Vaginal Protection and Immunity after Oral Immunization of Mice with a Novel Vaccine Strain of Listeria monocytogenes Expressing Human Immunodeficiency Virus Type 1 gag

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Natural transmission of human immunodeficiency virus (HIV) occurs at mucosal surfaces. During acute infection, intestinal and other mucosa are preferential sites of virus replication and rapidly become depleted of CD4 T cells. Therefore, mucosal immunity may be critical to control both initial infection and the massive early spread of virus. An attenuated β-alanine-requiring strain of the oral intracellular microorganism Listeria monocytogenes expressing HIV type 1 gag was shown to induce protective cell-mediated immunity in mice against viruses that express HIV gag when immunization occurs in the presence of a transient supply of β-alanine. In this study, we examined the efficacy of new attenuated strains that are able to synthesize β-alanine from a heterologous dal gene tightly regulated by an actA-promoted resolvable recombination system. In the absence of β-alanine, Gag-specific cytotoxic T lymphocytes (CTLs) were induced systemically after intravenous immunization, and one strain, Lmdv-gag/pARS, induced strong dose-dependent Gag-specific CTLs after oral immunization. A significant level of Gag-specific CD8+ T cells was induced in the mucosal-associated lymphoid tissues (MALTs). Upon intravaginal challenge of these orally immunized mice with recombinant vaccinia virus (rVV) expressing HIV gag, gamma interferon- and tumor necrosis factor alpha-secreting Gag-specific CD8+ T cells were dramatically increased in the spleen and MALTs. Oral immunization with Lmdv-gag/pARS led to complete protection against vaginal challenge by a homologous clade B gag-expressing rVV. In addition, strong cross-clade protection was seen against clades A and C and partial protection against clade G gag-expressing rVV. These results suggest that Lmdv-gag/pARS may be considered as a novel vaccine candidate for use against HIV/AIDS.

With over 42 million people currently infected, human immunodeficiency virus (HIV)/AIDS is now the most widespread pandemic in human history (52). Although HIV spreads systemically, 90% of infections are contracted through sexual transmission, with the virus crossing the mucosal lining of the genital tract, rectum, and oropharyngeal cavities (35). HIV not only subverts the immune system by rapidly infecting and depleting CD4+ T cells, predominantly memory CD4+ T cells of mucosal surfaces and other tissues (5, 19, 28, 31, 33), but with time becomes an integral part of the genetic material of its host’s cells. Thus, there is an urgent need for rapid intervention.

Vaccinology has made a major contribution to the eradication or management of infectious diseases (21). In developing a vaccine against HIV/AIDS, it is important to induce protective local immunity that can act at mucosal surfaces as well as in the systemic compartment. While neutralization antibodies have shown the ability to block HIV/simian immunodeficiency virus (13, 51), they are difficult to elicit. However, cellular immunity plays a critical role in controlling acute infection and maintaining a low chronic viral set point. Therefore, protective antiviral cytotoxic T lymphocytes (CTLs), particularly in the mucosal compartment, should be a major component of any immune response induced by an effective vaccine against HIV/AIDS.

Listeria monocytogenes is a gram-positive, facultative, intracellular, food-borne bacterium that has served for decades as a model pathogen for the study of cell-mediated immunity (12, 26). Subsequent to infection and uptake by phagocytic cells, unlike other intracellular organisms such as Salmonella spp. and Mycobacterium tuberculosis, a number of bacteria escape the endocytic vacuoles and replicate in the cytoplasm. Since foreign genes can be easily and stably inserted into the L. monocytogenes chromosome and express at high levels, such gene products are efficiently delivered via the major histocompatibility complex (MHC) class I and MHC class II pathways and induce strong CTL and CD4 help (4, 37, 43, 47, 50). L. monocytogenes has been explored as a live vaccine against M. tuberculosis (34), model cancers (6, 7, 20, 23, 36, 39), lymphocytic choriomeningitis (17, 48), and influenza virus infection (22). Since the natural route of L. monocytogenes infection is through the intestinal mucosa following oral ingestion of the organism, the induction of mucosal immunity is a natural consequence of infection. In fact, mice immunized orally with recombinant L. monocytogenes that expressed influenza nucleoprotein showed regression of established renal and colorectal tumors that expressed the same nucleoprotein antigen (38). Also, orally administered recombinant L. monocytogenes strains that express the HIV type 1 (HIV-1) gag gene result in protection against challenge by recombinant vaccinia virus (rVV) expressing HIV-1 gag (rVV-gag) (46).
Preexisting immunity against a vector could interfere with the generation of an immune response against a foreign antigen expressed by that vector. However, the annual incidence of listeriosis is very low, and few individuals are expected to have preexisting immunity. In addition, existing antilisterial immunity does not appear to inhibit the development of an *L. monocytogenes*-specific primary CTL response or diminish its therapeutic efficacy (2, 49).

Nevertheless, *L. monocytogenes* is itself a pathogen that can cause fatal infections, particularly in immunocompromised or pregnant individuals. Therefore, we have focused on methods to attenuate the organism while still retaining its immunogenicity. We initially constructed an attenuated strain, Lmdd, by deleting the two genes, *dai* and *dat*, used for d-alanine biosynthesis and cell wall formation. A recombinant, Lmdd-gag, was shown to be a safe and promising AIDS vaccine candidate (46). However the immunogenicity of this strain is achieved by a transient supply of d-alanine provided along with the vaccine. To obviate this requirement, we constructed several derivative strains of Lmdd that are able to supply their own d-alanine but under highly restrictive conditions (29, 55). In this study, we evaluated a d-alanine-independent strain of Lmdd-gag designed to generate Gag-specific CTL responses in mucosal-associated lymphoid tissues (MALTs), which are critical for the initial control of HIV infection and spread. We showed that intragastic inoculation of mice with this new attenuated strain, Lmdd-gag/pARS, generated potent mucosal and systemic Gag-specific CTL responses. In addition, we provide new evidence that CTLs induced in the mucosal and systemic lymphoid compartments result in cross-clade protection against intravaginal virus challenge.

**MATERIALS AND METHODS**

*Bacteria and plasmids.* The double deletion strain Lmdd-gag and its wild-type parent, Lm-gag, have been described previously (15, 16). Plasmids pRRR, pKRS, and pARS (see below) were individually introduced into Lmdd-gag by electroporation according to established protocols (40), and the resulting transformants, Lmdd-gag/pRRR, Lmdd-gag/pKRS, and Lmdd-gag/pARS, were selected on brain heart infusion (BHI) agar plates containing 10 μg/ml chloramphenicol. All bacterial strains were maintained as 80°C stocks, streaked onto BHI agar plates with or without d-alanine (200 μg/ml) or chloramphenicol (10 μg/ml) as necessary, and grown in BHI medium at 30°C or 37°C for aeration. For cultures used for infection of mice, an overnight culture of bacteria was diluted 1:10 into appropriate BHI medium, grown with shaking at 37°C for 3 to 4 h, harvested in 10% FCS. 

**Mouse immunization.** Six to 8-week-old female BALB/c mice were obtained through the National Cancer Institute (Frederick, MD). The animals were immunized with recombinant *L. monocytogenes* systemically (intravenous [i.v.]) with ~10^7 CFU for primary infection and with ~10^4 CFU for a booster infection. Oral immunization was performed intragastrically by gavage to deliver ~2 × 10^9 CFU in 200 μl of BHI for primary infections and/or boosted once or twice with ~10^6 CFU of bacteria. The interval between immunizations was 3 weeks. Actual CFU was assessed following infection by plating dilutions of the inoculum. The animals were not fasted or neutralized for pH before immunization. All procedures used in this study complied with federal guidelines and institutional policies of the University of Pennsylvania Animal Care and Use Committee.

**Isolation of lymphocytes.** Spleens, lymph nodes (cervical, mesenteric, and iliac) and Peyer’s patches (PP) were removed from sacrificed mice. Splenocyte suspensions were obtained by pressing the tissue through a nylon mesh screen, followed by lysing of red blood cells using ACK lysis buffer (0.15 M NH_4Cl, 1 mM KHCO_3, 0.1 mM EDTA [pH 7.2]). Lymphocytes from pooled lymph nodes or PP were prepared as described previously (46, 54). Briefly, tissues were digested with collagenase D and DNase I at 37°C for 30 min and then incubated in the presence of 5 mM EDTA for 5 min. A single-cell suspension was prepared by teasing the digested tissues into suspension and filtering through nylon mesh to remove debris.

**Cytometric analysis.** Surface staining was performed using freshly prepared single-cell suspensions of splenocytes. The cells were stained in 1% (wt/vol) fetal calf serum (FCS)–phosphate-buffered saline (PBS) for 60 min at 4°C with fluorescent isothiocyanate-conjugated anti-CD11a (clone M17/4; eBioscience, San Diego, CA), phycoerythrin-conjugated anti-CD8 (clone 53-6.7; BD Pharmingen, San Diego, CA), and allophycocyanin-conjugated H-2Kk–MHC class I–listeriolysin O (LLO)–ssrA or Gag<sup>197–205</sup> tetramers (MHC Tetramer Core Facility, National Institute of Allergy and Infectious Diseases, Atlanta, GA). Cells were being stained, the cells were washed with PBS, fixed in 2% (wt/vol) paraformaldehyde-PBS, and analyzed with a FACS caliber flow cytometer. The data were further analyzed using Flowjo software (Tree Star, Inc.).

**Intracellular detection of IFN-γ and TNF-α.** Intracellular cytokine staining was performed using a BD cytotoxic/cytokine kit according to the manufacturer’s protocol. Briefly, lymphocytes were isolated from the indicated tissues and cultured for 5 h with Golgistop, with or without 5 μg/ml peptide. After culture, cells were stained for surface molecules and fixed and cell membranes were permeabilized in cytofix/cytoperm solution and stained with fluorescein isothiocyanate-conjugated anti-gamma interferon (IFN-γ) (clone XMG 1.2; BD Pharmingen, San Diego, CA) and allophycocyanin-conjugated anti-tumor necrosis factor alpha (TNF-α) (clone MIP-6-T2X22; eBioscience, San Diego, CA) in Perm/Wash solution. Cells were then washed with Perm/Wash solution, and the fluorescence intensity was measured with a FACS caliber flow cytometer.

**CFSE labeling of cells.** Splenocytes isolated from naive BALB/c mice were suspended in PBS at a concentration of 2 × 10<sup>7</sup> cells/ml and warmed to 37°C. Cells were incubated for 10 min with 3 μM or 0.3 μM 5-(and 6-)carboxyfluorescein diacetate, succinimidyl ester (CFSE) (Molecular Probes, Eugene, OR) in the dark, and then an equal volume of 100% FCS was added and incubated at room temperature for 1 min, followed by two washes with RPMI 1640 medium with 10% FCS.

**In vivo CTL assay.** The in vivo CTL assay was performed essentially as described previously (9, 53). BALB/c splenocytes were labeled with either a low (0.3 μM) (CFSE<sub>low</sub>) or a high (3 μM) (CFSE<sub>high</sub>) concentration of CFSE, and CFSE<sub>high</sub> cells were further incubated with 5 μg/ml HIV-1 Gag<sup>197–205</sup> LLO<sub>99–99</sub> or lymphocytic choriomeningitis virus nucleoprotein 118–126 (LCMV) (CFSE<sub>low</sub>) or LCMV NP<sub>118–126</sub> peptides for 1 h at 37°C in the dark. Following incubation, the cells were washed and equal numbers (1 × 10<sup>6</sup>) of each population were mixed in 200 μl of PBS and injected i.v. into previously immunized or naive mice. Sixteen hours later, spleens from recipients were analyzed for the presence of CFSE<sub>low</sub> and CFSE<sub>high</sub> populations. In vivo killing was indicated by percent specific lysis, calculated according to the following formula: percent lysis = (1 – ratio unprimed/ratio primed) × 100, where the ratio is percent CFSE<sub>low</sub>/CFSE<sub>high</sub>.

**ELISPOT assay for IFN-γ-secreting cells.** Enzyme-linked immunospot (ELISPOT) assays were conducted according to the manufacturer’s protocol (R&D Systems, Minneapolis, MN), with minor modifications. Briefly, ELISPOT 96-well plates (Millipore, Bedford, MA) were coated with anti-mouse IFN-γ capture antibody (1:100 in PBS) and incubated overnight at 4°C. The following day, plates were washed and blocked for 2 h with 5% FCS. Suspensions containing 2 × 10<sup>5</sup> splenocytes in RPMI 1640 (with 10% FCS and 1 ng/ml interleukin-2) were added to each well and stimulated in triplicate, with or without peptide (5 μg/ml) or concanavalin A (2.5 μg/ml, positive control; Sigma) at 37°C. After 18 h, plates were washed and incubated overnight with biotinylated anti-mouse IFN-γ detection antibody (1:100 in 1% bovine serum albumin (BSA) at 4°C). The following day, plates were washed extensively, and BCIP (5-bromo-4-chloro-3-indolylphosphate)–ni troblue tetrazolium chromogen was added to each well to develop color. The plates were then rinsed with distilled water and dried at room temperature. Spots were counted by use of an automated ELISPOT reader (CTL Limited).
RESULTS

New vaccine strain of recombinant *L. monocytogenes* expressing HIV-1 gag. In earlier studies, a highly attenuated strain of *L. monocytogenes*, Lmdd-gag, that expressed HIV-1 gag and carried deletions of two chromosomal genes (*dal* and *dat*) was shown to induce long-lasting protective cell-mediated immunity against a viral agent expressing Gag (44–46). *Dal* and *dat* are required by *L. monocytogenes* for *d*-alanine biosynthesis, cell wall formation, and growth. The immunogenicity of Lmdd-gag depended on a minimal level of bacterial replication that was achieved with a transient exogenous supply of *d*-alanine provided along with the vaccine. Consequently, comparable infection of monkeys or humans would require parental administration of significant quantities of *d*-alanine. While this compound appears to be safe in the small rodent and in rhesus macaques (unpublished observations), its safety in humans is unknown.

These considerations prompted us to devise ways to allow transient endogenous synthesis of *d*-alanine while retaining a high level of attenuation and immunogenicity (29, 55). We describe here the immunogenicity and protection generated by a derivative of Lmdd-gag in which the transient supply of *d*-alanine is provided by a *dal* gene-carrying plasmid from which the *dal* gene is excised during cytosolic growth of the bacteria through the action of the res/resolvase recombination system. As shown later, in order to achieve a sufficiently robust cellular immune response following oral vaccination, we needed to reduce the high activity of the resolvase enzyme (on plasmid pRRR) by introducing into the protein a destabilizing peptide sequence encoded by *ssrA* (18, 25) (generating plasmid pKRS). We also transferred this recombination system onto the more stable plasmid pAM401 (generating plasmid pARS). The resulting bacteria were designated Lmdd-gag/pRRR, Lmdd-gag/pKRS, and Lmdd-gag/pARS. All three recombinant *L. monocytogenes* strains contain the same clade B HIV-1 gag gene in their chromosomes, express a *Bacillus subtilis* *dal* gene, and synthesize *d*-alanine under the stringent regulation of an actA-promoted resolvase recombination system (55).

Figure 1A shows that all of the new strains grew in bacteriological media in the absence of *d*-alanine at only slightly lower rates than wild-type Lm-gag (15) or Lmdd-gag plus *d*-alanine (16, 46). Nevertheless, these organisms were attenuated in mice, as shown in Fig. 1B, which indicates that they survived for only 3 days following i.v. infection. Thus, while the wild-type Lm-gag recombinant bacteria were increasing in number in spleen at days 2 and 3 after infection, the viability of the attenuated strains had fallen 3 to 5 log_{10}. As anticipated, the presence of the destabilizing *ssrA* peptide sequence on the resolvase enzyme of Lmdd-gag/pARS allowed these bacteria to survive slightly longer in mice than Lmdd-gag/pRRR. Additional characterization of these strains revealed that whereas they could replicate for 10 h or longer within the cytoplasm of macrophage-like J774 cells, they did not survive sufficiently long (3 to 4 days) to form plaques (55) on monolayers of mouse L2 fibroblasts (data not shown).

Induction of antigen-specific CTLs in the spleen after systemic immunization with Lmdd-gag derivative strains. The
were coated with either Gag197–205 peptide or a control peptide. Splenocytes labeled with a high concentration of CFSE were isolated and stained for the presence of antigen-specific CD8+ T cells. In summary, like Lmdd-gag with exogenous D-alanine, Lmdd-gag/pKRS induced comparable levels of GAG-specific CD8+ T cells retained effector functions, we measured the in vivo cytolytic activity of CD8+ T cells in mice immunized systemically with Lmdd-gag/pRRR as described for panel A. Six days after the boost, a total of 2 × 10^7 cells containing equal numbers (#) of CFSE^high (peptide Gag197–205- or LCMV-NP118–126-coated) and CFSE^low (uncoated) spleen cells were coinjected into unimmunized or immunized BALB/c mice, and in vivo cytosis of CFSE-labeled donor cells was assessed 16 h later. The percent lysis value was calculated as described in Materials and Methods. Individual percentages of CFSE^high or CFSE^low splenocytes in the recipient mice are shown (as gated on CFSE^+ cells) in small font.

FIG. 2. Comparable Gag-specific and LLO-specific CTLs are generated in the spleens of mice immunized i.v. with Lmdd-gag and derivative strains. (A) BALB/c mice were left unimmunized or immunized twice through the tail vein with 10^7 (primary) and then 10^8 (boost) CFU of the recombinant L. monocytogenes bacteria. Only Lmdd-gag was given along with 10 mg D-alanine in the inoculum. The interval between the prime and boost was 3 weeks. The frequencies of CD8+ T cells specific for LLO_91–99 and Gag197–205 were determined by tetramer staining at 6 days after the boost. Plots shown are of gated CD8^+ cells and are representative of two to three mice used for each group. (B) Mice were immunized as described for panel A. Six days after the boost, a total of 2 × 10^7 cells containing equal numbers (#) of CFSE^high (peptide Gag197–205- or LCMV-NP118–126-coated) and CFSE^low (uncoated) spleen cells were coinjected into unimmunized or immunized BALB/c mice, and in vivo cytosis of CFSE-labeled donor cells was assessed 16 h later. The percent lysis value was calculated as described in Materials and Methods. Individual percentages of CFSE^high or CFSE^low splenocytes in the recipient mice are shown (as gated on CFSE^+ cells) in small font.

50% lethal dose of Lmdd-gag as well as that of the three new derivative strains was approximately 1 × 10^7 to 2 × 10^8 CFU/mouse (data not shown). To determine whether the new strains, like Lmdd plus D-alanine, could induce protective CD8+ T cells in the absence of D-alanine, we first examined CD8+ T-cell activation in the spleens of systemically immunized mice. Groups of mice were immunized through the tail vein with a sublethal dose (10^7) of recombinant bacteria and boosted with 10^8 of the same bacteria. Six days later, splenocytes were isolated and stained for the presence of antigen-specific CD8+ T cells by use of Gag197–205 or LLO_91–99 H-2K^b tetramers to detect the dominant CD8^+ T-cell response to each of these antigens in BALB/c mice (8, 30). As shown in Fig. 2A, robust expansion of antigen-specific CD8+ T cells was seen. Most of these cells showed high expression of CD11a, a β2 integrin up-regulated upon activation of CD8+ T cells and present at high levels on memory CD8+ T cells (27). In the presence of D-alanine, Lmdd-gag induced 1.12% Gag-specific and 10.2% LLO-specific CD8+ T cells in the spleen, while in the absence of D-alanine, Lmdd-gag/pRRR and Lmdd-gag/pKRS induced comparable levels of GAG-specific (1.09% and 1.35%) and LLO-specific (10.1% and 12.1%) CD8+ T cells. Lmdd-gag/pARS induced the highest percentages of antigen-specific (1.55% Gag-specific and 13.1% LLO-specific) CD8+ T cells. In summary, like Lmdd-gag with exogenous D-alanine, Lmdd-gag derivative strains were all able to stimulate substantial antigen-specific CD8+ T-cell responses without exogenous D-alanine, despite showing a 50% lethal dose similar to that of Lmdd-gag.

To determine whether the induced Gag-specific CD8+ T cells retained effector functions, we measured the in vivo epitope-specific cytolytic activities of CD8+ T cells in mice immunized systemically with Lmdd-gag/pRRR as described above. Splenocytes labeled with a high concentration of CFSE were coated with either Gag197–205 peptide or a control peptide, LCMV-NP118–126, and coinjected with equal numbers of non-peptide-coated splenocytes labeled with a lower concentration of CFSE into immunized mice at day 6 after the booster infection. After 16 h, the ratio of CFSE^high (peptide-coated): CFSE^low (no peptide) cells served as an indication of CTL activity. As shown in Fig. 2B, robust Gag-specific killing occurred in both Lmdd-gag (79% lysis of targets-) and Lmdd-gag/pRRR (79.5% lysis of targets-)immunized mice, while no killing was detected in unimmunized mice. As expected, we detected little lysis of LCMV-NP118–126-coated splenocytes. These results suggested that systemic immunization with Lmdd-gag derivatives in the absence of D-alanine could generate functional CTLs.

Induction of antigen-specific CTLs in the spleen and MALTs after oral immunization with Lmdd-gag/pARS. To examine the induction of antigen-specific CD8+ T-cell responses in the spleens of orally immunized mice, groups of mice were immunized by gavage to deliver a single oral dose of 2 × 10^9 CFU of recombinant L. monocytogenes. Six days later, as shown in Fig. 3A, both Lmdd-gag/pKRS and Lmdd-gag/pARS, but not Lmdd-gag/pRRR, induced significant levels of Gag-specific (0.27% and 0.33%) CD8+ T cells. Levels of LLO-specific CD8+ T cells were high in all immunized mice, but again Lmdd-gag/pRRR showed the lowest response. The response produced by Lmdd-gag/pARS was comparable to that produced by Lmdd-gag plus D-alanine.

Therefore, subsequent experiments focused on Lmdd-gag/pARS. Figure 3B illustrates the relationship between the immunization dose and the magnitude of antigen-specific CD8+ T cells induced in the spleen and shows that increased numbers of recombinant Lmdd-gag/pARS delivered orally resulted in increased numbers of antigen-specific CD8+ T cells. With the lowest dose (2 × 10^8 CFU), although LLO-specific CD8+ T cells were readily detected, Gag-specific CD8+ T cells scarcely exceeded background levels. The highest dose (10^9 CFU)
induced 0.61% Gag-specific and 1.06% LLO-specific CD8+ T cells. Based on these results, in subsequent oral immunization experiments we used $2 \times 10^9$ CFU of Lmdd-gag/pARS for primary immunizations and $10^{10}$ CFU for boosts.

Since protection against HIV infection is likely to require immunity at mucosal sites, we further examined whether antigen-specific CD8+ T cells could be induced in MALTs, such as mesenteric lymph nodes (MLN) and PP. Groups of mice were fed $2 \times 10^9$ CFU bacteria for a primary immunization and $10^{10}$ CFU bacteria for a boost. Six days later, lymphocytes were isolated from MLN, PP, and spleens and analyzed for antigen-specific CD8+ T cells by use of tetramer staining. As shown in Fig. 3C, all tissues of immunized mice contained significant numbers of Gag-specific and LLO-specific CD8+ T cells, with spleens containing the highest percentages. In MLN and PP, antigen-specific CD8+ T cells were detectable only after the boost despite the fact that large numbers of Lmdd-gag/pARS were found in these tissues at days 1 and 3 after initial infection (data not shown). Unlike results for spleen, the numbers of Gag- and LLO-specific CD8+ T cells were equivalent in MLN and PP. In unimmunized mice, only background levels of tetramer-positive cells were detected in any tissue.

To test whether the antigen-specific CD8+ T cells induced by oral immunization with Lmdd-gag/pARS possess effector functions, we measured the in vivo cytolytic activities of these animals. Groups of mice were fed $2 \times 10^9$ CFU bacteria and boosted twice with $10^{10}$ CFU Lmdd-gag/pARS. At day 6 after the last immunization, a total of $2 \times 10^7$ cells containing equal numbers (#) of peptide-coated CFSEhigh and peptide-uncoated CFSELFow spleen cells were coinjected into unimmunized or immunized BALB/c mice, and in vivo cytolysis of CFSE-labeled donor cells was assessed 16 h later. The percent lysis value was calculated as described in Materials and Methods. Individual percentages of CFSEhigh or CFSELFow splenocytes in the recipient mice are shown (small font). Peptides used to coat spleen cells were Gag197–205, LLO91–99, and LCMV-NP118–126.

**FIG. 3.** Antigen (Ag)-specific CD8+ T cells are generated in the spleens and MALTs of mice orally immunized with Lmdd-gag/pARS. BALB/c mice were orally immunized with $2 \times 10^9$ CFU of various bacterial strains (A) or with the indicated doses of Lmdd-gag/pARS (B). The percentages of Ag-specific CD8+ T cells in the spleens were determined by Ag-specific tetramer staining 9 days after immunization. (C) BALB/c mice were orally immunized with $2 \times 10^9$ CFU and boosted once with $10^{10}$ CFU Lmdd-gag/pARS. Six days after the boost, the frequencies of Ag-specific CD8+ T cells in the spleens (SP), mesenteric lymph nodes (MLN), and Peyer’s patches (PP) were determined by Ag-specific tetramer staining. Open bars, Gag-specific CD8+ T cells; filled bars, LLO-specific CD8+ T cells.

**FIG. 4.** In vivo CTL activity is generated in mice orally immunized with Lmdd-gag/pARS. BALB/c mice were orally immunized with $2 \times 10^9$ CFU and boosted twice with $10^{10}$ CFU Lmdd-gag/pARS. At day 6 after the last immunization, a total of $2 \times 10^7$ cells containing equal numbers (#) of peptide-coated CFSEhigh and peptide-uncoated CFSELFow spleen cells were coinjected into unimmunized or immunized BALB/c mice, and in vivo cytolysis of CFSE-labeled donor cells was assessed 16 h later. The percent lysis value was calculated as described in Materials and Methods. Individual percentages of CFSEhigh or CFSELFow splenocytes in the recipient mice are shown (small font). Peptides used to coat spleen cells were Gag197–205, LLO91–99, and LCMV-NP118–126.
lated spleen cells from mice that had been immunized orally three times as described above and rested for 8 weeks after the last vaccination. As shown in Fig. 5, substantial and comparable numbers of Gag-specific and LLO-specific IFN-γ-secreting cells were detected in the Lmdd-gag/pARS (without D-alanine)- and Lmdd-gag (with D-alanine)-immunized mice, whereas only a few nonspecific spots were detected in response to the unrelated LCMV-NP peptide. Depletion of CD8+ T cells from the splenocytes before antigen-specific stimulation resulted in complete loss of IFN-γ secretion. These data indicate that oral immunization with Lmdd-gag/pARS in the absence of D-alanine induced a pool of systemic antigen-specific memory CD8+ T cells.

We subsequently determined whether these memory CD8+ T cells could mount a recall response following challenge with an HIV-1 gag-expressing virus. Since mice are not susceptible to HIV infection, we used a surrogate virus—recombinant vaccinia virus expressing HIV-1 gag from clade B (rVV-gag, VVK1). Mice that received three oral doses of Lmdd-gag/pARS were challenged with VVK1 either systemically (i.p., 1.2 × 10^6 PFU), intravaginally (IVAG, 8 × 10^7 PFU), or intranasally (i.n., 1 × 10^6 PFU) with vaccinia virus expressing HIV-1 gag from clade B (VVK1). Controls were unimmunized (Naïve) or age-matched animals that had been infected only with rVV-gag (VVK1). Six days after challenge, splenocytes from unchallenged (A) or challenged (B) mice were isolated and measured for Gag-specific CD8+ T cells by tetramer staining. (C) Splenocytes from IVAG-challenged mice were assayed for IFN-γ and TNF-α production following Gag 197–205 peptide stimulation using intracellular staining. Plots shown in panels B and C are of gated CD8 cells and are representative of two to three mice per group.

FIG. 5. Oral immunization with Lmdd-gag/pARS leads to a pool of Gag-specific memory CD8+ T cells. BALB/c mice were primed orally with 2 × 10^9 CFU bacteria and then boosted twice with 10^9 CFU bacteria. The interval between immunizations was 3 weeks. The mice were allowed to rest for 8 weeks. Splenocytes from immunized mice were isolated and measured for antigen-specific CD8+ T cells by IFN-γ ELISPOT assay. Peptides used for splenocyte stimulation were Gag197–205, LLO118–126, and LCMV-NP118–126. Values represent the means (± standard deviations) of the triplicate cultures.

FIG. 6. Gag-specific memory CD8+ T cells of orally immunized mice expand rapidly following rVV-gag challenge. BALB/c mice were immunized as described in the legend for Fig. 5 and were allowed to rest for 4 weeks. Mice were challenged either systemically (i.p., 1.2 × 10^6 PFU), intravaginally (IVAG, 8 × 10^7 PFU), or intranasally (i.n., 1 × 10^6 PFU) with vaccinia virus expressing HIV-1 gag from clade B (VVK1). Controls were unimmunized (Naïve) or age-matched animals that had been infected only with rVV-gag (VVK1). Six days after challenge, splenocytes from unchallenged (A) or challenged (B) mice were isolated and measured for Gag-specific CD8+ T cells by tetramer staining. (C) Splenocytes from IVAG-challenged mice were assayed for IFN-γ and TNF-α production following Gag197–205 peptide stimulation using intracellular staining. Plots shown in panels B and C are of gated CD8 cells and are representative of two to three mice per group.

FIG. 6B shows that at 6 days after i.p. virus challenge there was a 25-fold increase in the frequency of activated Gag-specific cells (24.7%) in the CD8+ T-cell population in the spleen, compared to 0.97% in unchallenged mice, which contained only the memory CD8+ T-cell population (Fig. 6A). Compared to the systemic challenge, 21-fold and 16-fold increases were seen after mucosal challenges by the intravaginal and intranasal routes, respectively. By contrast, VVK1 infection of unimmunized naïve mice, regardless of the route of infection, resulted in the induction of a primary effector population that contained only a small number (0.14 to 0.37%) of Gag-specific CD8+ T cells.

When the CD8+ T cells were restimulated with Gag197–205 peptide and analyzed for IFN-γ and TNF-α production, we found that the majority of activated CD8+ T cells produced both of these inflammatory cytokines in response to peptide (Fig. 6C). There was no response in the absence of peptide or response to the unrelated LCMV peptide (data not shown). Consistent with the tetramer staining results, cells from mice infected only with VVK1 produced only low levels of cytokine.
Thus, the Gag-specific memory CD8⁺ T cells induced by oral immunization with Lmdd-gag/pARS were capable of dramatically expanding into effector CD8⁺ T cells upon rVV-gag challenge.

To further address mucosal immunity after virus challenge, we examined the presence of Gag-specific CD8⁺ T cells in the MALTs, including PP, MLN, cervical lymph nodes (CLN), and iliac lymph nodes (ILN), at day 6 after challenge. The experiments illustrated in Table 1, carried out using tetramer staining, showed that both intravaginal and intranasal challenges induced significant levels of Gag-specific CD8⁺ T cells in all tissues examined. The response in CLN was higher after intranasal challenge than after intravaginal challenge, while intravaginal challenge induced much higher responses in all other tissues (PP, MLN, and ILN). Moreover, intravaginal challenge induced the highest level (4.6%) of Gag-specific CD8⁺ T cells in the ILN, which enlarged dramatically after virus challenge. The majority of Gag-specific CD8⁺ T cells in the MALTs resulted from rapidly expanded memory CD8⁺ T cells. Data shown are mean virus titers ± standard deviations for groups of three to five mice. The detection limit was 50 PFU. The dose of VVK1 was 10⁷ PFU for i.v. challenge. For VP1287, 6 × 10⁷ PFU was used for IVAG challenge and 1 × 10⁶ PFU for i.p. challenge.

**TABLE 1. HIV-1 Gag-specific CD8⁺ T-cell responses in MALTs of orally immunized mice after rVV-gag challenge by the routes shown**

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Challenge route</th>
<th>Lmdd-gag/pARS plus VVK1</th>
<th>VVK1 only</th>
<th>Naïve</th>
</tr>
</thead>
<tbody>
<tr>
<td>CLN</td>
<td>Nasal</td>
<td>1.18</td>
<td>0.08</td>
<td>0.06</td>
</tr>
<tr>
<td></td>
<td>Vaginal</td>
<td>0.51</td>
<td>0.11</td>
<td>0.09</td>
</tr>
<tr>
<td>ILN</td>
<td>Nasal</td>
<td>0.75</td>
<td>0.13</td>
<td>0.11</td>
</tr>
<tr>
<td></td>
<td>Vaginal</td>
<td>4.62</td>
<td>0.22</td>
<td>0.09</td>
</tr>
<tr>
<td>MLN</td>
<td>Nasal</td>
<td>0.96</td>
<td>0.12</td>
<td>0.03</td>
</tr>
<tr>
<td></td>
<td>Vaginal</td>
<td>3.7</td>
<td>0.11</td>
<td>0.06</td>
</tr>
<tr>
<td>PP</td>
<td>Nasal</td>
<td>2.55</td>
<td>0.14</td>
<td>0.05</td>
</tr>
<tr>
<td></td>
<td>Vaginal</td>
<td>3.66</td>
<td>0.32</td>
<td>0.13</td>
</tr>
</tbody>
</table>

* Mice were orally primed with 2 × 10⁶ CFU Lmdd-gag/pARS and boosted twice with 10⁶ CFU bacteria. Four weeks later, they were challenged vaginally or nasally with rVV-gag (VVK1). Six days after challenge, the percentages of Gag-specific CD8⁺ T cells were determined by Gag tetramer staining.

Protection against systemic and mucosal virus challenges. Finally, we determined the efficacy of the immunity induced by the oral Lmdd-gag/pARS vaccine to protect against a mucosal challenge by rVV-gag by either the intravaginal or intranasal route. Mice were orally immunized three times and then challenged with rVV-gag (VVK1 or VP1287) expressing a homologous clade B gag gene. Six days after the challenge, mice were sacrificed and tissues were assayed for virus content.

In naïve, nonimmune mice, high titers of virus were consistently found in ovaries after i.p. infection (Fig. 7A) and intravaginal infection (Fig. 7B), in agreement with previous reports that the virus has a tropism for the ovary (1). However, following intranasal infection, no virus was found in ovaries. Rather, a modest level of virus (4 log₁₀ PFU) was seen in the lung (Fig. 7A) and occasionally in the draining cervical lymph node (data not shown). Surprisingly, we found that intravaginal infection, but not i.p. infection, resulted in a log₁₀-higher titer of virus in the oviduct than in the ovaries (Fig. 7B), indicating that this tissue is an even more preferred site for virus replication following vaginal infection. Little virus was detected in the oviduct after i.p. infection (Fig. 7C).

Figure 7A compares the effects of oral and systemic immunization with Lmdd-gag/pARS on the level of virus in ovaries after a systemic virus challenge. Although mice immunized by the i.v. route were completely protected against i.p. challenge, orally immunized mice showed only a 2- to 3-log₁₀ reduction in virus titer in the ovaries. This is consistent with previous observations that oral immunization with recombinant L. monocytogenes expressing HIV-1 gag produced complete protection populations, since the tissues contained significant numbers of memory T cells prior to challenge (0.38 to 0.48%), and VVK1 infection of naïve mice resulted in levels of Gag-specific CD8⁺ T cells that were only slightly higher than the background staining levels for the naïve animals (Table 1). Taken together, these data indicate that mucosal challenge of mice immunized orally with Lmdd-gag/pARS induced strong Gag-specific CD8⁺ T-cell recall responses in the MALTs.

**FIG. 7. Protection against systemic or mucosal rVV-gag challenge after oral immunization with attenuated Lmdd-gag/pARS.** Naïve BALB/c mice were unimmunized (open bars), immunized orally (filled bars) as described in the legend for Fig. 5, or immunized i.v. (hatched bar) as described in the legend for Fig. 2. Four weeks after their final immunizations, mice were challenged with rVV-gag expressing the homologous HIV gag from clade B (VVK1 or VP1287) by various routes: systemically (i.p.) (A, left columns [L], and C), intranasally (i.n.) (A, right columns [R]), and intravaginally (B). Six days after challenge, rVV-gag titers in various tissues (indicated above the columns) were assayed by infection of BSC-1 cells. Data shown are mean virus titers ± standard deviations for groups of three to five mice. The detection limit was 50 PFU. The dose of VVK1 was 10⁷ PFU for i.n., 1.2 × 10⁶ PFU for i.p., and 8 × 10⁵ PFU for IVAG challenge. For VP1287, 6 × 10⁷ PFU was used for IVAG challenge and 1 × 10⁶ PFU for i.p. challenge.
against mucosal but not systemic challenge by rVV-gag (46).

Figure 7A also shows that oral immunization led to partial protection against virus replication in the lung, the apparent target organ after nasal infection by the virus. Not discernible from the mean value shown in the figure is that four of the five intranasally challenged mice in this experiment were fully protected.

As shown in Fig. 7B, oral immunization with Lmdd-gag/pARS completely protected mice against an intravaginal challenge, as indicated by the 6-log10 reduction in virus titer in oviduct and the 4- to 5-log10 reduction in titer in ovaries, compared with titers in unimmunized control animals. As expected, no protection against challenge with vaccinia virus expressing a nef gene was observed in immune mice (data not shown). Figure 7C further confirms that virus replication in ovaries following systemic virus challenge (using two different clade B gag-expressing viruses) is only partially inhibited after oral immunization. Figure 7C also shows that there was little replication of virus in oviduct following this i.p. route of challenge.

Finally, to determine whether the oral immunization with Lmdd-gag/pARS could induce cross-clade protection, orally immunized mice were challenged intravaginally with rVV expressing HIV-1 gag from several different viral clades, supporting the important role of cell-mediated immunity in the control of initial virus infection, replication, and persistence.

This study addressed several questions about the mechanisms of L. monocytogenes-based mucosal vaccines. We first determined the effectiveness of three d-alanine-independent recombinant L. monocytogenes vaccine strains expressing HIV-1 gag in the stimulation of Gag-specific CD8+ T-cell responses following mucosal and systemic immunization (Fig. 2

**DISCUSSION**

HIV transmission occurs predominantly through sexual exposure of mucosal surfaces of the genital tract and rectum to virus and virus-infected cells. Recent studies have shown that the mucosal system, particularly the gastrointestinal immune system, is the major target for HIV infection and replication at early stages, with more than 50% of memory T cells destroyed at this site within the first 2 weeks of infection (5, 19, 28, 31, 33). These findings imply that the induction of strong virus-specific immunity at mucosal sites may be crucial for an effective AIDS vaccine. Consequently, while the challenge remains to induce neutralizing antibodies, there is now a major effort to develop vaccines that maximize cell-mediated mucosal immunity, especially in the intestine and genital tract. Although mucosal immunity can be induced by many parenterally administered vaccines, a more robust response is usually elicited by vaccines that act at the oral, nasal, vaginal, or rectal mucosa.

L. monocytogenes is therefore an attractive vaccine vehicle since it naturally infects after oral ingestion and induces strong innate immune responses and potent mucosal and systemic cell-mediated adaptive immunity. In the present study, mice orally immunized with attenuated recombinant L. monocytogenes bacteria responded with functional Gag-specific CTLs, both systemically and mucosally, and were completely protected against vaginal challenge with rVV expressing HIV-1 gag from several different viral clades, supporting the important role of cell-mediated immunity in the control of initial virus infection, replication, and persistence.
We and others (15, 41, 45, 46) have shown that antigen-specific CD8\(^+\) T cells are induced by wild-type and attenuated *L. monocytogenes* strains by use of \(^{51}Cr\) release in vitro CTL measurements, Gag/LLO tetramer binding assays, and peptide-stimulated IFN-\(\gamma\) intracellular staining. In this study, we also sought to determine whether the new constructs could elicit a CTL response detectable in vivo, an activity likely to be necessary in the control of an HIV infection. This study is the first to use this more sensitive assay to evaluate the endogenous Gag-specific CTL activity induced by an oral *L. monocytogenes* vaccine. In fact, in vivo lysis of antigen-labeled target cells was detected after oral immunization with both Lmdd-gag and Lmdd-gag/pARS (Fig. 4). Interestingly, unlike a previously reported study (41), we detected similar levels of Gag-specific and LLO-specific CD8\(^+\) T cells in MLN and PP (Fig. 3C), whereas a higher level of LLO-specific CD8\(^+\) T cells was consistently observed in the spleen after oral immunization (Fig. 3A to C). This suggested that Gag and LLO antigen presentation might be organ specific after oral immunization and that an attenuated strain of *L. monocytogenes* expressing HIV-1 Gag might disseminate systemically differently from wild-type Lm-gag.

Although *L. monocytogenes* has been explored as an oral vaccine against several infectious diseases, it is not known whether it induces effective immune responses at mucosal surfaces other than the gastrointestinal tract, particularly since the respiratory and genital tracts are major routes of entry of many human pathogens. Since CD8\(^+\) T-cell responses in mucosal tissues are difficult to assess, such responses are often measured in the lymph nodes draining mucosal tissues. Expansion of Gag-specific CD8\(^+\) T cells in the spleen and lymph nodes draining the respiratory tract (cervical lymph node) and the genital tract (iliac lymph node) was observed following oral immunization with Lmdd-gag/pARS (Table 1). These responses were strikingly augmented following infection with an rVV expressing a homologous gag gene (VVK1 or VP1287), administered through either the intranasal or the intravaginal route. This suggests that effective mucosal immune responses can be induced at those sites after oral immunization. Importantly, the dramatically increased frequency of Gag-specific CD8\(^+\) T cells in the iliac lymph node would be expected to translate into protection against intravaginal virus challenge. Our preliminary studies have shown that the nasal mucosa is more sensitive than the gut to infection and translocation by *L. monocytogenes* (unpublished data). It is therefore of interest to determine whether nasal infection with *L. monocytogenes* induces vaginal and intestinal responses in mice.

HIV genetic diversity has been a major challenge to the development and evaluation of effective HIV vaccines, since HIV exhibits a high frequency of mutation and leads to recombinant formation following superinfection. The three major groups of HIV have been subdivided into 11 clades. In this study, we showed that immunization with Lmdd-gag/pARS expressing HIV-1 clade B gag induced cross-clade protection against intravaginal challenge with rVV expressing HIV-1 gag from clades A and C but only weak protection against clade G (Fig. 8). In addition, when orally immunized mice were systemically challenged with virus, a 4-log\(_{10}\) reduction in virus titer of clade A gag-expressing rVV was observed, comparable to that of clade B gag-expressing rVV, whereas only a minor reduction in virus titer occurred in mice challenged with clade C gag- or clade G gag-expressing virus. The cross-protection is most likely mediated by CTLs recognizing closely similar H2-K\(^d\)-restricted Gag-specific CTL epitopes shared between clades A, B, and C but presented less efficiently by clade G. Our data support but are slightly different from a previously published report that a clade G Gag-specific cell-mediated immunity was found to be cross-protective with clades A and C but not clade B (24). Nevertheless, a clade B-based HIV-1 vaccine was shown to be capable of inducing cross-clade immunity in uninfected volunteers (14). Cross-clade immunity to Gag, Nef, and Env has also been reported to occur in HIV-1-infected individuals (10, 32).

In conclusion, this mouse study makes the novel observation that a new oral *L. monocytogenes*-based vaccine expressing the HIV gag gene induced strong antigen-specific protective CTL responses in the mucosal immune system and cross-clade protection against intravaginal virus challenge. Recent studies have already revealed that oral immunization of rhesus macaques with recombinant strains of *L. monocytogenes* can be effective inducers of cellular immune responses in this nonhuman primate model (3; S. Jiang, R. Rasmussen, K. McGeehan, F. Frankel, J. Lieberman, H. McClure, K. Williams, U. Babu, R. Raybourne, E. Strobert, and R. Ruprecht, submitted for publication). While the mouse model has served to prove our concept, in general it is unreliable for assessing immunogenicity in nonhuman primates and humans. We therefore look forward to further evaluation of Lmdd-gag/pARS in monkeys, where it may prove to be a particularly safe and effective mucosal vaccine against HIV/AIDS. These results may also have important implications in the development and evaluation of mucosal vaccines against other sexually transmitted diseases, such as herpes simplex virus.
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