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The Induction of HIV Gag-Specific CD8$^+$ T Cells in the Spleen and Gut-Associated Lymphoid Tissue by Parenteral or Mucosal Immunization with Recombinant *Listeria monocytogenes* HIV Gag[1]

Christian Peters,[2] Xiaohui Peng, Dennis Douven, Zhen-Kun Pan, and Yvonne Paterson[3]

The induction of mucosal immunity is crucial in controlling viral replication during HIV infection. In this study we compare the ability of a recombinant *Listeria monocytogenes* that expresses and secretes the HIV Ag Gag to induce CD8$^+$ T cells against this Ag in the spleen, mesenteric lymph nodes, and Peyer's patches and the ability to provide effector Gag-specific CD8$^+$ T cells to the lamina propria after i.v., oral, or rectal administration of the vaccine. The levels of Ag-specific CD8$^+$-activated T cells were measured ex vivo using intracellular cytokine staining for IFN-γ and H-2K^d Gag peptide tetramer staining. We found that all routes of immunization induced Gag-specific CD8$^+$ T cells in the spleen. After secondary infection, we observed substantial increases in splenic levels of CD8$^+$ T cells, and levels of Gag-specific cells were similar to those against listeriolysin O, the immunodominant Ag of *L. monocytogenes*. Both primary and secondary oral immunization resulted in abundant Gag-specific CD8$^+$-activated T cells in the lamina propria that constituted ~35% of the CD8 compartment. However, significant levels of Gag and listeriolysin O-specific CD8$^+$ T cells were observed in mucosal lymphoid tissue only after two immunizations, perhaps because they had already entered the lamina propria compartment after a single immunization. In the context of HIV, a mucosally administered vaccine seems best calculated to prompt an immune response that is capable of preventing infection. The data presented in this report demonstrate that mucosally administered *Listeria* can prompt such a response and that booster doses can maintain this response. *The Journal of Immunology,* **2003, 170: 5176–5187.**

Effective vaccines against HIV are urgently needed. But key elements of an effective vaccine approach remain unclear: neither the kind of immunity to be induced nor the best route of immunization has been identified. Our laboratory has been developing the intracellular bacterium, *Listeria monocytogenes,* as a vaccine vector for passenger Ags (Reviewed in Refs. 1–3). Currently accumulating evidence suggests that this approach may be effective against HIV infection.

First, our vaccine construct induces CD8$^+$ T cells, which appear to play an important role in the control of viral replication during HIV infection (4–9). Second, our approach seeks to induce immunity at mucosal sites, specifically through oral or rectal administration of the vaccine. We emphasize mucosal sites not only because they host significant immunological activity, but also because HIV-specific immune elements at mucosal sites will be critical for the initial (and timely) control of infection in many or most individuals exposed to the virus (10–12). Studies of candidate HIV vaccines that are designed to elicit mucosal immune responses have so far focused mainly on HIV-induced B cell (IgA) and Th cell responses (13–15) or CTLs in the spleen (16). While recent findings underscore the need for vaccines capable of inducing CTL in mucosal sites (17–22), the knowledge necessary to create them remains scarce. Only a few vaccine approaches for HIV have been shown to induce a CTL response in the mucosa (19–22). In this study, we address this issue with studies of mucosal CTL responses to recombinant *L. monocytogenes* Gag vaccines.

L. monocytogenes is a β-hemolytic Gram-positive, facultative intracellular bacterium that has been extensively used for decades to study cell-mediated immunity (23). Unlike other intracellular organisms such as *Salmonella* and *Mycobacterium bovis* bacillus Calmette-Guérin, a fraction of the bacteria escape into the cytoplasm of the host cell by disrupting the phagosomal membrane, mainly through the action of hemolysin, also known as listeriolysin O (LLO)[4] (24). Because the bacteria replicate in the cytoplasm without coming into contact with the extracellular compartment, humoral immunity (neutralizing Ab) does not play a major role in resistance to listerial infections (23, 24). Peptides derived from *L. monocytogenes* in the phagolysosome can be presented via both the MHC class I and class II pathways, and thus induce both CD4$^+$ and strong CD8$^+$ T cell responses (1, 2).

Although the gastrointestinal tract is the natural site of infection, most studies of experimental listeriosis have delivered the organism i.p. or i.v. Therefore, comparatively little is known about the immune response to *L. monocytogenes* at mucosal sites. This study

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[4] Abbreviations used in this paper: LLO, listeriolysin O; HPV, human papilloma virus; Lm-E7, recombinant *Listeria monocytogenes* strain that secretes HPV-16 E7; Lm-Gag, recombinant *Listeria monocytogenes* strain that secretes HIV-1 Gag; LP, lamina propria; LPL, LP lymphocytes; MLN, mesenteric lymph nodes; PP, Peyer's patches.

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aims to redress that lack of information because the oral and rectal routes, if practicable, have distinct advantages. As the most widely used mucosal vaccination route, oral immunization has proven both practical and reliable in large-scale public health vaccination programs and generally elicits a high level of compliance from clinical cohorts enrolled in oral vaccine field trials (25–28). Despite this successful background, the poor immunogenicity of most Ags when given orally has hindered the development of oral vaccines against other pathogens. By contrast, live *L. monocytogenes* traverses the natural defenses of the gastrointestinal tract and delivers Ags to inductive sites in the mucosal immune compartment. Further, live *Listeria* vaccines possess attributes that make them well suited for large-scale use in both developed and developing countries.

Listeria has received increasing attention as a vaccine vector because of its unusual characteristics and immunological properties. *L. monocytogenes* has been shown to be a potent vaccine vector for infectious and neoplastic disease (29–37).

We have constructed several wild-type *L. monocytogenes* recombinants that secrete HIV Ags (2, 38–40). In an earlier study, we demonstrated that one of these constructs, *L. monocytogenes*-Gag (Lm-Gag), mounted a strong, specific, long-lasting CTL response against the HIV-1 Gag protein. The induction of CTLs was measured through chromium release assays and indirectly through T cell depletion after animals were immunized via the parenteral route (39). In this study, we take advantage of recent technological advances to quantify the strength of the $CD8^+$ T cell response using tetramer and intracellular cytokine staining. These visualization techniques helped us to establish that *L. monocytogenes* administered orally is an effective vaccine vector for HIV Ags and that it induces Ag-specific lymphocytes in peripheral and mucosal tissue.

Materials and Methods

Bacteria

Recombinant Lm-Gag is based on strain 10403S serotype 1 and carries a copy of the HIV-1 strain HXB (subtype B laboratory strain with a syncytia forming phenotype) *gag* gene stably integrated into the listerial chromosome (39). The Gag protein is expressed and secreted by *L. monocytogenes* as determined by Western blot. The strain has a LD_{50} of 5×10^6 in BALB/c mice compared with the parental *L. monocytogenes* strain 10403S, which has an LD_{50} of $\sim 5 \times 10^4$ CFU when injected i.p. into BALB/c mice. The recombinant *L. monocytogenes*-E7 (Lm-E7) was used as a control and is isogenic with Lm-Gag except that it carries the human papilloma virus (HPV) E7 gene (37) instead of *gag* and expresses and secretes this protein. The growth medium used in this study was beef heart infusion (Difco, Detroit, MI). Bacterial aliquots were stored at −70°C.

Before their use, all strains of bacteria were mouse-passaged to stabilize their behavior in vivo (40). Bacteria were grown from spleen homogenates in tryptic soy broth, harvested in log phase, dispensed in 0.5 ml aliquots, and frozen at −70°C until needed. An inoculum of bacteria was prepared for i.v. by thawing an aliquot and appropriately diluting it in PBS.

Mice

Female BALB/c ($H\text{-}2^d$) mice were purchased from Charles River Breeding Laboratories (Wilmington, MA) or The Jackson Laboratory (Bar Harbor, ME). All mice were maintained in a pathogen-free microisolator environment. Mice used in this study were 6–12 wk old.

Immunization

BALB/c mice were immunized by one of three routes, either a single dose or one boost was given as indicated. For i.v. immunization, 10^5 live bacteria (0.02 LD_{50}) were used. Oral immunization was performed intragastrically using a feeding tube to deliver 10^8 live bacteria in 200 μl of beef heart infusion. The animals were not fasted before immunization. A similar dose of bacteria was used for intrarectal immunization except that the volume was reduced to 20 μl. A flexible cannula attached to a syringe needle was used to deliver the inoculum to the rectum.

Bacterial translocation studies

The spleen, mesenteric lymph nodes (MLN), and Peyer's patches (PP) were removed on days 1, 2, 3, 5, 10, and 14 after intragastric or intrarectal immunization with 10^8 *L. monocytogenes* or i.v. immunization with 10^5 live bacteria. Three mice were used for each time point. Each type of tissue was pooled for each mouse and homogenized in 5 ml of medium. Bacterial growth was determined by plating 10-fold serial dilutions of the organ homogenates on tryptic soy agar. The detection limit of this procedure was 10^2 CFU per organ. Colonies were counted after 24 h of incubation at 37°C. Bacterial levels in each tissue were calculated as CFU per milliliter of tissue homogenate for each mouse at each time point.

Preparation of tissue for T cell analysis

Single-cell suspensions were prepared using tissue from the spleen, PP, and MLN by homogenizing in ice cold, sterile PBS and filtration through a nylon mesh. Cells were pelleted gently and washed three times with RPMI.

To isolate lamina propria lymphocytes (LPL), small intestine segments from eight mice per experimental group were isolated, and the luminal content was flushed with HBSS. Visible PP were excised, and the intestine was opened longitudinally and cut into 2-mm pieces. The pieces were then incubated for 30 min with stirring at 37°C in 30 ml of PBS with 1 mM EDTA (Sigma-Aldrich, St. Louis, MO) and 1 mM DTT (Fisher Scientific, Fair Lawn, NJ). The supernatant was discarded, fresh PBS-EDTA-DTT was added, and the incubation procedure was repeated. The cells were washed three times with RPMI supplemented with 10% FCS and 1% penicillin and streptomycin (complete RPMI). LPL were then liberated from remaining sediment by placing the intestinal debris in 30 ml of complete RPMI medium with 0.5 mg/ml collagenase (Sigma-Aldrich) and 15 μg/ml DNase I (Amersham Pharmacia Biotech, Piscataway, NJ) for 90 min at stirring at 37°C. The supernatants were collected and the cells were washed three times with complete RPMI. The cells were then resuspended in 2 ml of 40% Percoll (Amersham Pharmacia Biotech) and underlayed with 2 ml of 70% Percoll. After centrifugation at 2000 rpm for 20 min, the interfaces between the 70% and 40% layers were removed, and the cells were washed three times with complete RPMI medium.

Analyses were performed on cell preparations from each tissue pooled from eight mice because that was the minimum number of animals required to give sufficient numbers of cells to analyze lamina propria (LP) responses.

Detection of Ag-specific $CD8^+$ T cells by intracellular cytokine stain for IFN-γ

Single-cell suspensions were cultured for 5 h in complete RPMI medium supplemented with 50 U/ml human recombinant IL-2 and 1 μl/ml brefeldin A (Golgistop; BD PharMingen, San Diego, CA) in the presence or absence of either the CTL immunodominant epitope for gag (AMQMLKETI) (41), the LLO epitope (GYKDGNEYI) (42), or a control epitope from the HPV (RAHYNIVTF) (43). The peptides were used at a concentration of 1 μM. The cells were first surface-stained, then washed and subjected to intracellular cytokine stain using the Cytofix/Cytoperm kit in accordance with the manufacturer's recommendations (BD PharMingen). For intracellular IFN-γ stain, we used FITC-conjugated rat anti-mouse IFN-γ mAb (clone XMG 1.2) and its isotype control Ab (rat IgG1; both from BD PharMingen). A total of 10^6 cells were stained in PBS containing 1% BSA and 0.02% sodium azide (FACS buffer) for 30 min at 4°C followed by three washes in FACS buffer. Samples were acquired on either a FACScan flow cytometer or FACSCalibur instrument (BD Biosciences, San Jose, CA).

Three-color flow cytometry for CD8 (PerCP-conjugated, rat anti-mouse, clone 53-6.7; BD PharMingen), CD62L (APC-conjugated, rat anti-mouse, clone MEL-14), and intracellular IFN-γ was performed using a FACSCalibur flow cytometer, and data were further analyzed with CellQuest software (BD Biosciences). Cells were gated on $CD8^{high}$ and $CD62L^{low}$ before they were analyzed for $CD8^+$ and intracellular IFN-γ.

Analysis of HIV Gag-specific $CD8^+$ T cells with MHC class I tetrameric K^d HIV peptide

A single-cell suspension from tissue harvested 9 days after the primary or 5 days after the secondary immunization was stained at room temperature with $H\text{-}2K^d$ tetramers loaded with the Gag peptide (AMQMLKETI) or the LLO peptide (GYKDGNEYI) or with D^b tetramers loaded with the HPV E7 peptide (RAHYNIVTF) as a control. Tetramers were provided at a monomer concentration of 2 mg/ml (i.e., before interaction with streptavidin) by the National Institute of Allergy and Infectious Diseases Tetramer Core Facility of Emory University under the direction of Dr. J. Altman and the National Institutes of Health AIDS Research and Reference Reagent Program. The tetramers were titrated and used at a 1:200 dilution.

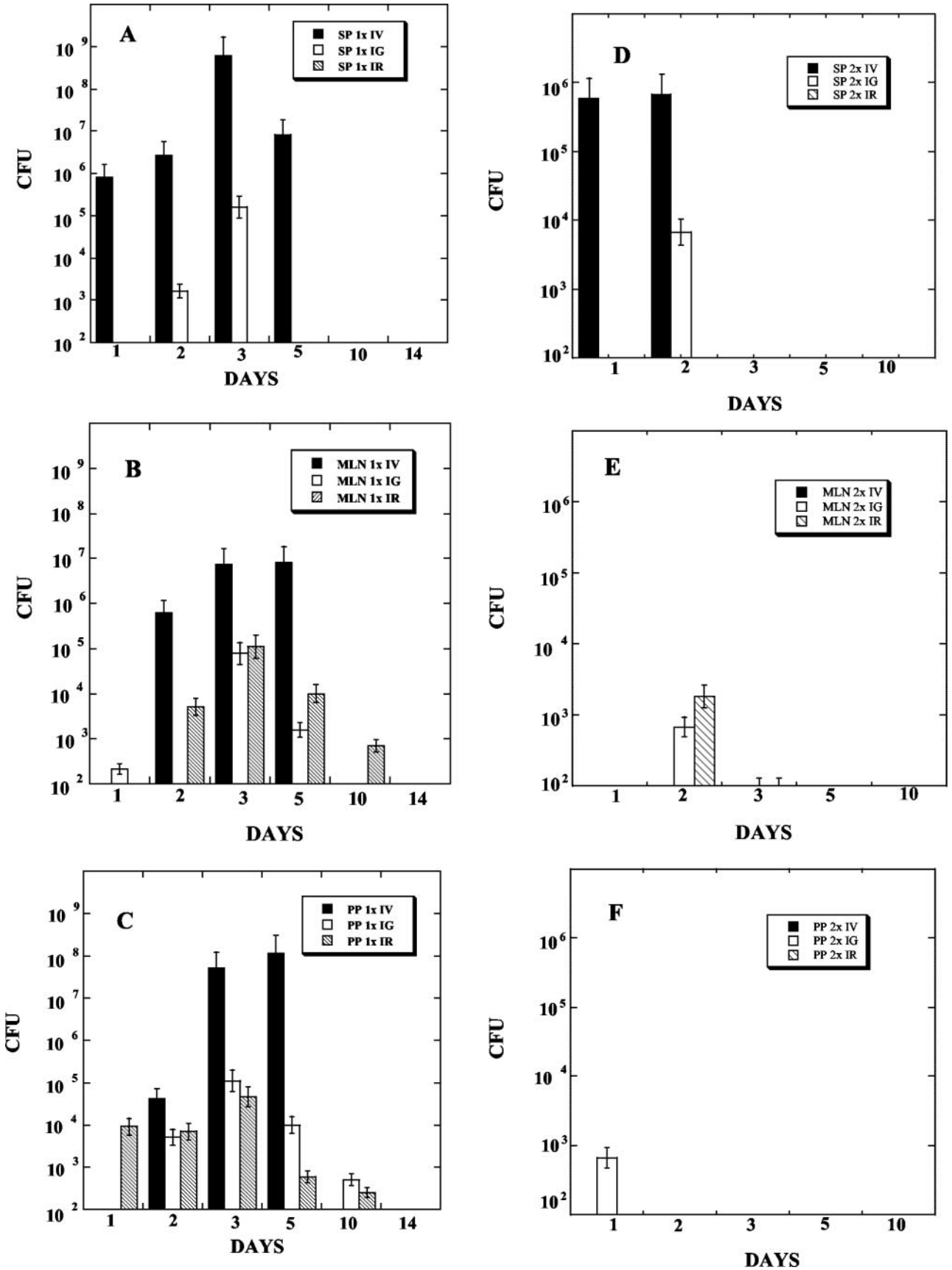

FIGURE 1. Time course of the bacterial translocation to spleen (*A* and *D*), MLN (*B* and *E*), and PP (*C* and *F*) after i.v. rectal and intragastric immunization of Lm-Gag. Data are shown as CFU per milliliter of organ homogenate after a single immunization (*A–C*) or two immunizations 30 days apart (*D–F*). Data for each time point are the averages of three mice per group.

Cells were analyzed as previously described for intracellular IFN-γ staining comparing tetramer-positive, $CD8^+$ and $CD62L^{low}$ cells, responsive to each peptide Ag, generated by Lm-Gag immunization. In some experiments, CD44 was used as the activation marker and cells were gated for high expression of CD44. Using either activation marker gave similar results.

Flow cytometry analysis

For flow cytometry analysis, $\sim 1 \times 10^6$ cells were added per staining to a well of a 96-well plate. Splenocytes were stained at 4°C for 20 min with FITC-conjugated anti-CD8 (BD PharMingen), and 40 min with PE-conjugated H2-K^d-Gag tetramers (0.25–0.5 mg/ml) in FACS staining buffer (PBS, pH 7.45, 0.5% BSA, and 0.02% sodium azide). Subsequently, cells were washed three times in staining buffer and then fixed in 1% paraformaldehyde/PBS (pH 7.45).

Two-color flow cytometry was performed using a FACSCalibur flow cytometer and data were further analyzed with CellQuest software (BD Biosciences). For the analysis of tetramer-positive $CD8^+$-activated ($CD44^{high}$) T cells in LP, single-color histograms of tetramer-positive cells gated from the $CD8^+CD44^{high}$ population are shown.

^{51}Cr release assay

Mice were immunized i.v. once only with 10^5 live recombinant Lm-Gag. Spleens were harvested on day 9 and set up in a 5-day primary culture with irradiated BALB/c splenocytes as feeder cells and 1 μM of either the immunodominant Gag or LLO epitope. Effector cells were used in a standard 4-h ^{51}Cr release assay with P815 cells pulsed with either peptide at 1 μM. Results are plotted as percentage of specific lysis = [(experimental-spontaneous)/(total-spontaneous)] × 100.

Results

Bacterial translocation studies

In the model of murine listeriosis, it has been proposed that the primary induction of $CD8^+$ T cells strongly depends on the presence of *Listeria* in immunologically inductive sites (44). In addition, for successful induction of T cell-mediated immunity, *Listeria* must be present for at least 3 days in the inductive sites (44). We therefore chose to investigate the kinetics of bacterial translocation into the inductive sites of the immune system for all routes of immunization used in this study, i.e., oral, rectal, and i.v., after primary and secondary immunization. When bacteria are given orally, they are challenged by the acidic environment of the stomach, the commensal flora of the intestinal tract, and the physical barriers of mucus and epithelial layers. To induce immunity, the bacteria must overcome these considerable challenges to translocate to immune-inductive sites.

As displayed in Fig. 1, *A–C*, i.v. immunization resulted in colonization of the spleen and the gut-associated lymphoid tissue with titers of bacteria peaking at days 3–5 and effectively being cleared after day 10. Oral immunization also resulted in colonization of all three tissues by day 3 following immunization, albeit at a three-logs lower level than for i.v. immunization. In contrast intrarectal immunization showed a similar level and kinetics of infection in the MLN and PP to immunization by the oral route but did not colonize the spleen at detectable levels. Secondary immunization 1 mo following the first (Fig. 1, *D–F*) showed some colonization in the spleen after i.v. and oral immunization (but not after intrarectal immunization) and only barely detectable levels in MLN and PP after mucosal immunization. The levels of bacteria colonization were very low and transient and were cleared by day 3 after secondary immunization.

Because the technique of oral gavage can result in the introduction of bacteria into the respiratory passages if not performed correctly, we also investigated the lung after intragastric immunization. We ascertained that the lung was free of bacteria on day 1; thus, oral immunization did not result in direct application into the lung. On days 2 and 3, however, we found a small but detectable number of bacteria in the lung. Bacterial numbers in the lung

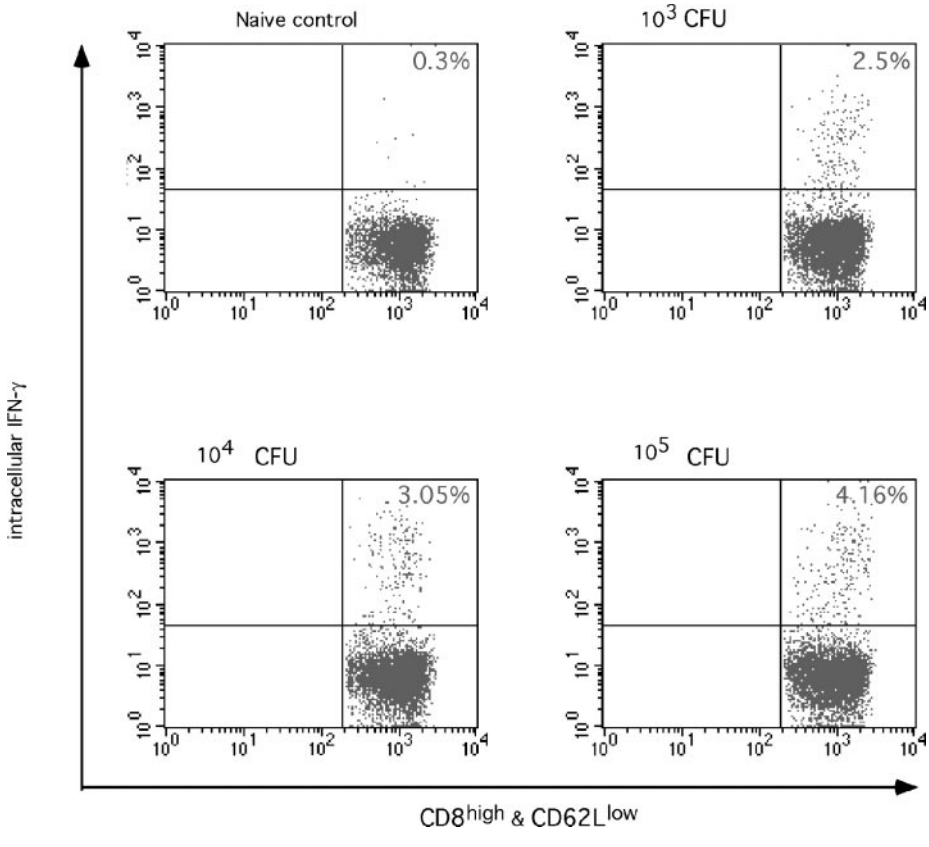

FIGURE 2. The induction of HIV Gag-specific $CD8^+$ T cells in the spleen after i.v. immunization with Lm-Gag depends on the immunizing dose. Spleens were removed on day 9 following i.v. immunization with the doses of Lm-Gag shown and analyzed for the presence of IFN-γ secreting $CD8^+$ T cells as described in *Materials and Methods*.

reached a level of 1.6×10^2/ml of organ homogenate on day 3, peaked on day 5 (5×10^2/ml of organ homogenate), and are eradicated by day 10. They showed similar kinetics to those observed in the other organs after intragastric immunization (Fig. 1, *B* and *C*), which suggests a systemic spread of the infection throughout the organs.

Analysis of Ag-specific CD8$^+$ T cells induced by immunization via the parenteral route

In this study, we were interested in investigating the quantitative aspects of CD8$^+$ T cell induction by both the parenteral and oral immunization routes to determine whether oral immunization can result in effective immunity. On day 9, when the primary immune response peaks in listeriosis after parenteral immunization (45), we performed an analysis using intracellular cytokine staining for IFN-γ. Fig. 2 illustrates the relationship between the immunizing dose (in numbers of bacteria delivered i.v.) and the number of CD8$^+$ T cells induced in the spleen: increased numbers of recombinant Lm-Gag administered i.v. result in increased numbers of Ag-specific CD8$^+$ T cells. With the lowest dose administered (10^3 Lm-Gag), we found that 2.5% of all activated CD8$^+$ T cells were Gag-specific (i.e., CD8^{high}, CD62L^{low}, IFN-γ^+ cells). The highest dose administered (10^5 Lm-Gag) produced 4.16% Gag-specific CD8$^+$ T cells.

Next, we compared the maximum induction of Gag-specific CD8$^+$ T cells to the maximum induction of T cells specific to LLO, produced by a gene endogenous to *L. monocytogenes*. In this series of experiments as shown in Fig. 3, after primary parenteral immunization, Gag-specific CD8$^+$ T cells constituted 4.85% of the activated total, whereas LLO$^+$-specific CD8$^+$ T cells constituted 7.99% of the total. After secondary immunization, however, the number of Gag-specific (14.85%) and LLO-specific (16.80%) T cells were equivalent (Fig. 3).

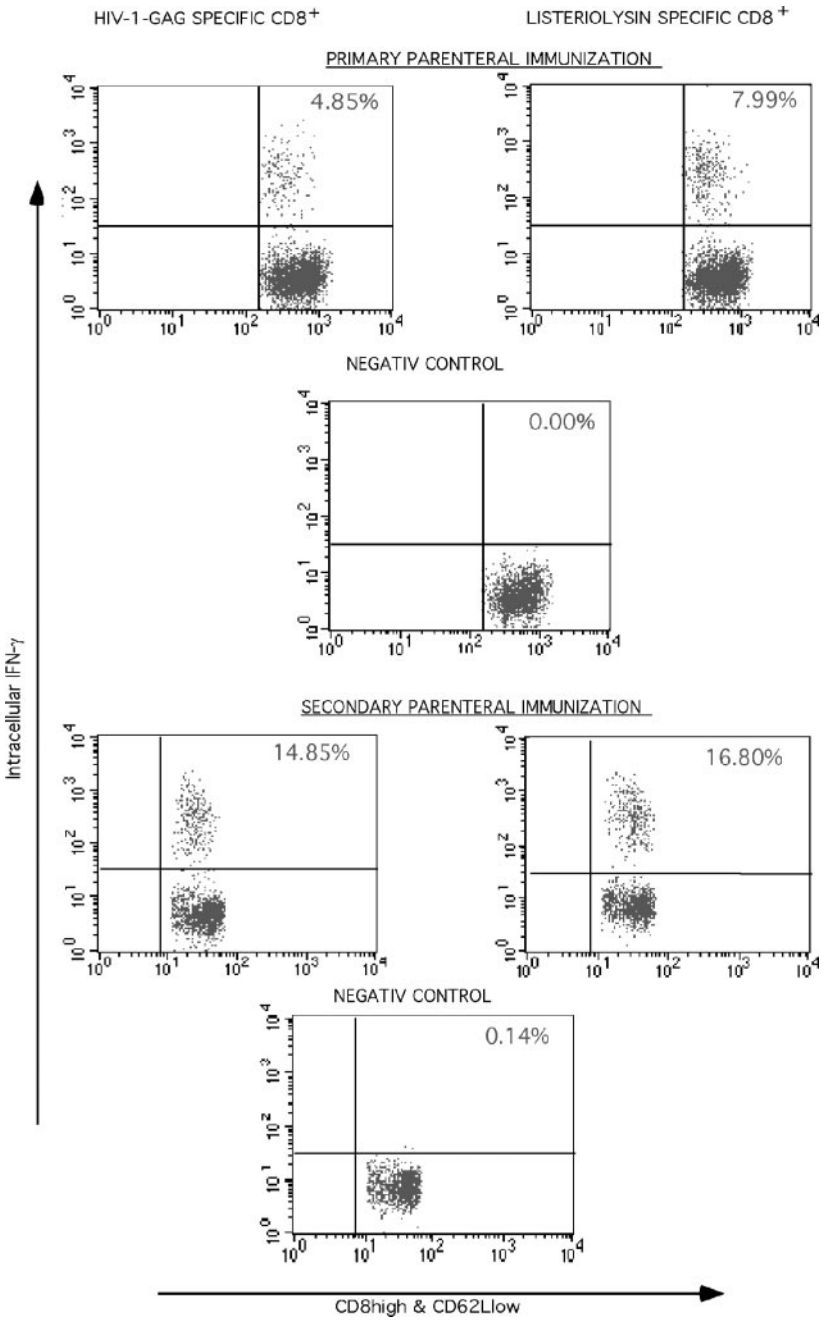

FIGURE 3. The induction of HIV-1 Gag-specific CD8$^+$ T cells in the spleen, compared with LLO-specific T cells, after i.v. immunization with 10^5 Lm-Gag. Primary responses were measured 9 days after immunization. Secondary i.v. immunization of 10^5 Lm-Gag took place 30 days following the primary dose and 5 days later splenocytes were analyzed for the presence of IFN-γ secreting CD8$^+$ T cells as described in *Materials and Methods*.

Assays for cytolytic function

We then deployed chromium release assays to determine whether the $CD8^+$ T cells induced by one immunization (Fig. 3) are cytolytic. The results in Fig. 4 show that HIV Gag-specific CTLs lyse P815 target cells as effectively as do LLO-specific CTLs. Specific lysis occurs at comparable levels as between the *trans*-gene and the endogenous gene despite the higher numbers of induced LLO-specific $CD8^+$ T cells after primary immunization.

Induction of specific $CD8^+$ T cells by immunization at mucosal surfaces

We then compared the ability of an *L. monocytogenes*-based vaccine to induce strong immunity when delivered either orally, intrarectally, or parenterally (i.v.). We wished to examine not only the specific number of $CD8^+$ T cells in the spleen, but also their presence in mucosally associated lymphoid tissue, such as MLN and PP. The experiments illustrated in Fig. 5 and Table I, using intracellular cytokine staining and tetramer staining, showed that both primary oral and i.v. immunization induced similar levels of Gag-specific $CD8^+$ T cells in the spleen (Fig. 5) and in the MLN (Table I). Responses in all tissues, including the spleen, were much lower after intrarectal immunization than by other routes (Table I and compare Figs. 5 and 6). In addition, no Gag-specific T cells were apparent in PP by any route of primary immunization (Table I). Primary immunization combined with a booster immunization 30 days later, however, resulted in higher levels of Gag-specific $CD8^+$ T cells in the spleen, MLN, and PP (Table I). With the booster immunization, the Gag-specific $CD8^+$ T cells constituted ~15% of all activated $CD8^+$ T cells in the spleen (Fig. 5), similar to the level of $CD8^+$ T cells induced to LLO, the immunodominant endogenous Ag. Interestingly, rectal immunization (Fig. 6 and Table I) induced similar numbers of Ag-specific (Gag or LLO) T cells in the spleen after two immunizations. However, only oral immunization induced significant levels of Gag-specific $CD8^+$ T cells in the PP. The results from intracellular cytokine staining are summarized in Table I. Some of these measurements were confirmed by tetramer staining and these are shown in parentheses in Table I. Some experimental variation in the actual numbers of Ag-specific $CD8^+$ T cells was detected between different experiments, however the trend in the data was similar. The values shown in Table I are representative of three to five separate experiments.

We also used tetramer technology to determine that Gag-specific $CD8^+$ T cells were present in the LP after both an oral primary immunization and an oral booster immunization 30 days later. Both primary and booster immunization produced a surprisingly large number of Gag-specific T cells in the intestine; in both cases, ~35% of all $CD8^+/CD62L^{low}$ T cells found in the small intestine were Gag-specific even after a single immunization (Fig. 7*A*). A second immunization did not significantly increase this number (Fig. 7*B*). In contrast, neither intrarectal immunization (Fig. 7*C*) nor i.v. immunization (Fig. 7*D*) induced Gag-specific T cells in the LP above the level detected with the control Lm-E7 vector. We did detect activated $CD8^+$ T cells in mice immunized with the control vector Lm-E7 in the LP by all routes of immunization but the largest number were present after oral immunization. The high number of Gag tetramer-reactive $CD8^+$ T cells found in LP after Lm-E7 immunization is in contrast to the other tissue analyzed where nonspecific activation by the Lm-E7 vector was always <10% of the Gag-specific $CD8^+$ T cells induced by Lm-Gag. The percentage of Gag-specific activated $CD8^+$ T cells present in LP after oral immunization was calculated based on the gate shown in Fig. 7, *A* and *B*, by subtracting the number of tetramer-positive $CD8^+$-activated ($CD44^{high}$) T cells present in this gate for mice immunized with Lm-E7 (~15%) from the number of events for mice immunized with Lm-Gag (~50%).

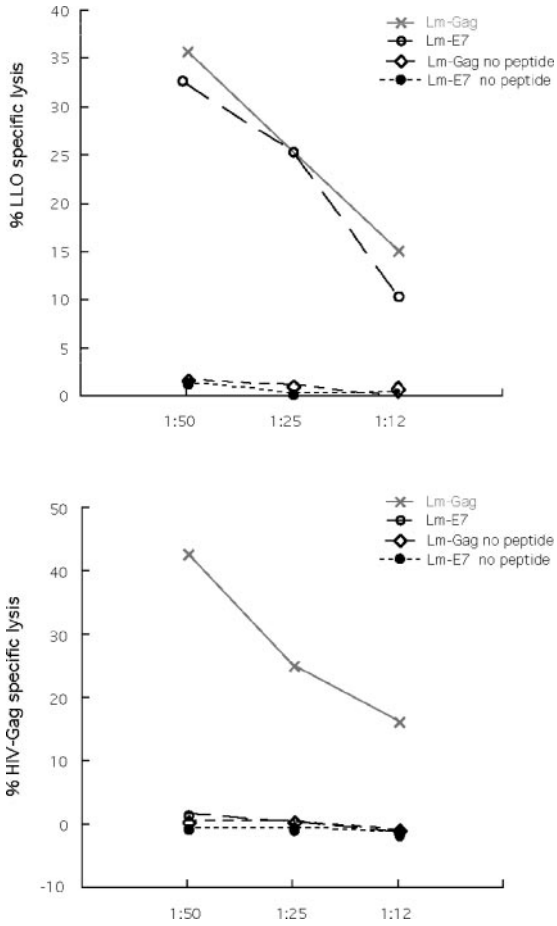

FIGURE 4. Cytotoxic T cell induction in splenocytes from mice immunized 9 days prior with 10^5 live recombinant Lm-Gag or Lm-E7. Splenocytes were incubated for 5 days with irradiated BALB/c splenocytes and 1 μM of either the immunodominant Gag (*bottom*) or LLO (*top*) epitope and analyzed for cytotoxicity as described in *Materials and Methods*.

Discussion

This study demonstrates that recombinant Lm-Gag can induce large numbers of HIV-1 Gag-specific $CD8^+$ T cells in mice that are immunized via the parenteral and mucosal routes. It also demonstrates that oral immunization induces a response in the LP of the small intestine that is absent after i.v. immunization. Further, we found that i.v. and orally administered *Listeria* can translocate to the inductive sites of the immune system to induce specific T cells in mucosally associated lymphoid tissue and in the spleen, whereas we could not detect *Listeria* outside the gut-associated lymphoid tissue when introduced intrarectally.

The reliance on bacterial translocation has been thought to be key for the induction of immunity. Indeed, a close relationship between the presence of *Listeria* at inductive sites and the induction of T cell-mediated immunity has long been suggested (44). Experiments that abrogated *Listeria* infection with antibiotics revealed that the persistence and number of viable microorganism are important parameters for efficient induction of T cell-mediated immunity (44). In addition in the present study, the level of response in the spleen when *L. monocytogenes* is delivered i.v. is dose dependent (Fig. 2), that is, the number of specific T cells rose with the number of *Listeria* used for immunization. These findings

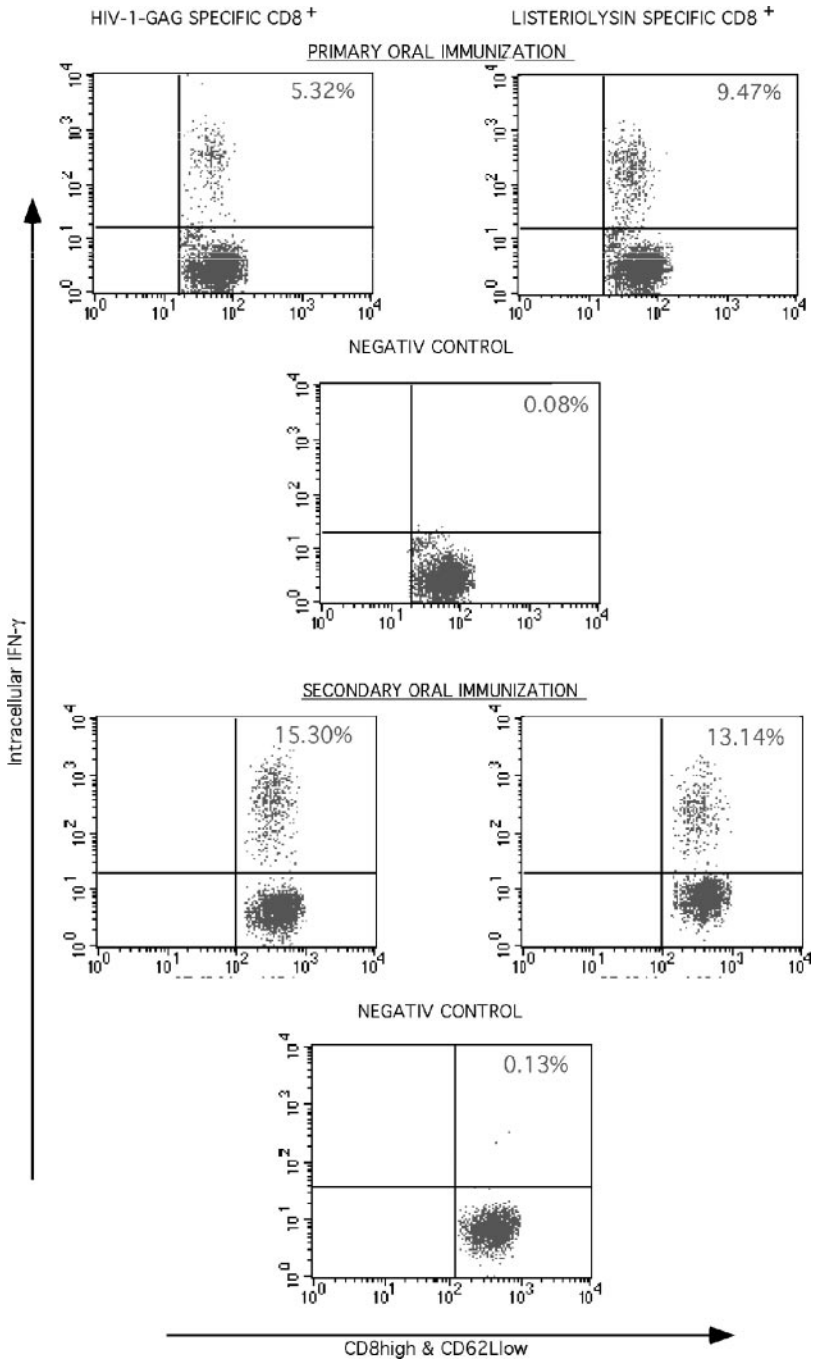

FIGURE 5. The induction of HIV-1 Gag-specific $CD8^+$ T cells in the spleen, compared with LLO-specific T cells, after oral immunization with 10^8 Lm-Gag. Primary responses were measured 9 days after immunization. Secondary oral immunization of 10^8 Lm-Gag took place 30 days following the primary dose, and 5 days later splenocytes were analyzed for the presence of IFN-γ secreting $CD8^+$ T cells as described in *Materials and Methods*.

imply that *Listeria* must cross the barriers of the intestinal tract to induce a vigorous immune response. Accordingly, the presence and growth kinetics in the mucosal-associated lymphoid tissue after mucosal immunization could determine the degree of T cell induction. When administered orally, *Listeria* disseminates throughout the entire gastrointestinal tract within 5–6 h; as a result, it is likely to engage most, if not all, inductive sites quickly after inoculation. Because orally administered *Listeria* has such strong translocation abilities and appears to engage the full range of inductive sites, it is difficult to determine whether some sites are more important than others for the successful induction of T cell-mediated immunity. *Listeria* is thought to translocate both directly through the intestinal wall as well as through M cells in the intestine to gain access to the PP (46). It is even less clear how *Listeria* spreads after intrarectal immunization. In this study we have shown that it does appear to be able to translocate into mucosally associated lymphoid tissue, but in contrast to oral immunization, we did not detect bacteria in the spleen after primary or secondary intrarectal immunization. Nevertheless, although levels of both Gag- and LLO-specific $CD8^+$ T cells were much lower in the spleen after intrarectal immunization compared with other routes, after secondary immunization the levels were very similar, although there was no evidence of persistent colonization of that tissue by the intrarectal route. In addition although all routes of immunization resulted in robust colonization of PP after primary immunization, no Ag-specific $CD8^+$ T cells were detected in that tissue until the animals received a booster dose, and only oral immunization produced significant numbers of detectable Gag-specific T cells. Thus, there does not seem to be a direct correlation between bacterial colonization of inductive sites and the level of

Table I. *HIV Gag-specific and LLO-specific CD8$^+$ T cell responses in the spleen and gut-associated tissue after primary and secondary immunization by the routes shown*[a]

	HIV-1 GAG Specific CD8$^+$ (% CD6$^+$/CD62L^{low}	Listeriolysin Specific CD8$^+$ (% CD8$^+$/CD62L^{low})	Negative Control[b] (% CD8$^+$/CD62L^{low})
After Primary infection			
Spleen			
i.v.	4.85 (4.83)	7.99	0.00
Oral	5.32 (4.43)	9.47	0.12
Rectal	1.43	4.16	0.13
MLN			
i.v.	1.10	0.53	0.00
Oral	0.69 (1.19)	1.72	0.05
Rectal	0.17	0.36	0.02
Peyer's Patches			
All routes	0.00 (0.00)	0.00	0.00
After Secondary infection			
Spleen			
i.v.	14.85	16.80	0.14
Oral	15.30	13.14	0.17
Rectal	13.85	15.08	1.78
MLN			
i.v.	1.88	4.50	0.50
Oral	1.63 (1.38)	2.54	0.05
Rectal	1.25	1.80	0.00
Peyer's patches			
i.v.	0.33	0.48	0.74
Oral	1.58 (1.95)	1.32	0.00
Rectal	0.60	1.47	0.00

[a] All measurements were made by intracellular cytokine staining for IFN-γ except for those shown in parenthesis that were made by staining with Ag-specific tetramers.

[b] As a negative control for nonspecific activation, single-cell suspensions were stimulated with HPV E7 peptide and stained for FACS, as described in *Materials and Methods*.

CD8$^+$ T cells found in these organs. There are a number of possible explanations for this finding. The first is that colonization may have occurred at levels below the detectable limit or too transiently to be detected in a 24-h period. In support of this hypothesis is the recent finding that clearance of a *Listeria* infection by day 1 after immunization does not shorten the duration of CD8$^+$ T cell proliferation (47). Secondly the T cells may have left the inductive site and entered local areas of infected tissue. However, we found Gag-specific CD8$^+$ T cells in the LP only after oral immunization, which correlates with the higher levels induced in the PP after this route of immunization. However, it should be noted that other workers have detected Ag-specific CD8$^+$ T cells in the LP after i.v. administration of *L. monocytogenes*. Significant (10%) numbers of LLO-specific CD8$^+$ T cells were found in the LP (48) after i.v. immunization with *L. monocytogenes*. I.v. immunization with *L. monocytogenes*-OVA also induced high levels of OVA-specific CD8$^+$ T cells in the LP (~15%) at the peak of the immune response (49). Nevertheless, oral immunization does appear to be associated with higher Ag-specific CD8$^+$ T cells in the LP because this route of administration resulted in about one-third of the CD8$^+$ T cells in the LP being Ag-specific in this study as well as when *L. monocytogenes*-OVA is administered this way (50), suggesting that this is the preferred route of immunization for the induction of protective immunity at mucosal surfaces.

In this study, we also sought to determine whether our vaccine construct elicited an immune response in vivo similar to that believed necessary to suppress an HIV infection. Current knowledge about the nature of protective immunity in HIV infection is incomplete, but accumulating evidence suggests that CTLs play an important role in the control of viral replication during HIV infection (4, 51). In primary infection, HIV-1 specific CTL can appear only a few days after clinical presentation and several weeks before neutralizing Abs (52). During acute HIV/SIV infection, the appearance of HIV/SIV CTL coincides with a marked decrease in plasma viremia (5). If CD8$^+$ T cells are depleted before macaques are experimentally challenged with a chimerical simian/human immunodeficiency virus, primary infection leads to much higher levels of virus in the blood and lymphoid tissue, together with a more profound immunosuppression (6). The importance of HIV-specific CTL in chronic HIV infection gains further support from the demonstration of a close inverse correlation between viral load and levels of circulating HIV-specific CTL (7). The emergence over time in an infected person of virus-escape mutants that elude CTL recognition suggests that strong CTL responses are a major influence on the survival of HIV populations within the host (8, 9). Finally, the demonstration of HIV-specific CTL in highly exposed but apparently uninfected individuals is consistent with a potential role for CTL in protection against HIV infection (4, 51).

Our vaccine construct is designed to elicit such a CTL response. Mainly using ^{51}Cr release CTL assays or protection with and without T cell depletion, our group and other researchers have shown that Gag expressing recombinant *Listeria* can induce Gag-specific CTL (36, 41, 53). Indeed, Rayevskaya and Frankel (36) employed a highly attenuated *Listeria* mutant, incapable of incorporating D-alanine into the cell wall, to deliver HIV Gag by the oral route with the induction of CTL activity in the spleen, MLN, and PP. However, this study is the first to use HIV Gag tetramers and HIV Gag

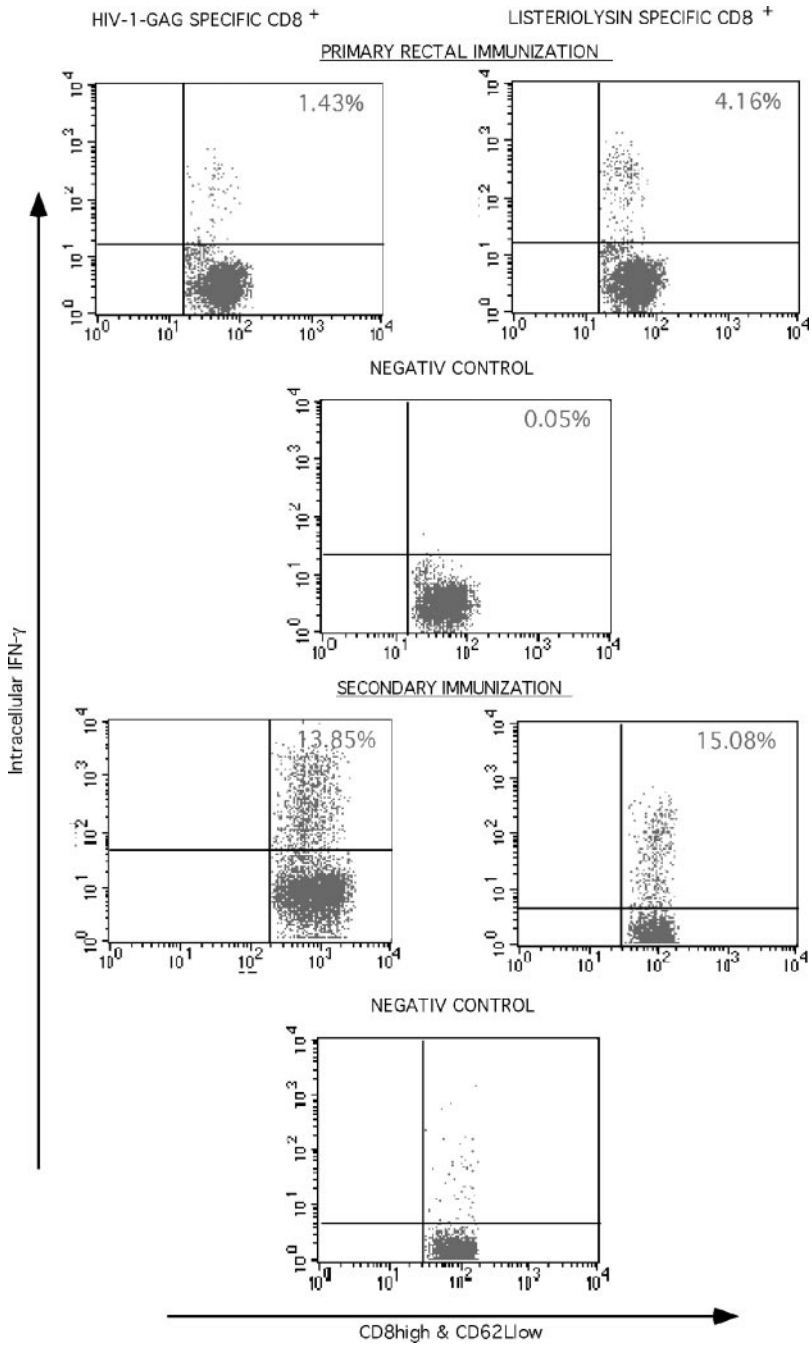

FIGURE 6. The induction of HIV-1 Gag-specific $CD8^+$ T cells in the spleen, compared with LLO-specific T cells, after rectal immunization with 10^5 Lm-Gag. Primary responses were measured 9 days after immunization. Secondary rectal immunization of 10^5 Lm-Gag took place 30 days following the primary dose, and 5 days later splenocytes were analyzed for the presence of IFN-γ secreting $CD8^+$ T cells as described in *Materials and Methods*.

MHC class I-restricted peptide-specific intracellular IFN-γ secretion to visualize ex vivo Gag-specific $CD8^+$ T cells induced by an oral *Listeria* vector. Moreover, these Gag-specific $CD8^+$ T cells were effectively induced via another mucosal route, i.e., intrarectal immunization.

In addition to quantifying the absolute level of induced HIV-1 Gag-specific $CD8^+$ T cells, we have also compared these levels to that of induced hemolysin-specific $CD8^+$ T cells as the immune response to LLO, coded by an endogenous gene, which represents the maximum response that *Listeria* can induce (54). After a single immunization at the one-time doses we administered, the number of HIV Gag-specific T cells induced varied from ~50 to 60% of the number of LLO-specific T cells. Although it has not been definitely determined that immune protection depends directly on the number of T cells induced, data generated using *Listeria* point to such a relationship (44). We have therefore deployed quantitative visualization techniques to measure T cell frequencies ex vivo rather than via ^{51}Cr release assays, which require expansion of induced T cells in vitro and thus may give a positive result even when T cell numbers might be too small for effective immune protection.

Surprisingly, a single booster administered 30 days later raised the number of HIV Gag-specific $CD8^+$ T cells to that of the endogenous gene benchmark. We speculate that the effectiveness of the booster immunization may be related to the absence of a strong Ab response to *Listeria*. Boosters are not effective for viral vectors of passenger Ags such as adenovirus or vaccinia, because Abs acting as a first-wave defense eliminate most of the invaders. Without this strong first-wave Ab defense, *Listeria* persists in numbers sufficient to trigger a T cell recall. The availability of effective booster immunization makes the *Listeria* vector uniquely attractive because booster immunizations can refresh specific immunity to maintain high numbers of Ag-specific $CD8^+$ T cells.

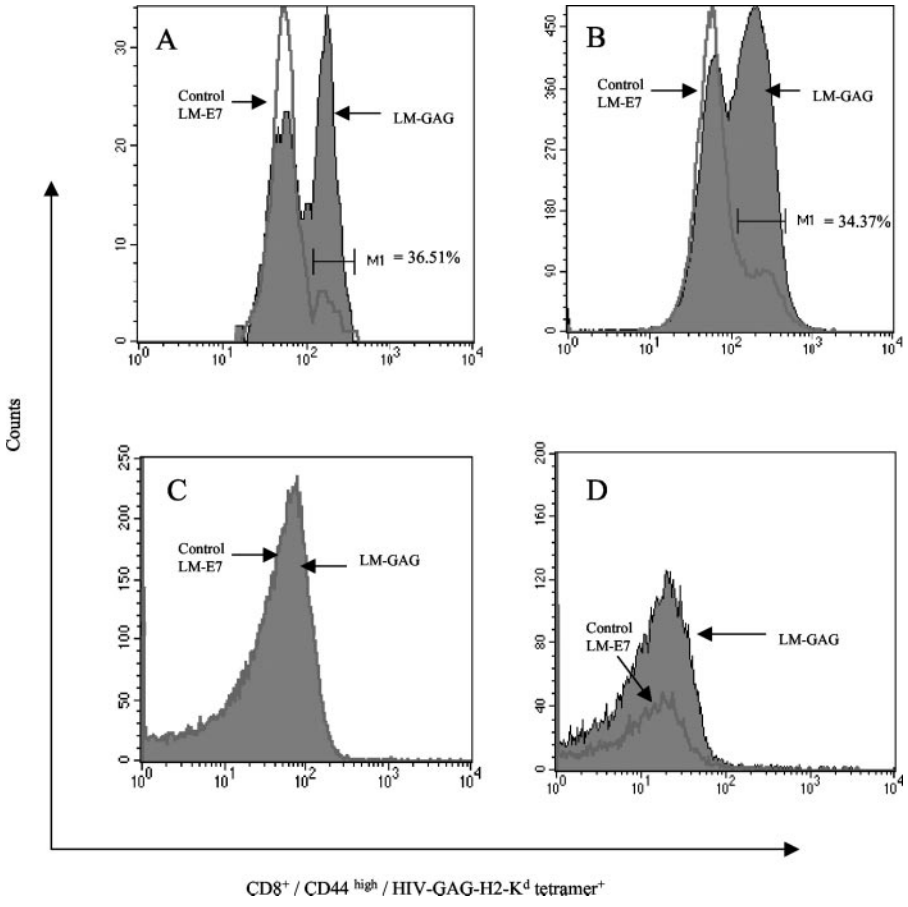

FIGURE 7. The induction of HIV-1 Gag-specific $CD8^+$ T cells in the LP, after oral or rectal immunization with 10^8 Lm-Gag or i.v. Primary responses were measured 9 days after immunization by the oral (*A*) or intrarectally (*C*) or i.v. (*D*) route. Secondary oral immunization of 10^8 Lm-gag took place 30 days following the primary dose, and responses were measured 5 days later (*B*). LP was analyzed for the presence of tetramer-positive $CD8^+$-activated ($CD44^{high}$) T cells as described in *Materials and Methods.* The percentage of Gag-specific activated $CD8^+$ T cells was calculated based on the gate shown in the panels by subtracting the number of tetramer-positive $CD8^+$-activated ($CD44^{high}$) T cells present in this gate from mice immunized in a similar manner with Lm-E7 (~15%) from the number of events from mice immunized with Lm-Gag (~50%).

This study suggests that *Listeria* can induce immunity at mucosal sites; indeed, both primary and booster immunizations proved effective when administered mucosally. This advantage is particularly important in the context of HIV infection. Recent studies have established that HIV-1 can enter the body via mucosal surfaces even if the latter are completely intact (10–12). This strongly implies that HIV-specific immune elements at mucosal sites will be critical for the initial (and timely) control of infection in many or most individuals exposed to the virus. Studies of candidate HIV vaccines that are designed to elicit mucosal immune responses have focused mainly on HIV-induced B cell (IgA) and Th cell responses (13–15) or CTLs in the spleen (16). Only limited information is available on mucosal vaccine induction of CTL responses in mucosal tissues themselves, by using either peptide-based vaccines (19), live viruses (20, 21, 55), DNA vaccination (56), or targeted lymph node immunization (13, 57). The development of vaccines capable of inducing CTL in mucosal sites, however, is becoming more critical with the emergence of recent evidence that natural genital infection with HIV-1 leads to the development of MHC class I-restricted cytotoxic T cells in the cervix (17) and that mucosal administration of live attenuated SIV, which protects against subsequent infection with virulent virus, elicits a CTL response in gut-associated lymph nodes (18).

We also investigated the presence of Gag-specific lymphocytes in the LP after a primary immunization and a booster immunization with recombinant Lm-Gag. After primary oral immunization, a surprisingly high number (~35%) of all-activated $CD8^+$ LPL were specific for HIV Gag. Boosting with Lm-Gag did not increase the level of Gag-specific CTL in the LP (Fig. 7). The discovery of high levels of Ag-specific $CD8^+$ T cells in LP are in contrast with the findings of other workers who have investigated the induction of LLO-specific $CD8^+$ T cells by wild-type *L. monocytogenes* in the same strain of mouse (BALB/c) as we used in this study where somewhat lower levels of tetramer-positive $CD8^+$ T cells were detected after oral infection (48). Indeed, the level of LLO-specific $CD8^+$ LPL T cells peaked at ~15% of the total after primary oral immunization. In addition, i.v. immunization also resulted in significant (10%) numbers of LLO-specific $CD8^+$ T cells in the LP (48), in contrast to our findings in this study (Fig. 7*D*). However, in another study in which an immunodominant, H-2^b-restricted OVA epitope was delivered by recombinant *L. monocytogenes* (50), oral immunization with *L. monocytogenes*-OVA did result in levels of OVA-specific $CD8^+$ T cells in the LP (~28%) (50) similar to the levels of Gag-specific $CD8^+$ T cells that we obtained in this study. In addition, although secondary oral immunization did produce a recall response to OVA in the LP, no increase in the level of OVA-specific LPL was detected either by OVA-tetramer staining or by intracellular IFN-γ staining (50) in agreement with our findings reported in this study.

We believe the induction of high numbers of Gag-specific $CD8^+$ T cells in the LP is potentially crucial: a vaccine against HIV must be able to provide large numbers of $CD8^+$ T cells to the LP if, as is currently believed, the vast majority of HIV infections occur via the mucosal route (e.g., sexual transmission and mother-to-child transmission via breastfeeding). The key role played by mucosal CTLs in protection against mucosally acquired HIV was demonstrated by Belyakov et al. (21) who showed that mucosal HIV-specific $CD8^+$ T cells conferred long-lasting immune resistance to mucosal viral transmission in mice while systemic CTLs were unable to protect against mucosal transmission. Furthermore, MHC class I-restricted CTLs directed against SIV-env in the jejunal LP are correlated with protection from colonic SIV challenge

(22) and from vaginal infection (58). These findings are consistent with the presence of a $CD8^+$ lymphocyte-mediated IFN-γ response to HIV epitopes in the cervix of highly exposed, uninfected Kenyan prostitutes. The immune response was also found systemically but was enhanced in the genital tract area at the likely site of virus exposure (51).

In the context of this mucosally transmitted disease, then, a mucosally administered vaccine seems best calculated to prompt an immune response capable of preventing infection. The data we present in this report demonstrate that mucosally administered *Listeria* can prompt an immune response of a kind promising in the context of HIV, and that booster doses can maintain this immune response. The recombinant construct we used in this study, however, is based on the *Listeria* wild type and may be virulent enough to pose a danger to immunocompromised individuals. To overcome this obstacle, we are working to develop appropriately attenuated mutants that retain the ability to induce a similar level of immunity when mucosally administered. If such a construct can be developed, *Listeria* may fulfill the promise suggested by the data presented in this report and by others.

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