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* is safe and immunogenic in primates.

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Keywords: Rhesus monkey; *Listeria monocytogenes*; d-alanine; Cellular immunity; Vaccine

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

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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. *Listeria monocytogenes* 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 *infB* 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 five 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]. *Lmdl* 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 × 104 organisms. The LD50 of *Lmdd* was >8 × 108 when inoculated i.v. alone or, when inoculated i.v. in the presence of 20 mg d-ala, was approximately 7 × 107 organisms [32] or higher (unpublished data). The LD50 following intragastric inoculation of mice is unknown for either organism, but probably exceeds 1010 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 in Ref. [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, RLg-8, RDh-8, RHl-8 and ROg-8) received 1 × 1012 *Lmdd-gag* in whipping cream orally at week 0 and 3 × 1012 *Lmdd-gag* at weeks 6 and 19. Group 2 (two animals, REg-8 and RMg-8) was given *Lmdd-gag* orally in whipping cream at weeks 0 and 6 (1 × 1012 organisms each) and at week 10, 1 × 1012 *Lmdd-gag* intramuscularly (i.m.). Group 3 (three animals, RSg-8, RUg-8 and RMh-8), received empty vector (*Lmdd*, 1 × 1012 organisms) in whipping cream orally at week 0 and 3 × 1012 organisms at weeks 6 and 19. All oral inocula of *Lmdd-gag* or *Lmdd* also contained d-ala (0.5 mg/ml in a
Fig. 1. Primary immunization schedule for Lmdd-gag administration to rhesus macaques. Animals were enrolled into three 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 h after each immunization.

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-alan 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^-4 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 h or at 56°C 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 in Refs. [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 x 10⁶/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 µg/ml) for 5 days. Cells were pulsed with ³H-thymidine (1 µCi per well) (Perkin-Elmer, 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 µl of HIV Gag (1 µg/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 ≥ a cut-off OD₄₁₀ value determined as 2 S.D. above the mean OD from sera of six 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 μg/well. Escherichia coli-produced recombinant 6× his-tagged LLO was purified from culture medium using a Ni-NTA column (Qiagen Inc., Valencia, CA) and tested for hemolytic activity as described in Ref. [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 Lmdg-gag expressing HIV gag (Fig. 1). Group 1, referred to as oral-only group, received Lmdg-gag orally three times. Group 2, referred to as oral/i.m. boost group, received Lmdg-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 Lmdg organisms deficient in endogenous d-ala synthesis. D-Ala was also present in all oral Lmdg-gag or Lmdg inocula. The pharmacokinetics of i.v. administered d-ala in two animals are shown in Fig. 2. Serum d-ala reached peak levels (>1000 μg/ml) approximately 15 min after the first i.v. injection followed by a second peak (>2000 μg/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 Lmdg 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 three rounds of oral Lmdg-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. 3(A)). 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 Lmdg-gag administration. The other two animals, Rlg-8 and ROg-8, only showed Gag-specific ELISPOT responses after the third Lmdg-gag immunization. Control animals given empty Lmdg and animals of the oral/i.m. group given two oral immunizations followed by i.m. immunization

Fig. 2. Pharmacokinetics of d-ala in rhesus macaques. Serum d-ala levels are shown for two representative animals receiving oral Lmdg-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 Lmdg-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 Lmdg-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).
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 three of the four animals of Group 1 (Fig. 4(A)); significant proliferative responses were detectable at the time of the second 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 three rounds of vaccination. In contrast, the two 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); two or three rounds of oral administration of either Lmdd-gag or Lmdd induced minimal anti-Listeria IgG responses that were only 2–4× above background and detectable in only five of the nine 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

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**Table 1**

<table>
<thead>
<tr>
<th>Groups</th>
<th>Vaccine</th>
<th>Weeks after the first immunization</th>
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<tr>
<td>Oral-only</td>
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<tr>
<td>Rlg-8</td>
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<tr>
<td>Rdh-8</td>
<td></td>
<td>&lt;100</td>
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<tr>
<td>Rlh-8</td>
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<td>&lt;100</td>
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<tr>
<td>Rog-8</td>
<td>&lt;50</td>
<td>&lt;50</td>
</tr>
<tr>
<td>Oral/i.m.</td>
<td>Lmdd-gag</td>
<td>&lt;100</td>
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<tr>
<td>Reg-8</td>
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<td>&lt;100</td>
</tr>
<tr>
<td>Rmg-8</td>
<td>&lt;50</td>
<td>&lt;50</td>
</tr>
<tr>
<td>Control (oral-only)</td>
<td>Lmdd (vector only)</td>
<td>&lt;50</td>
</tr>
<tr>
<td>Rsg-8</td>
<td>&lt;50</td>
<td>&lt;50</td>
</tr>
<tr>
<td>Rmg-8</td>
<td>&lt;50</td>
<td>&lt;50</td>
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</tbody>
</table>

Monkey #: Rlg-8, Rdh-8, Rlh-8, Rog-8, Reg-8, Rmg-8, Rsg-8, RUG-8 and RMh-8.
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 week 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 $3 \times 10^{12}$ 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 (Figs. 3 and 4(B)). Oral boosting with Lmdd-gag induced a significant rise of Gag-specific IFN-γ-secreting T cells in two of four animals (RDh-8 and ROg-8) at 2 weeks after this late boost (Fig. 3(B)). IFN-γ ELISPOT reactivity of these two animals decreased slightly by wk 8 post-boost, at which time ELISPOTs were detected in a third 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. 4(B)). 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 fourth 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

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**Table 2**

<table>
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**Table 3**

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<td>ROg-8</td>
<td>&lt;100</td>
</tr>
<tr>
<td>Oral/i.m.</td>
<td>REg-8</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>RMg-8</td>
<td>100</td>
</tr>
</tbody>
</table>

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Monkey #: RIg-8, RDh-8, RLh-8, ROg-8, REg-8, RMg-8, RSg-8, RUg-8 and RMh-8. ND, not done.
This study are: (1) vaccination with Lmdd-gag solely by oral administration generated Gag-directed cellular immune responses, (2) 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 Ln vector is an important issue. Ln poses potential risks for pregnant women, neonates and immunocompromised individuals [44]. The attenuated Ln 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 Ln as part of a vaccine regimen in macaques [45]. The Ln vectors were modified to secrete SIV Gag and Env. Following oral administration of recombinant Ln, 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 Ln 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 Ln 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 Ln. 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 Ln 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 immun-
odeficiency virus infection [46,47]. Oral immunization in many systems, including immunodeficiency virus transmis-
sion models, can generate high levels of mucosal immune 
responses [48–50]. As our pilot study was undertaken pri-
marily to assess the safety of live attenuated Lmdd-gag in
monkeys, we did not directly sample mucosal sites for cellu-
lar 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 pre-
vious 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 boost-
ing 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 con-
sistent 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. Our data suggest that
prior antibody responses to the vaccine vector, but not cel-
lar responses, likely interfere with development of cellular
immune responses to neoantigens.

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-
gamma 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 pre-
dominant 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/i.m. 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/i.m.
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 cellu-
lar versus humoral immunity. Any generalizations, however,
need to take into account the nature, dose and timing of anti-
gen exposure. For example, in two separate studies measuring
either hepatitis B virus or Hemophilus influenzae hemagglu-
tinin 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 immu-
nity [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.

Disclosures

Dr. Fred Frankel is listed as co-inventor on a University
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