors allows intracellular antigen expression, processing through the endogenous pathway, and presentation by MHC class I molecules. *Lm* is an intracellular bacterium with properties that make it an attractive vaccine vector to deliver foreign genes encoding either tumor antigens or genes of infectious agents, including HIV [18–22]. First, because *Lm* specifically infects and induces maturation of dendritic cells (DC), it is a good agent to stimulate innate as well as adaptive immune responses. Second, foreign antigens encoded by *Lm* are efficiently presented by both MHC class I and MHC class II molecules after being processed to peptides [23,24]; as *Lm* vectors deliver antigens directly to the DC cytosol, both CD8⁺ and CD4⁺ antigen-specific T cells can thus be activated [22]. Third, because the natural route of *Lm* infection involves oral exposure, *Listeria*-derived vaccine vectors may be given orally [25–28]. Fourth, evidence with a *Listeria* vector containing two genes of feline immunodeficiency virus (FIV) showed that pre-existing immunity against *Lm* does not preclude the generation of immunity to foreign antigens expressed by the *Listeria* vector [28].

As *Lm* can cause serious infections in neonates, pregnant women and immunocompromised hosts, different attenuation strategies have been considered: a) to delete the *Lm actA* gene (either alone or with the *plcB* or *inlB* genes) so that both intracellular movement and cell-to-cell spreading of bacteria is stopped but immunogenicity is maintained [27,29,30]; b) to delete the *uvrA* and *uvrB* genes, which encode nucleotide excision repair genes [31]; and c) to delete essential genes in the D-alanine (D-ala) synthesis pathway. We have chosen the latter approach to develop a highly attenuated vaccine vector, called *Lmdd*. Two genes required for the biosynthesis of bacterial cell walls, the alanine racemase (*dal*) and D-amino acid aminotransferase (*dat*) genes, were deleted from the bacterial chromosome [32]. *Lmdd* replication thus critically depends on exogenous D-ala.

Lmdd was first tested as a vaccine vector by inserting HIV *gag* [33]. The resulting bacteria, *Lmdd-gag*, generated HIV Gag-specific CD8⁺ CTL responses, both systemically and within Peyer's patches and mesenteric lymph nodes, in vaccinated mice [34]. Furthermore, *Lmdd-gag*-immunized mice were protected from challenge with recombinant vaccinia virus expressing HIV *gag* [34]. Importantly, *Lmdd-gag* was attenuated at least 5 logs in neonatal mice [35]. However, mice are not optimal for testing the safety and efficacy of *Lmdd-gag* as they are neither susceptible to lentivirus infection nor encode the proper E-cadherin receptor on intestinal epithelial cells to allow *Lm* to enter enterocytes [36].

Here we evaluated the safety and immunogenicity of *Lmdd-gag* in nonhuman primates. This pilot study showed that oral administration of *Lmdd-gag* induced cellular immune responses to HIV Gag and combined oral/intramuscular administration induced strong Gag-specific antibody responses.

2. Materials and Methods

2.1. *Lmdd-gag*

A double-deletion mutant of *Lm* 10403S (*Lmdd*) was engineered in which 82% of the *dal* and 31% of the *dat* genes were removed [32]. *Lmdd* was grown in brain/heart infusion medium (Difco Labs, Detroit, MI) with 100–200 µg/ml of D-ala. The 50% lethal dose (LD₅₀) of the wild-type *Lm* strain 10403 in female BALB/c mice following intravenous (i.v.) or intraperitoneal (i.p.) infection was approximately 1×10^4 organisms. The LD₅₀ of *Lmdd* was $>8 \times 10^8$ when inoculated i.v. alone or, when inoculated i.v. in the presence of 20 mg D-ala, was approximately 7×10^7 organisms [32] or higher (unpublished data). The LD₅₀ following intragastric inoculation of mice is unknown for either organism, but probably exceeds 10^{10} organisms. Recombinant *Lmdd-gag* was constructed by stable modification of the bacterial chromosome using the shuttle vector pKSV7 [37] and a protocol modified from Camilli et al. [38], as described [32], to insert HIV-*gag* into the *sepA* gene of *Lmdd*.

2.2. Animals and primary immunization schedule

Three groups of Chinese origin rhesus monkeys were immunized with *Lmdd-gag* or *Lmdd* (Fig. 1). Group 1 (four animals, RIg-8, RDh-8, RLh-8 and ROg-8) received 1×10^{12} *Lmdd-gag* in whipping cream orally at week 0 and 3×10^{12} *Lmdd-gag* at weeks 6 and 19. Group 2 (2 animals, REg-8 and RMg-8) was given *Lmdd-gag* orally in whipping cream at weeks 0 and 6 (1×10^{12} organisms each) and at week 10, 1×10^{12} *Lmdd-gag* intramuscularly (i.m.). Group 3 (3 animals, RSg-8, RUg-8 and RMh-8), received empty vector (*Lmdd*, 1×10^{12} organisms) in whipping cream orally at week 0 and 3×10^{12} organisms at weeks 6 and 19. All oral inocula of *Lmdd-gag* or *Lmdd* also contained D-ala (0.5 mg/ml in a volume of 20 ml). A final boost of the same animals after resting for at least 27 weeks is described in the text.

2.3. Measurement of serum D-ala levels

D-ala levels in mouse and monkey sera were determined with a spectrophotometric method that coupled oxidative deamination of D-ala by D-aminoacid oxidase to the reduction of the resulting product, pyruvate, in the presence of lactic dehydrogenase and NADH [39]. Catalase was present to prevent the alternative conversion of pyruvate to acetic acid and carbon dioxide by accumulated hydrogen peroxide. The reaction was linear from 10 µg/ml to 1 mg/ml of D-ala. Protein was removed from serum samples by extraction with perchloric acid followed by potassium bicarbonate prior to assay.

The D-ala assay was tested as follows: measurement of a known D-ala concentration was unaffected by the presence of naive mouse or monkey sera or by extraction of D-ala in the

presence of such sera. Maintaining mouse or monkey serum samples at room temperature or at 37° C for up to 2 hr or at 56° for 30 min prior to extraction did not alter D-ala content.

2.4. Collection of the mucosal fluids

Oral, rectal and vaginal secretions were collected using Weck-Cel sponges as described [40, 41].

2.5. Interferon- γ (IFN- γ) ELISPOT assay

ELISPOT assays for antigen-specific IFN- γ -secreting T cells were performed using ELISPOT kits (BioSource International, Camarillo, CA). Briefly, monkey peripheral blood mononuclear cells (PBMC) were restimulated with HIV-1 HXB2 Gag overlapping synthetic peptides (NIH AIDS Research and Reference Reagent Program) for 7 days (PBMC without peptides were used as control). PBMC were then washed, resuspended and added to anti-IFN- γ antibody-precoated plates. After overnight incubation (37°C, 5% CO₂), cells were washed away and biotinylated anti-IFN- γ antibodies were added. Plates were incubated for 1 h at 37°C, followed by incubation at 37°C with anti-biotin antibody labeled with enzyme. After color reaction with ELISPOT kit substrate, spots were counted under a microscope. Results were expressed as spot-forming units (SFU)/10⁶ cells after subtracting background spots (PBMC cultured with no peptide stimulation).

2.6. T cell proliferation assay

Monkey PBMC (2×10^6 /ml) were cultured in RPMI 1640 plus 15% fetal calf serum (FCS) plus antibiotics in the presence of HIV Gag protein (Immunodiagnostic Inc. Woburn, MA) (7.5 μ g/ml) for 5 days. Cells were pulsed with ³H-thymidine (1 μ Ci per well) (PerkinElmer, Boston, MA) for 16 h before harvesting, and thymidine incorporation was measured in a β -counter (Beckman Coulter, Inc., Miami, FL). Results were expressed as stimulation index (SI), which is the ratio of counts per min (CPM) from PBMC with HIV Gag stimulation to CPM from PBMC cultured in medium only.

2.7. ELISA for antibody responses

To test for anti-Gag antibody responses, 96-well Nunc ELISA plates (Fisher Scientific Co, Pittsburgh, PA) were coated with 50 μ l of HIV Gag (1 μ g/ml) in sodium bicarbonate buffer (pH 9.6) overnight at 4°C, washed with water and blocked with PBS containing 2% bovine serum albumin (2% PBS-BSA) for 2 h at 37°C. After washing, plates were incubated with serial dilutions of monkey sera in 0.5% Tween20 in 2% PBS-BSA (dilution buffer) for 1 h at 37°C and washed. Plates were then incubated with alkaline phosphatase-conjugated anti-monkey IgG (Sigma, St Louis, MO) for 1 h at 37°C, and enzyme activity was detected by adding substrate solution prepared using Fast p-Nitrophenyl phosphate tablets (Sigma). OD₄₁₀ values were read by an ELISA plate reader (Dynex Technologies, Chantilly, VA). Antibody titers are expressed as the end-point dilution giving an OD₄₁₀ value $\geq$ a cutoff OD₄₁₀ value determined as 2 SD above the mean OD from sera of 6 naïve animals. Similar tests were performed for mucosal anti-IgG and IgA, except the secondary antibody to test for IgA was replaced with alkaline phosphatase-conjugated anti-monkey IgA (Rockland Immunochemicals Inc., Gilbertsville, PA). To test for anti-*Lm* antibodies, an ELISA using either whole bacteria (*Lm* strain 12443) [42] or recombinant listeriolysin-O (LLO) as antigen was employed; Immulon 2 microtiter plates were coated with LLO at 0.1 μ g/well. *E. coli*-produced recombinant 6X his-tagged LLO was purified from culture medium using a Ni-NTA column (Qiagen, Inc., Valencia, CA) and tested for hemolytic activity as described [43].

3. Results

3.1. Primary immunization: Pharmacokinetics of D-ala and vaccine safety

Two experimental and one control group of macaques were enrolled to test safety and immunogenicity of attenuated *Lmdd-gag* expressing HIV *gag* (Fig. 1). Group 1, referred to as oral-only group, received *Lmdd-gag* orally 3 times. Group 2, referred to as oral/i.m. boost group, received *Lmdd-gag* twice orally followed by a single i.m. boost. Controls were given empty vector only by oral administration. All animals were given D-ala (640 mg/kg) i.v. 15 min before and 2.5 h after each immunization to allow replication of the double-mutant *Lmdd* organisms deficient in endogenous D-ala synthesis. D-ala was also present in all oral *Lmdd-gag* or *Lmdd* inocula. The pharmacokinetics of i.v. administered D-ala in two animals are shown in Fig 2. Serum D-ala reached peak levels ($>1000 \mu\text{g/ml}$) approximately 15 min after the first i.v. injection followed by a second peak ($>2000 \mu\text{g/ml}$) 15 min after the second i.v. injection. Serum D-ala levels in both animals decreased within 24 h and were undetectable after 48 h.

None of the animals showed adverse clinical effects from the immunization. Stools were tested for bacterial growth on D-ala-containing plates and were negative. There were some minor increases in muscle enzymes in some animals (data not shown). However, these were believed due to i.m. ketamine anesthesia procedures, as similar increases were seen in non-immunized, anesthetized animals. These results demonstrated that attenuated *Lmdd* and D-ala administration was safe and well tolerated in macaques.

3.2. Cellular immune responses

All animals in the oral-only group that received 3 rounds of oral *Lmdd-gag* showed Gag-specific T-cell responses as measured by IFN- γ ELISPOT analysis after prolonged in vitro stimulation with pooled HIV Gag peptides (Fig. 3A). Two animals, RDh-8 and RLh-8, began to develop Gag-specific T-cell responses after the second immunization. These responses decreased with time but rebounded with a third *Lmdd-gag* administration. The other two animals, RIg-8 and ROg-8, only showed Gag-specific ELISPOT responses after the third *Lmdd-gag* immunization. Control animals given empty *Lmdd* and animals of the oral/i.m. group given 2 oral immunizations followed by i.m. immunization with *Lmdd-gag* had no detectable ELISPOT responses to HIV Gag peptide stimulation at any time points tested.

HIV Gag-specific T-cell proliferative responses were observed in 3 of the 4 animals of Group 1 (Fig. 4A); significant proliferative responses were detectable at the time of the 2nd oral boost and were maintained following the boost. One of the two oral/i.m. boost animals (RMg-8) showed a transient but significant HIV Gag-specific T-cell proliferative response after the second oral immunization, which rose following i.m. boosting. The ELISPOT data and the proliferation results demonstrate that oral administration of macaques with attenuated *Lmdd-gag* induced Gag-specific cellular immune responses.

3.3. Antibody responses

Immunized animals were tested for antibodies to HIV Gag and to *Lm* (Tables 1 and 2). Oral immunization alone induced no detectable anti-Gag antibodies, even after 3 rounds of vaccination. In contrast, the 2 animals that received oral/i.m. immunization developed anti-Gag IgG responses, which were detectable only after the i.m. boost. This demonstrates that, in addition to inducing cellular immunity, *Lmdd-gag* inoculation can also induce Gag-specific antibody production, which appears dependent upon the route of vaccine administration.

Antibody responses to the vector itself were also measured in each animal (Table 2); 2 or 3 rounds of oral administration of either *Lmdd-gag* or *Lmdd* induced minimal anti-*Listeria* IgG

responses that were only 2–4x above background and detectable in only 5 of the 9 total animals. In contrast, i.m. boosting with *Lmdd-gag* raised anti-*Listeria* IgG titers substantially. These results demonstrate that the route of administration also affects the induction of anti-vector humoral immune responses.

3.4. Boosting after prolonged rest

The above data indicated that macaques inoculated with *Lmdd-gag* developed antibody responses to the *Lmdd* vector itself, albeit at different levels depending upon the route of inoculation. With the possibility that such antibodies may limit the development of a robust antigen-specific immune response to *Lmdd-gag* boosting, we sought to test if anti-vector immunity might prevent an anamnestic Gag-specific boosting effect upon *Lmdd-gag* boosting after a prolonged rest period. To evaluate this possibility, after waiting at least 27 weeks since the last *Lmdd-gag* inoculation, each animal from the primary immunization experiments was re-immunized exactly as it had been at the wk 19 time point of the primary set of immunizations shown in Fig. 1. Thus, the oral-only group was boosted orally and oral/i.m boost group was boosted i.m. with 3×10^{12} and 10^{12} *Lmdd-gag* organisms, respectively.

At the time of this late *Lmdd-gag* boosting after a >27 week rest period (week 39 overall), Gag-specific T-cell immune responses in all animals were at background levels (Fig. 3B and Fig. 4B). Oral boosting with *Lmdd-gag* induced a significant rise of Gag-specific IFN- γ -secreting T cells in 2 of 4 animals (RDh-8 and ROg-8) at 2 weeks after this late boost (Fig. 3B). IFN- γ ELISPOT reactivity of these 2 animals decreased slightly by wk 8 post-boost, at which time ELISPOTs were detected in a 3rd animal (RLh-8). However, one animal that showed high ELISPOT reactivity following the 3rd immunization 20 weeks earlier, RIg-8, had no increase in Gag peptide-reactive ELISPOT counts with this last boost. This animal, along with RDh-8 and RLh-8, had low but detectable levels of anti-*Lm* IgG prior to boosting. None of the animals that were boosted i.m. with *Lmdd-gag* showed an increase in IFN- γ ELISPOTs.

Gag-specific T-cell proliferative responses in all animals, whether boosted with *Lmdd-gag* orally or i.m., increased from an SI of 1 at the time of boost to 4.2 – 20 at 2 weeks post-boost (Fig. 4B). It is notable that ROg-8, the animal with the highest Gag-specific T-cell proliferative response at both weeks 2 and 8 post-boost, had no evidence of anti-vector IgG.

3.5. Serum and mucosal antibody levels after 4th immunization (following prolonged rest)

Similar to the first three immunizations, serum anti-HIV Gag antibodies were only detected in animals boosted i.m. (Table 3, animals REg-8 and RMg-8). The antibody titers reached peak levels at week 2 and declined to baseline by week 8.

The induction of Gag-specific antibodies at mucosal sites was also examined. Saliva, vaginal and rectal fluids were tested for HIV Gag-specific IgG and IgA before and after oral or i.m. boosting with *Lmdd-gag* following the prolonged rest. Animals in the oral/i.m. group (REg-8 and RMg-8) developed significant levels of vaginal anti-HIV Gag IgG antibodies by 2 weeks after the i.m. *Lmdd-gag* boost (Fig. 5A); monkey REg-8 also had measurable anti-Gag IgG levels in rectal fluid (Fig. 5B). These antibody titers subsequently dropped to background levels by 8 weeks post-boost (Fig. 5A and 5B). In contrast, only borderline or undetectable levels of anti-HIV Gag IgG were seen in vaginal or rectal fluids of the oral-only group of animals (data not shown). Anti-HIV Gag-specific IgA antibodies in vaginal and rectal fluids and in saliva of all animals were negative as were anti-HIV Gag IgG antibodies in saliva of all animals (data not shown).

4. Discussion

Here we examined the use of the live attenuated *Lmdd* vector as an HIV antigen delivery vehicle in a relevant animal model of AIDS vaccine development. The major findings of this study are: 1) *Lmdd-gag* and D-ala were safe in monkeys, 2) vaccination with *Lmdd-gag* solely by oral administration generated Gag-directed cellular immune responses, 3) vaccination with *Lmdd-gag* by oral priming followed by i.m. boosting predominantly generated HIV Gag-specific humoral immunity, with anti-HIV Gag IgG also detected in vaginal fluids.

A large body of evidence suggests that the generation of strong cellular immune responses will be an important requisite for any HIV/AIDS vaccine to be optimally effective. The basic premise of the current study, to deliver HIV antigens by using a microbial vector that naturally induces high levels of mucosal and systemic CD4⁺ and CD8⁺ T-cell mediated immune responses, therefore represents an intriguing approach for vaccine development. However, the safety of any attenuated *Lm* vector is an important issue. *Lm* poses potential risks for pregnant women, neonates and immunocompromised individuals [44]. The attenuated *Lm* vector used here, *Lmdd-gag*, was shown previously to be safe and immunogenic in adult and neonatal mice [34,35]. Here, we demonstrated that *Lmdd-gag*, as well as accompanying D-ala, were safe in monkeys when administered at levels that were able to induce Gag-specific immune responses.

Recently, Boyer et al. tested recombinant *Lm* as part of a vaccine regimen in macaques [45]. The *Lm* vectors were modified to secrete SIV Gag and Env. Following oral administration of recombinant *Lm*, moderate antigen-specific T-cell responses were noted but no significant protection from intrarectal challenge with SIVmac239 was obtained. Stronger T-cell reactivity and a moderate level of virus containment were noted when the recombinant *Lm* were used to boost DNA-primed animals. Unlike the *Lmdd-gag* used in our study, the vectors used by Boyer et al. were not attenuated and while healthy monkeys tolerated the recombinant *Lm* inoculations, the use of such a vaccine vector could raise safety concerns in pregnant women or immunocompromised hosts. A direct comparison of that study with ours is made difficult by the fact that we were able to immunize with approximately 100-fold higher numbers of bacteria due to the lower virulence of *Lmdd-gag* compared to wild-type *Lm*. Furthermore, our vector encoded HIV, not SIV *gag*. Therefore, we were unable to perform challenge experiments with SIV or simian-human immunodeficiency virus (SHIV).

An appealing aspect of using attenuated *Lm* as vaccine delivery vehicles is that the natural means of transmission is oral. An oral anti-HIV vaccine would have certain advantages over one requiring parental administration. Administering an efficacious vaccine to large, diverse populations, especially in third world countries where HIV infection rates may be as high as 20%, would be significantly easier with an oral vaccine. As the predominant means of HIV transmission is through mucosal exposure, a potential secondary benefit of our *Lmdd* vector would be the possibility of inducing HIV-directed mucosal immunity through oral vaccination. Of note, evidence in both humans and nonhuman primates has shown that CD4⁺ memory T cells in the gastrointestinal tract are a major target for infection and depletion during acute immunodeficiency virus infection [46] [47]. Oral immunization in many systems, including immunodeficiency virus transmission models, can generate high levels of mucosal immune responses [48–50]. As our pilot study was undertaken primarily to assess the safety of live attenuated *Lmdd-gag* in monkeys, we did not directly sample mucosal sites for cellular immunity and were limited to assessing PBMC, which clearly showed Gag-directed cellular immune responses. Future studies will investigate mucosal immune responses to determine whether the same high levels of antigen-specific cellular immune responses can be generated at mucosal sites in primates as have been demonstrated in mice [34].

One issue of concern for potentially using live vectors for vaccination is whether pre-existing anti-vector immunity, either occurring naturally or through prior vaccination, will affect the immunogenicity of the neoantigen. Indeed, a previous macaque study found no boosting of T-cell responses following a 3rd immunization using SIV *gag* and *env*-encoding *Lm*; while anti-*Listeria* antibody levels were not reported, the authors suggested that anti-vector immunity induced by the previous two immunizations prevented boosting of SIV antigen-specific responses [45]. In our study, we detected minimal induction of anti-vector antibodies in animals given *Lmdd-gag* or *Lmdd* orally. In addition, and of importance for designing practical vaccine scheduling, after a rest period of at least 5 months, three of four re-immunized animals were able to mount Gag-specific cellular immune responses, despite the earlier presence of low-level but detectable anti-vector antibodies. These results are consistent with those seen by Stevens et al. [28] and suggest that pre-existing anti-*Lm* immunity does not preclude the use of this vector for vaccine delivery.

An important finding in this study is that the route of *Lmdd-gag* delivery directly affected the generation of either cellular or humoral immune responses in macaques. Thus, by IFN- γ ELISPOT analysis, animals of the oral/i.m. boost group failed to develop significant Gag-specific T-cell immunity, yet mounted Gag-specific antibody responses following i.m. boosting. In contrast, animals of the oral-only group failed to develop detectable anti-Gag IgG, yet developed Gag-directed T-cell immune responses. The results are consistent with the idea that the i.m. administration shifted the HIV Gag-directed immunity from a predominant Th1 cellular response to a predominant Th2-type humoral response. Indeed, in some of our preliminary tests, we found that while oral/oral immunization induced mostly Th1 cytokines (IFN- γ , IL-2), some of the oral/im immunized animals did generate Th2 cytokines (IL-4 and IL-10) in addition to Th1 cytokines (data not shown). The rise in Gag-specific T-cell proliferation in the oral/im immunization group is consistent with this premise as both Th1 and Th2-type Gag-specific CD4⁺ helper T-cell subsets would show proliferative responses to whole protein antigen. In many systems, the route by which animals are exposed to foreign antigens influences the induction of Th1 versus Th2-mediated immune responses and corresponding cellular versus humoral immunity. Any generalizations, however, need to take into account the nature, dose and timing of antigen exposure. For example, in two separate studies measuring either hepatitis B virus or *Hemophilus influenza* hemagglutinin antibody responses in mice, DNA immunization given i.m. induced Th1-like antibody responses while gene-gun delivery (with much lower DNA doses) to skin induced Th2-like antibody responses [51,52]. Additionally, exposure to proteins via mucosal surfaces tends to induce humoral, rather than cell-mediated immunity [53,54] yet natural exposure to live *Lm* through oral infection induces strong cellular immunity [55]. Our results provide additional data indicating that the route of antigen exposure influences the nature of the predominant immune response.

We chose live attenuated *Lm* as a vaccine delivery vehicle in order to induce HIV Gag-directed cellular immunity. Our results open the possibility that this same vector may be used to induce either cellular or humoral immunity, depending on the route of vaccination. However, we have not induced both types of immunity in the same host, because the immune responses are so highly skewed by the immunization route toward either cellular or humoral responses.

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Fig. 1.

Primary immunization schedule for *Lmdd-gag* administration to rhesus macaques. Animals were enrolled into 3 groups and given *Lmdd-gag* or *Lmdd* at 0, 6 and 19 weeks (vertical arrows) as noted. The number of organisms administered at each time point is shown in parentheses for each group. All animals were given D-ala (640 mg/kg) i.v. at 15 min prior to and 2.5 hrs after each immunization.

Fig. 2. Pharmacokinetics of D-ala in rhesus macaques. Serum D-ala levels are shown for two representative animals receiving oral *Lmdd-gag* and accompanying i.v. and oral D-ala as noted in Fig. 1.

Fig. 3.

Gag-specific IFN- γ -secreting T cells by ELISPOT assay in *Lmdd-gag*-immunized macaques. **A.** PBMC from individual monkeys were tested at the indicated time points for Gag-specific IFN- γ secreting T cells after in vitro stimulation with overlapping HIV Gag peptides. **B.** Gag-specific IFN- γ -secreting T cells in *Lmdd-gag*-boosted macaques following prolonged rest (the interval between the week 19 boost and the final boost ranged from 27–64 weeks because of staggered enrollment).

Fig. 4.

Gag-specific T-cell proliferative responses in *Lmdd-gag*-immunized macaques. *A*, PBMC from individual monkeys were tested for Gag-specific T-cell proliferative responses at the indicated time points during and after vaccination. Stimulation indices (SI) were calculated as described. *B*, Gag-specific T-cell proliferative responses in *Lmdd-gag* boosted macaques following prolonged rest. The dotted horizontal line at SI of 4 denotes the cut-off for a positive response.

Fig. 5. Gag-specific antibody titers in vaginal and rectal fluids. Vaginal and rectal fluid samples were obtained from animals REg-8 (circles) and RMg-8 (triangles) at the indicated time points following the final i.m. inoculation with *Lmdd-gag*. Fluid samples were tested for anti-Gag IgG levels by ELISA. *A*, Vaginal fluid samples. *B*, Rectal fluid samples. The background OD readings (determined as 2 SD above the mean OD from samples of 6 naïve animals) are indicated by the horizontal dotted line.

Table 1

Serum Anti-HIV Gag IgG ELISA Titers

Groups (Monkey #)	Vaccine	Weeks after the first immunization							
		0	6	10	14	20	21	25	30
Oral Only	<i>Lmdd-gag</i>	<100	<100	<100	<100	<100	<100	<100	<100
Rig-8		<100	<100	<100	<100	<100	<100	<100	<100
RDh-8		<100	<100	<100	<100	<100	<100	<100	<100
RLh-8		<100	<100	<100	<100	<100	<100	<100	<100
ROg-8		<50	<50	<50	<50	<50	<50	<50	<50
Oral/Lm.	<i>Lmdd-gag</i>	<100	<100	<100	<100	3,200	3,200	3,200	3,200
REg-8		<50	<50	<50	<50	51,200	51,200	3,200	3,200
Control (oral only)	<i>Lmdd</i> (vector only)	<50	<50	<50	<50	<50	<50	<50	<50
RSg-8		<50	<50	<50	<50	<50	<50	<50	<50
RUG-8		<50	<50	<50	<50	<50	<50	<50	<50
RMh-8		<50	<50	<50	<50	<50	<50	<50	<50

Table 2
Serum Anti-*Lm* IgG ELISA Titers

Groups (Monkey #)	Vaccine	Weeks after the first immunization							
		0	6	10	14	20	21	25	30
<i>Lmddl-gag</i>									
Oral Only									
RIg-8		<400	<400	<400	<400	<400	<400	800	800
RDh-8		ND	1,600	800	800	800	ND	800	800
RLh-8		ND	1,600	1,600	1,600	ND	ND	<400	800
ROg-8		<800	ND	ND	<800	<800	<800	ND	ND
Oral/Lm.									
REg-8	<i>Lmddl-gag</i>	<800	ND	ND	<800	1,600	24,000	ND	ND
RMg-8		<400	<400	<400	<400	25,600	25,600	ND	24,000
<i>Lmddl</i> (vector only)									
Control (oral only)									
RSg-8		1,600	ND	ND	1,600	<800	1,600	ND	ND
RUG-8		<800	ND	ND	<800	<800	1,600	ND	ND
RMh-8		<800	ND	ND	1,600	<800	ND	ND	ND
ND, not done									

ND, not done

Table 3
Serum Anti-HIV Gag titers after the 4th immunization (after prolonged rest)

Groups (Monkey #)	Vaccine	Weeks after the 4 th immunization			
		0	2	4	8
Oral Only					
RIg-8	<i>Lmddt-gag</i>	<100	<100	<100	<100
RDh-8		<100	<100	<100	<100
RLh-8		<100	<100	<100	<100
ROg-8		<100	<100	<100	<100
Oral/Im.					
REg-8	<i>Lmddt-gag</i>	100	3,200	800	<100
RMg-8		100	3,200	1,600	<100
Control (oral only)					
RSg-8	<i>Lmdd</i> (vector only)	<100	<100	<100	<100
RUg-8		<100	<100	<100	<100
RMh-8		<100	<100	<100	<100

ND, not done