

# Oral Immunization with Recombinant *Listeria monocytogenes* Controls Virus Load after Vaginal Challenge with Feline Immunodeficiency Virus

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Received 23 December 2003/Accepted 2 April 2004

**Recombinant *Listeria monocytogenes* has many attractive characteristics as a vaccine vector against human immunodeficiency virus (HIV). Wild-type and attenuated *Listeria* strains expressing HIV Gag have been shown to induce long-lived mucosal and systemic T-cell responses in mice. Using the feline immunodeficiency virus (FIV) model of HIV we evaluated recombinant *L. monocytogenes* in a challenge system. Five cats were immunized with recombinant *L. monocytogenes* that expresses the FIV Gag and delivers an FIV Env-expressing DNA vaccine (LMgag/pND14-Lc-env). Control cats were either sham immunized or immunized with wild-type *L. monocytogenes* (LMgag-wt). At 1 year after vaginal challenge, provirus could not be detected in any of the nine tissues evaluated from cats immunized with the recombinant bacteria but was detected in at least one tissue in 8 of 10 control animals. Virus was isolated from bone marrow of four of five LMgag/pND14-Lc-env-immunized cats by use of a stringent coculture system but required CD8<sup>+</sup> T-cell depletion, indicating CD8<sup>+</sup> T-cell suppression of virus replication. Control animals had an inverted CD4:CD8 ratio in mesenteric lymph node and were depleted of both CD4<sup>+</sup> and CD8<sup>+</sup> intestinal epithelial T cells, while LMgag/pND14-Lc-env-immunized animals showed no such abnormalities. Vaginal FIV-specific immunoglobulin A was present at high titer in three LMgag/pND14-Lc-env-immunized cats before challenge and in all five at 1 year postchallenge. This study demonstrates that recombinant *L. monocytogenes* conferred some control of viral load after vaginal challenge with FIV.**

Because natural transmission of human immunodeficiency virus (HIV) occurs at the mucosa and because mucosa-associated lymphoid tissues may be the earliest site for virus replication (31, 74), successful induction of robust mucosal immunity may require vaccination by a mucosal route. Live vaccines tend to be more immunogenic than killed or subunit vaccines and are generally more effective because of the antigen load amplification by the replicating agent. Given the safety concerns regarding live attenuated HIV vaccines, vectored immunization strategies are under intense investigation as a means to deliver an adequately immunogenic antigen load. Many viral and bacterial vectors are being evaluated for use in HIV vaccine strategies, and each has unique benefits and drawbacks (76). *Listeria monocytogenes* has received relatively little attention as an HIV vaccine vector but possesses many characteristics thought to be necessary for a successful HIV vaccine strategy.

*L. monocytogenes* is a gram-positive, facultative intracellular bacterium that escapes the phagolysosome of host target cells, replicates in the cytosol, and efficiently delivers antigens to the major histocompatibility complex class I presentation pathway (54, 59, 67). The host response to *L. monocytogenes* has been studied for more than 40 years and is considered the paradigm for cell-mediated immunity induction (42). The natural route

of infection by *L. monocytogenes* is at the mucosa upon oral ingestion, and targeted cells during infection include dendritic cells and macrophages (29, 38, 52, 55, 60). *L. monocytogenes* infection activates the innate immune system, induces a type 1 cytokine profile, and is cleared by a characteristically strong cytotoxic T lymphocyte (CTL) response (33, 50). As a vector, foreign genes can be stably inserted into the *L. monocytogenes* genome and express at high levels into the cytoplasm of host cells for processing and presentation by the major histocompatibility complex class I pathway (27). *L. monocytogenes* is also an effective delivery vehicle for plasmids designed for eukaryotic expression of immunogens (21, 32). With regard to safety, several strategies have been employed to restrict but not eliminate replication of *L. monocytogenes*, thereby allowing amplification of the antigenic load and induction of an immune response at the mucosa as well as systemically (25, 40). *L. monocytogenes* also has the advantage of being highly susceptible to many inexpensive and readily available antibiotics in the case of an adverse response to vaccination. Thus, *L. monocytogenes* can be delivered orally, infects antigen-presenting cells, and stimulates strong cell-mediated immunity. In addition, *L. monocytogenes* is easily engineered to express antigens of interest, is inexpensive to produce, and can be attenuated and controlled.

Recombinant *L. monocytogenes* bacteria have been successfully used as biologic vaccine vectors against lymphocytic choriomeningitis virus, cottontail rabbit papillomavirus, murine influenza, and murine neoplasms (40, 68, 77). Several studies using the mouse model have demonstrated the potential of *L.*

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*monocytogenes* as an HIV vaccine vector (24, 30, 43–45, 58, 62, 64). It has been shown that recombinant *L. monocytogenes* can induce a strong CTL response and long-lived memory response to HIV Gag and that oral administration with recombinant *L. monocytogenes* stimulates a strong intestinal mucosal cellular immune response (24, 45, 58, 62, 64). Given the potential of *L. monocytogenes* as a biologic vaccine vector against HIV, recombinant *L. monocytogenes* needs to be investigated in a challenge system.

In the present study, we employed the feline immunodeficiency virus (FIV) model of HIV to evaluate recombinant *L. monocytogenes* as a biologic vaccine vector. FIV is a pathogen of cats that induces a disease syndrome similar to that of HIV infection in humans (78). Like HIV, FIV infection leads to chronic immune dysfunctions, including depletion of CD4<sup>+</sup> T cells, inversion of CD4/CD8 T-cell ratios, decreased lymphocyte proliferation, and increased susceptibility to opportunistic infections (2, 53, 72). The feline model of infection and disease progression is uniquely relevant for the evaluation of vaccine design and immune response upon challenge (22, 34, 61). Cats are the natural hosts for FIV and can be infected by the vaginal route with either cell-free or cell-associated virus, thereby mimicking the natural route of infection by HIV (11). We show here that a single oral immunization with a novel recombinant *L. monocytogenes* strain conferred some control of viral load after vaginal challenge with FIV.

#### MATERIALS AND METHODS

**Animals, bacterial stock, and challenge inoculum.** A total of 15 female 16-week-old specific-pathogen-free (SPF) cats were randomly divided into three groups of 5 cats each. Data from three additional age-matched SPF cats from a separate study provided comparative normal values of intraepithelial lymphocyte (IEL) subset percentages (these animals were part of a larger study performed by K. Howard to fully characterize IEL subsets; unpublished data). Animals were housed and cared for in accordance with the standards of the Association for Assessment and Accreditation of Laboratory Animal Care and with all guidelines of the Institutional Animal Care and Use Committee. *L. monocytogenes* strain 10403S was the wild-type (LM-wt) organism used in these studies. *L. monocytogenes* bacteria were grown in brain heart infusion medium (Difco, Detroit, Mich.). LMgag/pND14-Lc-env is the recombinant strain of *L. monocytogenes* that carries a full-length copy of the FIV molecular clone NCSU<sub>1</sub> gag gene stably integrated into the *L. monocytogenes* genome. The LMgag/pND14-Lc-env strain also carries a DNA vaccine plasmid with the NCSU<sub>1</sub> FIV envelope (surface glycoprotein and ectodomain of transmembrane glycoprotein) cloned into pND14-Lc for eukaryotic expression of the FIV Env under the control of a cytomegalovirus promoter. The construction and validation of LMgag/pND14-Lc-env have been previously described (G. A. Dean, A. LaVoy, R. Stevens, S. Nordone, M. J. Burkhard, unpublished data). Cats were either sham immunized with phosphate-buffered saline (PBS) or given a single oral immunization consisting of  $5 \times 10^6$  CFU of LM-wt or LMgag/pND14-Lc-env. At 12 weeks postimmunization, all 15 cats were challenged by vaginal inoculation with  $7.5 \times 10^4$  FIV-infected feline peripheral blood mononuclear cells (PBMC) and  $7.5 \times 10^4$  TCID<sub>50</sub> cell-free virus. The challenge virus was FIV-NCSU<sub>1</sub>, a pathogenic subgroup A molecular clone. Animals were sacrificed 52 weeks postchallenge.

**Sample collection and processing.** Serum, saliva, and vaginal wash fluids for enzyme-linked immunosorbent assays (ELISA) were collected at 0, 4, 8, 16, 20, 24, and 60 weeks postimmunization and processed as previously described (10). PBMC prepared by centrifugation ( $600 \times g$ , 30 min) over Histopaque (Sigma, St. Louis, Mo.) were washed in LBT medium (RPMI medium supplemented with 10% fetal bovine serum [FBS], 15 mM HEPES, 1 mM sodium pyruvate, 4 mM L-glutamine, 10 IU of penicillin/ml, and 10 µg of streptomycin/ml) and cryopreserved in 90% FBS–10% dimethyl sulfoxide. Retropharyngeal, mesenteric, and medial iliac lymph nodes as well as thymus, bone marrow, and spleen were harvested at necropsy and processed as previously described (16) and cryopreserved as for PBMC. Bronchial alveolar lavage was performed at necropsy. Alveolar macrophages were isolated by adherence to a plastic flask for 4 h at

37°C and 5% CO<sub>2</sub>. Nonadherent cells were washed off, and macrophages were gently removed with a 1:3 solution of lidocaine, washed, and pelleted for DNA extraction. IELs were isolated from a 20-cm section of distal jejunum. The intestine was flushed with wash medium (PBS supplemented with 20% FBS, 4 mM L-glutamine, 2 mM penicillin-streptomycin, 1.25% Fungizone [Bio-Whittaker, Walkersville, Md.], and 10 µg of gentamicin/ml) and cut into 0.5-cm strips following excision of Peyer's patches and lymphoid follicles. Cut intestinal sections were stirred vigorously for 30 min at 37°C in spin medium (Hanks balanced salt solution without Ca<sup>2+</sup> or Mg<sup>2+</sup> and supplemented with 20% FBS, 4 mM L-glutamine, 2 mM penicillin-streptomycin, 1.25% Fungizone [Bio-Whittaker], 10 µg of gentamicin/ml, 2 mM EDTA, and 2 mM dithiothreitol). The supernatant was collected and then centrifuged at  $1,000 \times g$  for 15 min at 16°C. The supernatant was removed, and the pellet was resuspended in 30% Percoll (Amersham Biosciences, Piscataway, N.J.) and then underlaid with 70% Percoll and centrifuged at  $400 \times g$  for 30 min at 25°C. Following centrifugation, the 30%-70% interface layer was collected and washed twice in wash medium and counting was performed.

**Flow cytometric analysis.** PBMC, IEL, and mesenteric lymph node samples ( $10^6$  cells) were stained with fluorescein isothiocyanate- or phycoerythrin-conjugated mouse monoclonal antibodies against feline CD8α (71) or CD4 (3-F4; Southern Biotechnology, Birmingham, Ala.) and analyzed using a FACScan flow cytometer (Becton Dickinson, San Jose, Calif.) and CellQuest software (Becton Dickinson). Data were compared using analysis of variance (ANOVA) with Bonferroni posttest (GraphPad Prism; GraphPad Software, San Diego, Calif.).

**FIV p24 antibody ELISA.** ELISA was performed as previously described (70), with minor modifications. Briefly, Immulon-2HB plates (Dynex Technologies, Chantilly, Va.) were coated with 1.0 µg of p24-GST fusion protein/ml (65). Antibody was detected with goat anti-cat immunoglobulin G (IgG) (Bethyl Labs, Montgomery, Tex.) or IgA (Serotec, Raleigh, N.C.) diluted 1:16,000 or 1:250, respectively, and incubated for 1 h at room temperature. Plates were developed with tetramethylbenzidine (Kirkegaard and Perry, Gaithersburg, Md.) for 10 min and stopped with 1 N H<sub>2</sub>SO<sub>4</sub> before the optical density was read at 450 nm. Titers of antibody were considered positive when they were twofold higher than preimmune values for washes and threefold higher than preimmune values for serum. Statistical differences in antibody endpoint titers between immunization groups were determined using ANOVA, Duncan's multiple range test, and the ANOVA and DUNCAN procedure of SAS (version 8.02; SAS Institute, Inc., Cary, N.C.). Kinetics of antibody responses, including group and time interactions, were analyzed using regression analysis. Significance was defined as  $P \leq 0.05$ .

**Virus isolation.** Virus isolation and CD8<sup>+</sup> T-cell depletion were performed as previously described (17), with minor modifications. Bone marrow mononuclear cells were stained for 20 min with a fluorescein isothiocyanate-conjugated anti-CD8 antibody cocktail consisting of monoclonal antibodies 357 (71) and FCD8 (Southern Biotechnology). Cells were washed with flow wash buffer (PBS supplemented with 10% horse serum and 0.1% sodium azide) and incubated with goat anti-mouse IgG beads (Miltenyi Biotec, Auburn, Calif.) for 20 min, washed again, and then run on an AutoMACS separation unit (Miltenyi Biotec) per the manufacturer's instructions. Sorting purity was higher than 98%, as determined by flow cytometric analysis (FACScan; Becton Dickinson). Unfractionated or CD8<sup>+</sup>-depleted bone marrow cells ( $2.5 \times 10^6$ ) were washed in LBT medium before being subjected to coculturing in triplicate with  $1 \times 10^6$  FCD4E cells (a feline CD4<sup>+</sup>, T-cell line) (9) in the presence of interleukin-2 (National Institutes of Health AIDS Research and Reference Reagent Program). Supernatants were analyzed for FIV p24 levels by antigen capture ELISA every 2 to 3 days over the 21-day culture period.

**FIV p24 antigen capture ELISA.** Maxisorp plates (Fisher Scientific, Houston, Tex.) were coated with 51G11.1 anti-p24 monoclonal antibody (a generous gift from Edward A. Hoover, Colorado State University) (0.45 µg/ml) and incubated overnight at 4°C. Plates were washed and blocked with TRIS-EDTA-NaCl–2% bovine serum albumin overnight at 4°C. Plates were washed, and 100 µl of coculture supernatants was added to the plate. Positive and negative controls were run on each plate and consisted of supernatants collected from persistently FIV-NCSU<sub>1</sub>-infected Crandell feline kidney cells or uninfected Crandell feline kidney cells, respectively. The plates were incubated for 2 h at 37°C. After washing, 100 µl of CG5 antibody (DEAE-purified, S-300-sized IgG from FIV<sup>+</sup> cat plasma; Custom Monoclonal Antibodies International, Sacramento, Calif.) (2 µg/ml) was added to each well and the plate was incubated at 37°C for 1 h. After washing, 100 µl of goat anti-cat IgG-horseradish peroxidase (Kirkegaard and Perry) diluted 1:1,000 in buffer containing 5% mouse serum and 2% goat serum was added to each well and incubated at room temperature for 45 min. Plates were developed with tetramethylbenzidine (Kirkegaard and Perry) for 10 min,

TABLE 1. Real-time PCR detection of proviral DNA in tissue

Immunization group and cat	No. of cells with proviral DNA detected <sup>a</sup>										
	PBMC at wk postchallenge:			Retro <sup>b</sup>	IEL	Bal <sup>c</sup>	Thymus	BM <sup>d</sup>	MLN <sup>e</sup>	Spleen	Iliac <sup>f</sup>
	4	32	52								
Sham											
IU3	8.20 × 10 <sup>1</sup>	8.00 × 10 <sup>2</sup>	5.48 × 10 <sup>2</sup>	6.63	1.07	2.78 × 10 <sup>1</sup>	5.48	1.29 × 10 <sup>4</sup>	4.03 × 10 <sup>4</sup>	1.32 × 10 <sup>2</sup>	1.70 × 10 <sup>3</sup>
IIS2	ND <sup>g</sup>	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
IHH4	2.42 × 10 <sup>3</sup>	5.75 × 10 <sup>4</sup>	2.07 × 10 <sup>4</sup>	1.20 × 10 <sup>3</sup>	5.70 × 10 <sup>4</sup>	1.39 × 10 <sup>9</sup>	2.24 × 10 <sup>9</sup>	5.74 × 10 <sup>9</sup>	7.22 × 10 <sup>6</sup>	7.24 × 10 <sup>5</sup>	1.16 × 10 <sup>6</sup>
IIG5	ND	ND	ND	ND	ND	ND	ND	1.35 × 10 <sup>2</sup>	ND	ND	ND
IIM3	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Wt-LM											
IIG4	ND	2.07 × 10 <sup>2</sup>	ND	9.37	5.42 × 10 <sup>2</sup>	3.28 × 10 <sup>3</sup>	7.11 × 10 <sup>4</sup>	5.29 × 10 <sup>1</sup>	ND	6.29 × 10 <sup>2</sup>	8.64 × 10 <sup>4</sup>
IIS1	ND	3.88 × 10 <sup>2</sup>	ND	3.46 × 10 <sup>1</sup>	1.03 × 10 <sup>3</sup>	5.36 × 10 <sup>3</sup>	1.24 × 10 <sup>3</sup>	1.14 × 10 <sup>2</sup>	4.23 × 10 <sup>5</sup>	7.69 × 10 <sup>3</sup>	6.38 × 10 <sup>2</sup>
IHH5	ND	5.03 × 10 <sup>2</sup>	3.60 × 10 <sup>2</sup>	5.60 × 10 <sup>2</sup>	5.28 × 10 <sup>3</sup>	3.14 × 10 <sup>2</sup>	1.20 × 10 <sup>5</sup>	1.30 × 10 <sup>3</sup>	3.93 × 10 <sup>2</sup>	ND	9.42 × 10 <sup>3</sup>
IIP4	1.40 × 10 <sup>1</sup>	5.75 × 10 <sup>2</sup>	1.85 × 10 <sup>2</sup>	3.55	2.63	2.42 × 10 <sup>1</sup>	6.38 × 10 <sup>2</sup>	7.95 × 10 <sup>1</sup>	6.11 × 10 <sup>1</sup>	9.40 × 10 <sup>1</sup>	2.92 × 10 <sup>1</sup>
IIM2	ND	1.88 × 10 <sup>2</sup>	ND	1.06 × 10 <sup>2</sup>	ND	ND	ND	ND	ND	ND	ND
LMgag/env <sup>h</sup>											
IHH2	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
IHH3	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
IIO5	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
IIO6	8.0	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
IIO7	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND

<sup>a</sup> Detection for all cell types other than PBMC at 52 weeks postchallenge.

<sup>b</sup> Retro, retropharyngeal lymph node.

<sup>c</sup> Bal, bronchoalveolar lavage mononuclear cells.

<sup>d</sup> BM, bone marrow.

<sup>e</sup> MLN, mesenteric lymph node.

<sup>f</sup> Iliac, medial iliac lymph node.

<sup>g</sup> ND, not detected.

<sup>h</sup> LMgag/env, LMgag/pND14-Lc-env.

and development was stopped with 1 N H<sub>2</sub>SO<sub>4</sub> before the optical density was read at 450 nm.

**Real-time DNA PCR.** Real-time PCR was performed to quantify FIV provirus as previously described (57). DNA was extracted from PBMC and tissue pellets by the use of a DNeasy tissue kit as directed by the manufacturer (QIAGEN, Valencia, Calif.). Each amplification reaction contained 0.5 µg of DNA. PCR primers and probe specific for the NCSU<sub>1</sub> gag gene were designed using Primer Express (PE Applied Biosystems, Foster City, Calif.). The primers (FIVNC.491f and FIVNC.617r) and probe (FIVNC.555p) have previously been described (12). A single copy of the CCR5 sequence exists in the feline genome and was used to normalize the FIV copy number. The CCR5 primers and probe (University of California at Davis Taqman Service, Davis, Calif.) for quantification of the feline CCR5 gene were used in the PCR at 400 and 80 nM, respectively. All standards and controls were run in triplicate, and unknowns were run in duplicate and averaged. Standard curves for FIV and CCR5 were accepted when the slopes were between -3.74 and -3.32. The limit of detection was ≤10 copies FIV per µg of DNA.

## RESULTS

**Immunization and challenge strategy.** A total of 15 female 16-week-old cats were either sham immunized or orally inoculated with 5 × 10<sup>6</sup> CFU of wt-LM or LMgag/pND14-Lc-env bacteria. At 3 months postvaccination, all cats were vaginally challenged with FIV-NCSU<sub>1</sub>, a pathogenic subgroup A molecular clone. At 52 weeks postchallenge, all cats were sacrificed and multiple tissues were collected.

**Assessment of proviral loads in tissues.** While sterilizing immunity may not be achievable, limiting proviral burden may provide protection against transmission and disease progression. To determine the effect of LMgag/pND14-Lc-env immunization on proviral burden after vaginal FIV challenge, we performed quantitative real-time PCR (sensitivity ≤ 10 provi-

rus copies) on DNA extracted from PBMC at 4, 32, and 52 weeks postchallenge (Table 1). At 4 weeks postchallenge, two sham-immunized cats and one LM-wt-inoculated cat were PCR positive; at 32 weeks postchallenge, two of five sham-

TABLE 2. Virus isolation from unfractionated and CD8<sup>+</sup> T-cell-depleted bone marrow 52 weeks after vaginal challenge with FIV

Immunization group and cat	Day of virus isolation for indicated bone marrow type <sup>a</sup>	
	Unfractionated	CD8 Depleted
Sham		
IU3	Neg	14 <sup>b</sup>
IIG5	14	14
IIM3 <sup>c</sup>	Neg	9
IIS2	14	14
IHH4	7	7
LM-wt		
IIG4	Neg	21
IHH5	Neg	Neg
IIS1	19	19
IIP4	8	8
IIM2	19	19
LM-gag/env <sup>d</sup>		
IHH2	Neg	14
IHH3	Neg	14
IIO5	Neg	19
IIO6	7	7
IIO7	Neg	21

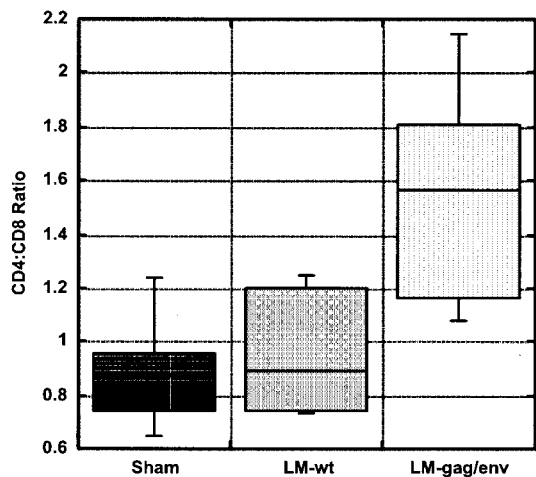
<sup>a</sup> Neg, virus isolation negative after 21 days of coculture.

<sup>b</sup> Day culture was FIVp24 positive by ELISA.

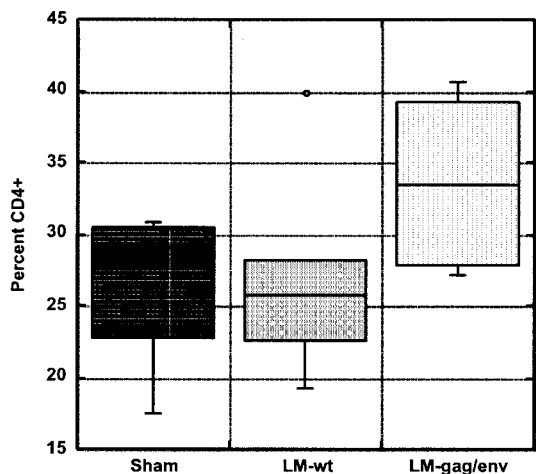
<sup>c</sup> For cat IIM3 mesenteric lymph node was evaluated.

<sup>d</sup> LM-gag/env, LMgag/pND14-Lc-env.

A.



B.



immunized and five of five LM-wt-inoculated cats were positive. Intestinal IELs and medial iliac lymph node, mesenteric lymph node, retropharyngeal lymph node, spleen, bone marrow, thymus, and bronchoalveolar lavage mononuclear cells (Table 1) were evaluated at the time of sacrifice (1 year post-challenge). By 52 weeks postchallenge, provirus was detected in the tissues of 8 of 10 control cats (3 of 5 sham-immunized cats and 5 of 5 LM-wt-immunized cats). Notably, provirus was not detected in any evaluated tissue from the LMgag/pND14-Lc-env-immunized cats.

C.

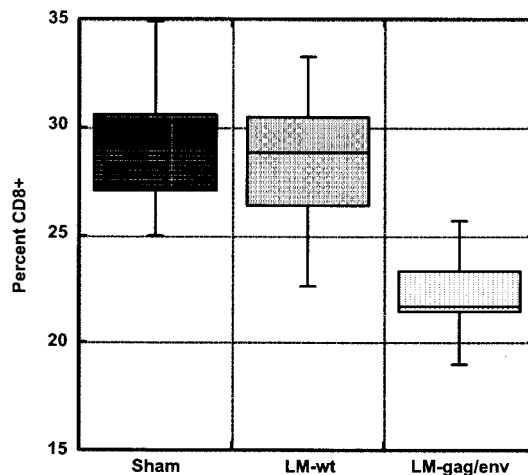


FIG. 1. Lymphocyte subsets in mesenteric lymph node. CD4:CD8 ratios (A) and percentages of CD4<sup>+</sup> (B) and CD8<sup>+</sup> (C) T cells are shown in a box (enclosing 50% of the data with the median value displayed as a line) and whisker (lines extending from the bottom and top of each box mark the minimum and maximum values) plot. An outlier is displayed as an individual point in panel B. The mean CD4:CD8 ratio for LMgag/pND14-Lc-env (LM-gag/env)-immunized group is significantly greater than that for the LM-wt- or sham-immunized group ( $P = 0.04$  [ANOVA]).

**Virus isolation.** A maximum of 0.5  $\mu$ g of DNA, representing approximately  $5 \times 10^5$  cells, can be assessed per reaction with real-time PCR. To further search for viral reservoirs in a greater number of cells, virus isolation was performed in triplicate on  $2.5 \times 10^6$  unfractionated or CD8<sup>+</sup> T-cell-depleted bone marrow cells. Bone marrow mononuclear cells were cocultured with highly permissive FCD4E cells, and FIVp24 levels were measured in culture supernatants by antigen capture ELISA every 2 to 3 days. Cells were cocultured for a total of 21 days. Due to unavailability of bone marrow for cat IIM3, virus isolation was performed on mesenteric lymph node mononuclear cells. Virus isolation results were positive on day 14 for four of five sham-immunized cats by coculture in both unfractionated and CD8<sup>+</sup>-depleted cultures, while results for cat IIU3 were positive only when CD8<sup>+</sup> T cells were depleted. Of the LM-wt-immunized cats, three of five gave positive results in unfractionated cultures and four of five gave positive results in CD8<sup>+</sup> T-cell-depleted cultures. Thus, all 10 sham- and LM-wt-immunized cats were FIV positive by PCR and/or virus isolation. Only one of five LMgag/pND14-Lc-env-immunized cat was positive by virus isolation in unfractionated bone marrow cells. However, virus was isolated from all five LMgag/pND14-Lc-env-immunized cats when CD8<sup>+</sup> T cells were depleted (Table 2). These results are consistent with suppression of virus replication by CD8<sup>+</sup> T cells.

**Lymphocyte subsets in lymph node and intestine.** Peripheral blood lymphocyte numbers and phenotype subpopulations remained within normal limits for all cats throughout the study

period (data not shown). However, significant differences in lymphocyte subsets in the mesenteric lymph node were observed among the three study groups. The mean CD4:CD8 ratio in the mesenteric lymph node of LMgag/pND14-Lc-env-immunized cats at 52 weeks post-vaginal FIV challenge was  $1.55 \pm 0.45$ . In contrast, sham- and LM-wt-immunized groups had inverted mean CD4:CD8 T-cell ratios of  $0.89 \pm 0.23$  and  $0.96 \pm 0.25$ , respectively (Fig. 1A). These ratios for the sham- and LM-wt-immunized groups were significantly lower than those of the LMgag/pND14-Lc-env-immunized group ( $P = 0.04$ ). The inverted ratios were the result of both CD4<sup>+</sup> T-cell loss and increased CD8<sup>+</sup> T-cell percentages (Fig. 1B and C).

To determine whether oral immunization might prevent lymphocyte subset perturbations in the intestine, we determined the phenotype of IELs isolated at necropsy. We found immunophenotypic analysis by flow cytometry of cryopreserved IELs was not comparable to results obtained from freshly isolated IELs. Unfortunately, freshly isolated IELs were analyzed for just three LMgag/pND14-Lc-env-immunized and three sham-immunized animals. Data from three FIV-naïve cats are included for comparison. Two of three LMgag/pND14-Lc-env-immunized cats analyzed maintained normal (compared to FIV-naïve values) CD4<sup>+</sup> percentages, while all three sham-immunized cats were CD4<sup>+</sup> T cell depleted (Fig. 2A). The three sham-immunized cats also had significant loss of CD8<sup>+</sup> T cells compared to LMgag/pND14-Lc-env-immunized cats ( $P = 0.0016$ ), whose results were similar to FIV-naïve control values (Fig. 2B). CD4 and CD8 percentages in IELs of LMgag/pND14-Lc-env-immunized cats compared to those naïve control cats were not significantly different ( $P = 0.05$ ).

**Mucosal and systemic induction of Gag-specific IgG and IgA.** FIVp24-specific IgG and IgA levels in vaginal washes, saliva, and serum were measured by ELISA. Table 3 lists titers for each cat at 8 weeks postimmunization and at study termination (52 weeks postchallenge [60 weeks postimmunization]). Immunization with LMgag/pND14-Lc-env induced vaginal anti-p24 IgA responses in three of five cats. Vaginal IgA was found in all 5 immunized cats after challenge, while only 2 of 10 control cats mounted a minimal anti-p24 vaginal IgA antibody response upon vaginal FIV challenge (Fig. 3). Interestingly, four of five cats immunized with LMgag/pND14-Lc-env mounted a postchallenge anti-p24 IgG vaginal antibody response and by 52 weeks postchallenge there was a strong statistical difference between LMgag/pND14-Lc-env-immunized cats and LM-wt-immunized cats ( $P = 0.009$ ). Two LMgag/pND14-Lc-env-immunized cats mounted anti-p24 IgG and IgA antibody responses in saliva; however, postchallenge antibody titers were not significantly different among the three groups. LMgag/pND14-Lc-env-immunized cats did not mount a prechallenge serum p24 IgG antibody response. However, by 52 weeks postchallenge the mean p24-specific serum IgG titer for LMgag/pND14-Lc-env-immunized cats was  $3,270 \pm 3,635$  versus  $716 \pm 857$  and  $1,024 \pm 1,024$  for sham- and LM-wt-immunized cats, respectively. Although the mean titer of the LMgag/pND14-Lc-env-immunized group was 3 to 5 times greater than that of the control groups, this was not statistically significant due to variations among individual cats.

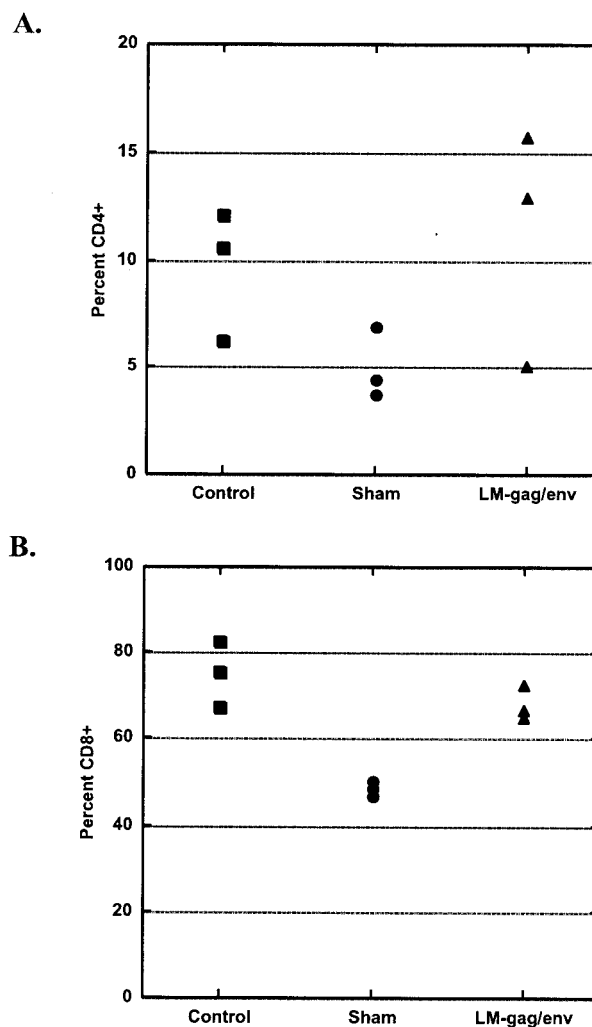


FIG. 2. Lymphocyte subsets in intestinal epithelium. Percentages of CD4<sup>+</sup> (A) and CD8<sup>+</sup> (B) T cells are shown for three FIV-naïve, SPF control cats, three sham-immunized cats, and three LMgag/pND14-Lc-env (LM-gag/env)-immunized cats.

## DISCUSSION

To limit transmission of HIV and combat the virus at its earliest target, it may be essential for an efficacious vaccine to induce a strong mucosal immune response. Several studies have demonstrated the importance of mucosal CTL (3, 4, 35, 51) and suggest that mucosal CTL may be at least as important as systemic CTL (5). Another key component of the mucosal immune barrier is secretory IgA (sIgA). Mucosal IgA production has been correlated with protection (6, 18, 36, 49), and IgA-mediated virus neutralization has been demonstrated (7, 18, 56). While many parenteral vaccine strategies have induced mucosal immune responses, in general a more robust mucosal response is seen when immunization occurs at the mucosa (39). Oral immunization is therefore an attractive approach to target induction of mucosal immunity and has the practical benefit of ease of delivery.

In the present study, cats immunized with a single oral dose of recombinant *L. monocytogenes* bacteria showed some con-

TABLE 3. Anti-p24 IgG and IgA antibody response

Immunization group and cat	Endpoint antibody titer <sup>a</sup>									
	Vaginal IgA		Vaginal IgG		Saliva IgA		Saliva IgG		Serum IgG	
	8 wpi	52 wpc	8 wpi	52 wpc	8 wpi	52 wpc	8 wpi	52 wpc	8 wpi	52 wpc
Sham										
IU3	0	0	0	32	0	0	0	64	0	2,048
IIG5	0	0	0	128	0	4,096	0	512	0	0
IIM3	0	64	0	64	0	128	0	64	0	512
IIS2	0	0	32	128	64	0	0	0	0	1,024
IIH4	0	0	0	0	0	0	0	0	0	0
LM-wt										
IIG4	0	0	0	0	0	0	0	512	0	1,024
IIH5	0	0	0	0	0	0	0	0	0	2,048
IIS1	0	0	0	0	0	0	128	64	0	0
IIP4	0	32	0	0	0	4,096	0	128	0	2,048
IIM2	0	0	0	0	0	1,024	0	512	0	0
LM-gag/env <sup>b</sup>										
IIH2	0	1,024	0	128	0	64	64	1,024	0	8,192
IIH3	0	1,024	0	0	512	1,024	4,096	128	0	0
IIO5	32	2,048	256	32	256	0	0	0	0	1,024
IIO6	256	2,048	0	256	0	128	0	128	0	2,048
IIO7	512	256	0	256	0	0	0	0	0	4,096

<sup>a</sup> wpi, weeks postimmunization; wpc, weeks postchallenge.

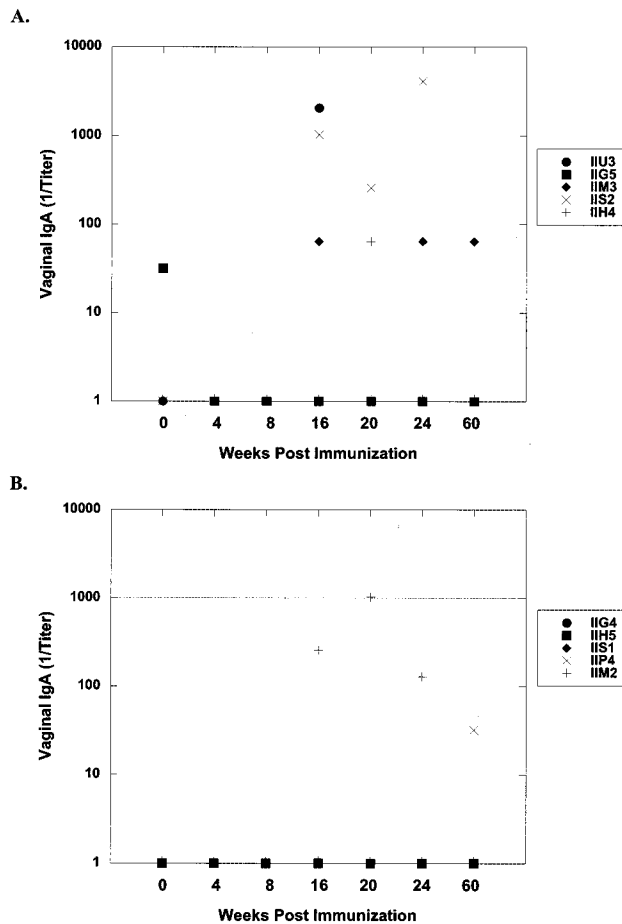
<sup>b</sup> LM-gag/env, LMgag/pND14-Lc-env.

trol of virus burden after vaginal challenge with pathogenic FIV. At 1 year after vaginal FIV challenge, immunized cats had undetectable provirus levels, as assessed by real-time PCR analysis of peripheral blood lymphocytes, multiple lymph nodes, intestinal mucosal lymphocytes, and several other tissues. Given the enormous difficulty in inducing sterilizing immunity, reducing or controlling the viral reservoirs may be the most important feature of a successful HIV vaccine. Prolonged virus isolation coculturing with large numbers of lymphocytes was required to reveal the presence of virus in the LMgag/pND14-Lc-env-immunized cats. Furthermore, CD8<sup>+</sup> T-cell depletion was necessary for four of five of the cats before virus replication was detected. This result emphasizes the importance of CD8<sup>+</sup> T cells in the control of viral replication, whether the control is mediated by direct cytotoxicity or through soluble factors.

Preventing lymphocyte depletion and dysfunction is another critical measure of vaccination success. Recent studies suggest that analysis of lymph nodes and mucosa-associated lymphocytes is a more sensitive approach to detecting alterations in lymphocyte subpopulations due to HIV or simian immunodeficiency virus infection (37, 47, 69, 73, 75). This was apparent in the present study, as no lymphocyte subset alterations were seen in the peripheral blood of any FIV-challenged animals. However, an inversion of the CD4:CD8 ratio in the mesenteric lymph node occurred in sham-immunized and LM-wt-immunized but not in LMgag/pND14-Lc-env-immunized cats. The loss of CD4<sup>+</sup> T cells and increase of CD8<sup>+</sup> T-cell levels observed is characteristic of FIV infection (78). Loss of CD4<sup>+</sup> T cells from the intestinal immune system occurs very early in infection and is known to precede losses in lymph nodes of simian immunodeficiency virus-infected macaques and HIV-infected people (28, 37, 46, 47, 69, 73, 75). In fact, the intestine appears to be a primary target organ for virus infection and

CD4<sup>+</sup> T-cell depletion (28, 37, 47, 73, 75). We observed a loss of both CD4<sup>+</sup> and CD8<sup>+</sup> T cells in sham- and LM-wt-immunized cats, while LMgag/pND14-Lc-env-immunized cats maintained IEL subpopulations at FIV-naïve control levels. Oral immunization of mice with HIV-Gag recombinant *L. monocytogenes* has resulted in Gag-specific responses in about 35% of lamina propria CD8<sup>+</sup> T cells (58). Induction of such a robust T-cell response in combination with sIgA may prevent the establishment of viral reservoirs and depletion of CD4<sup>+</sup> T cells in the intestine. Given the pivotal role of the intestinal immune system in HIV pathogenesis, this type of protection may be sufficient to prevent disease.

Immunized cats demonstrated a durable vaginal IgA response to FIV Gag. Vaginal IgA has been well studied in exposed, uninfected individuals and has been shown to neutralize primary HIV-1 isolates from multiple clades (8, 18–20, 36, 41). The neutralizing effect may be due to inhibition of virus transcytosis across the epithelial membrane (8, 18). However, IgA titers have not necessarily been correlated with protection and this may be due to differences in epitope specificity or levels of the IgA (14, 48). Mapping of IgA-neutralizing activity has identified the coiled-coil pocket in the alpha-helical region of gp41, the transmembrane protein, as an important target (14). However, simply coating virus particles with sIgA, regardless of epitope specificity, may promote entrapment in the vaginal mucus (15). We did not determine whether IgA from immunized cats recognized the transmembrane glycoprotein or surface glycoprotein of FIV, so the role of IgA in the protection of the cats in this study is uncertain. *L. monocytogenes* is recognized as a strong inducer of cellular immunity and a weak inducer of humoral immunity. Our observation that recombinant *L. monocytogenes* induced mucosal sIgA that was enhanced upon vaginal challenge is significant and raises optimism that an oral vaccine strategy utilizing *L. monocytogenes*



may be able to stimulate a useful antibody response at the vaginal mucosa.

Demonstration by ELISPOT of antigen-specific gamma interferon (IFN- $\gamma$ ) production in cats immunized with *L. monocytogenes* was confounded by dramatic cell activation associated with *L. monocytogenes* exposure (data not shown). We have previously observed high-level constitutive IFN- $\gamma$  production by PBMC that persists 30 to 60 days postimmunization with *L. monocytogenes* (Dean et al., unpublished). This is not surprising given that *L. monocytogenes* is well known to be a strong inducer of cell-mediated immunity and as such is part of the appeal of *L. monocytogenes* as a vaccine vector. Some difficulty dissecting the antigen specificity of the IFN- $\gamma$  response after immunization with recombinant *L. monocytogenes* has also been experienced in the mouse model but was nicely resolved using tetramer staining (58). Unfortunately tetramers are not available for the study of IFN- $\gamma$  responses in cats. Several studies employing recombinant *L. monocytogenes* with HIV *gag* integrated into the bacterial genome have previously demonstrated induction of anti-Gag responses (23, 24, 44, 58, 62, 64). The CD8<sup>+</sup> T-cell suppression of virus replication in the virus isolation assay provided some evidence for cell-mediated immunity in this study.

Bacteria have received much less attention than viruses as

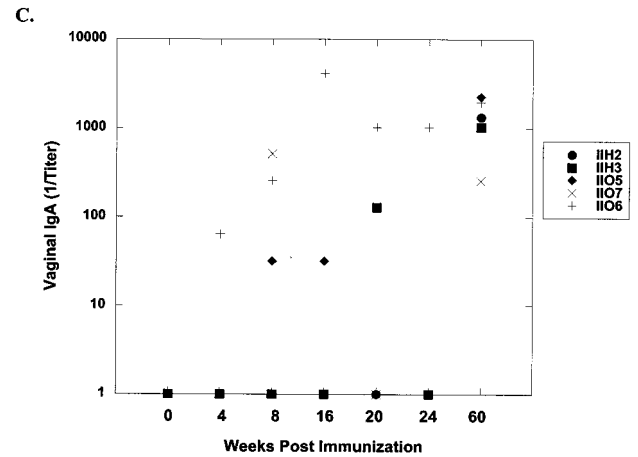


FIG. 3. Induction of FIV anti-p24 IgA in vaginal secretions. FIV Gag-specific IgA levels were measured in vaginal washes from sham-immunized (A), LM-wt-immunized (B), and LMgag/pND14-Lc-env-immunized (C) cats by ELISA at 0, 4, 8, 16, 20, 24, and 60 weeks postimmunization. Cats were vaginally challenged with FIV at week 12. Titers were determined by endpoint dilution.

potential HIV vaccine vectors. However, bacteria have several attributes making them worthy of consideration in this respect. *L. monocytogenes* may have advantages over other bacterial vectors, since it replicates in the cytoplasm of the host cell and this improves the likelihood that antigens are protected against enzymatic degradation. Additional advantages of *L. monocytogenes* are that the natural route of infection is at the intestinal mucosa and that antigen-presenting cells are target cells. Furthermore, it has been proven that *L. monocytogenes* can transfer a plasmid to mammalian cells (21, 32) and, as reported here, can be employed to deliver an FIV Env-expressing DNA vaccine plasmid. As with any live vaccine vector, safety issues must be addressed. Towards this end, several promising and immunogenic attenuated *L. monocytogenes* have been reported previously (1, 13, 21, 24–26, 30, 63, 64, 66). With further development, recombinant *L. monocytogenes* may prove to be a safe and efficacious vaccine vector against HIV.

#### ACKNOWLEDGMENTS

This work was supported by Public Health Service grants AI40407, AI47046, and AI01529.

We acknowledge the expert assistance of Ingrid Fisher, Julie McCormick, and Alora LaVoy.

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