ABSTRACT

Stevens, Rosemary. *Listeria monocytogenes* as a Biologic Vaccine Vector Against Feline Immunodeficiency Virus (FIV). (Under the direction of Dr. Gregg A. Dean)

Listeria monocytogenes is attractive as a vaccine vector against human immunodeficiency virus (HIV) for multiple reasons. It induces strong cell-mediated immunity, can be delivered mucosally, is easily manipulated to express foreign antigens, is amenable to transport plasmids, is inexpensive to produce and is responsive to antibiotic treatment. Recent murine studies with orally delivered recombinant *L. monocytogenes* expressing HIV Gag resulted in strong CTL responses to Gag in the spleen and gut-associated lymphoid tissues. As the gut is the site of early immune defects caused by HIV infection, a successful vaccine will need to target this vulnerable site. Using the feline immunodeficiency virus (FIV) model of HIV we evaluated a recombinant *L. monocytogenes* in an FIV challenge system. In the first study, five cats were immunized with recombinant *L. monocytogenes* that expresses FIV Gag and delivers an FIV Env-expressing DNA vaccine (LM*gag*/pND14-Lc-*env*). Control cats were either sham immunized or immunized with wild-type *L. monocytogenes* (10403S, LM-wt). All cats were challenged vaginally three months post immunization with FIV strain NCSU$_1$. One year post challenge all animals were sacrificed and tissues were harvested. Provirus was not detected by real-time PCR in any tissue evaluated from cats immunized with the recombinant bacteria. These cats also maintained normal CD4:CD8 ratios in mesenteric lymph nodes while control cats had inverted ratios. Recombinant *L. monocytogenes* vaccinated cats also maintained normal percentages of CD4 and CD8 T

cells in intestinal epithelium while control cats showed depletion of both populations. Vaginal FIV-specific immunoglobulin A was present in three immunized cats before challenge and in all five at one-year post challenge. This study demonstrates that while a single, low-dose immunization with LM*gag*/pND14-Lc-*env* did not prevent FIV infection, it did reduce viral loads and enabled immunized cats to control viral replication.

The second study addresses two questions, 1) vaccine efficacy in the face of pre-existing vector immunity, and 2) evaluation of peripheral blood as an indicator of vaccine-induced immune responses. Pre-existing immunity is a critical consideration in light of diminished effectiveness of adenovirus and recombinant vaccinia virus upon repeated exposure. We examined the immunogenicity of LM*gag*/pND14-Lc-*env* in cats previously infected with LM-wt. Eight subcutaneously infected cats were divided into two groups of four. One group received $5x10^6$ cfu LM-wt orally followed two months later with $1x10^8$ cfu oral LM*gag*/pND14-Lc-*env* (SQ/Oral). The other group received only $1x10^8$ cfu oral LM*gag*/pND14-Lc-*env*. After a single oral dose of LM*gag*/pND14-Lc-*env*, cats with existing anti-*L. monocytogenes* immune responses developed anti-FIV Gag IgA titers in vaginal secretions, saliva, and feces. Similarly, FIV Gag and Env specific IFN-γ ELISPOT responses were measurable in spleen and lymph node, but at a statistically higher frequency in cats exposed to a single subcutaneous dose of wild-type *L. monocytogenes* versus those cats exposed both subcutaneously and orally.

These two groups of cats were also used to answer the question of appropriateness of peripheral blood as a measurement of systemic and mucosal vaccine-specific immune responses. Our data show that extended culture was required to measure low-frequency

antigen-specific immune responses in peripheral blood. Our results also show that there is no relationship between response measured in PBMC and that observed in splenic or lamina propria lymphocytes.

Together, these studies show the ability of the oral vaccine LM*gag*/pND14-Lc-*env* to control viral load upon vaginal challenge and to generate anti-FIV antibodies and CD8 T cell response in the face of existing anti-listerial immunity.

Listeria monocytogenes as a Biologic Vaccine Vector against Feline Immunodeficiency Virus (FIV)

By

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Dedication

I would like to dedicate this work to my daughters Allison, Katherine and Megan.

Their love and patience make all things possible.

Biography

Rosemary Greer Stevens was born in Brooklyn, NY to Joseph and Eileen Greer. As the only girl in a family of boys, she often spent her time dreaming of world domination. As a young girl, she lived in Pakistan and traveled extensively, thus beginning her love of boxes and packing shells. In 1982, she married Sam Stevens and they began a life together and raised three beautiful daughters. Realizing the necessity of a higher education, Rosemary returned to school when her youngest daughter began to walk. In 1998, Rosemary graduated from NC State with a degree in Biochemistry. She began working in a reference laboratory where she immunophenotyped leukemias and lymphomas. It was there that her love of immunology developed. Eventually, this desire to know more about immunology drove her back to school to pursue a doctorate in immunology under the direction of Dr. Gregg Dean. And the rest, as they say, is history.

Acknowledgements

First of all, I would like to express my appreciation to my major advisor, Dr. Gregg Dean, for accepting me into his laboratory, putting up with me, and helping me pursue my dreams. I would also like to thank the members of my committee: Dr. Frederick Fuller, Dr. Edward Havell, and Dr. Mary Jo Burkhard, for their support, encouragement and thoughtful suggestions towards my work. I am grateful to Dr. Wayne Tompkins, Director of the Immunology Graduate Program, for his interest in my scientific development and for scaring me smart in journal club. I would also like to acknowledge the technical support (and friendship) provided by Alora LaVoy, Ingrid Fisher, Julie Long, Glicerio Ignacio and Erin Peters. Their kindness and technical help kept me going when life was darkest. A special thanks to Dr. Shila Nordone and Dr. Kristina Howard, without their questioning, prodding, and (sometimes irritating) in-your-face attitude, I would not be half the scientist I am. Thanks to Linda Costine for making life run smoothly, you are amazing. Last, but certainly not least, thanks to Dr. Jacquelyn Schechter for her friendship and expertise. Without her help, this dissertation would not have existed.

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1) Introduction

Since 1981 more than 20 million people worldwide have died of AIDS and greater than 40 million people are currently living with HIV. Every day more than 14 thousand people are infected, with the preponderance of these new cases occurring in developing nations (1). In developed nations, anti-retroviral drug regimens have had a tremendous impact on both quality of life and slowing of disease progression. However, the cost and complexity of these drug therapies are prohibitive for most people in third-world countries. Preventative programs such as health education and condom and needle distribution can slow the spread of disease, but to truly stem the pandemic a safe and effective HIV vaccine must be developed.

Twenty years of HIV research have advanced our knowledge in basic immunology and retrovirology and have improved vaccine design and use of animal models. Early HIV vaccine design initially focused on the generation of anti-HIV antibodies, based on successes in neutralization of laboratory-adapted viral strains (2). Observation of cohorts of people who were highly exposed to HIV but persistently seronegative (HEPS) and long-term non-progressors (LTNP) revealed HIV-specific CD8 cytotoxic T lymphocytes (CTL) to be important players in HIV protection and led to the second wave of vaccines designed to generate HIV-specific CD8 T cells (3,4,5,6). Results were initially promising, however, macaque vaccine experiments and clinical experience with viral CTL escape variants with ensuing rapid progression to disease has led to our current understanding that both arms of the immune system will be needed to combat this global epidemic (7,8,9,10,11).

Novel vaccine strategies and animal models will have to be exploited to generate high risk/high gain results. *Listeria monocytogenes* (LM) is a Gram positive, intracellular enteric

pathogen that has been extensively studied for over thirty years and has many attractive characteristics as a vaccine vector against HIV. It induces a strong cell mediated immune response, can be delivered by mucosal routes, is amenable to genetic manipulation to express viral antigens, can carry plasmids to enhance exogenous antigen exposure and is easy and inexpensive to produce (7,8,9,10,12,13,14,15,16). Clearance of LM involves a concerted effort between CD8^{+} CTLs and CD4^{+} Th1 cells, making LM infection the paradigm of cell-mediated immunity (17,18,19). While the humoral immune response appears to play a negligible role in clearance of LM infection, anti-Listerial antibodies are generated upon infection (20,21,22). Our studies show that antibodies are also generated against the exogenous antigens carried by recombinant LM (23).

Previous murine studies utilizing recombinant LM that expresses HIV Gag proteins have had success in generating long-lived anti-gag mucosal and systemic T-cell responses that were protective against a vaccinia-gag challenge (24,25,26).

In order to further our understanding of LM as a vaccine vector in a feline model of AIDS, we designed experiments to address two hypotheses:

1. A single oral immunization with recombinant *Listeria monocytogenes* that expresses the FIV Gag and delivers an FIV Env-expressing DNA vaccine (LMgag/pND14-Lc-env) will control viral replication and disease progression in a feline challenge model.

2. Previous exposure to *L. monocytogenes* will not affect the ability of LM*gag*/pND14-Lc-*env* to generate an anti-FIV immune response.

Specific questions to be addressed include:

1. Can orally delivered LM*gag*/pND14-Lc-*env* prevent infection upon vaginal FIV challenge?

2. If infection is not prevented, will immunization limit viral load and prevent immune dysregulation?

3. Will pre-exposure to *L. monocytogenes* limit the efficacy of LM*gag*/pND14-Lc-*env* vaccine?

4. What tissue sites are most informative for evaluation of immune response generated by orally delivered LM*gag*/pND14-Lc-*env* vaccine?

Literature Review

HIV, a Global Epidemic

In 1981, the first clinical cases of human immunodeficiency virus (HIV) were recognized (27,28). Twenty-three years later, more than 20 million people have died and there are over 40 million people infected with HIV.1 The majority of new infections occur in impoverished developing countries where access to anti-viral regimens is severely limited. A vaccine is desperately needed to control the spread of HIV.

HIV is a single strand ribonucleic acid (RNA) positive-sense *Lentivirus* in the family *Retroviridae*. All retroviruses encode for Gag, Pol and Env, replicate by reverse transcription and integrate into the host genome. Lentiviruses are genetically more complex than other retroviruses as they encode for additional accessory proteins.

Animal models being used to investigate HIV vaccine strategies include murine, non-human primate and feline. Of the non-human primate lentiviruses, SIV chimpanzee (SIVcpz) is the most phylogenetically closest to HIV-1 (29,30). Chimpanzees, the natural host for SIVcpz, are both endangered and refractory to disease progression except under some experimental conditions, thus limiting the value of this model (30). Sooty mangabeys in the wild are naturally infected with SIV (SIVsm), but like chimpanzees, sooty mangabeys fail to progress to AIDS due to an apparent lack of immune response to virus (31,32). This lack of response is characterized by decreased expression of CCR5 on CD4 T cells (33). Currently, macaques are the primates of choice for vaccine research as they can be experimentally infected with SIVsm. However, since macaques are not the natural hosts for SIVsm, infection can lead to abnormally rapid immune dysregulation and disease progression (34,35,36,37). In addition, SIVsm has significantly divergent envelope sequences compared to HIV (38). To

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overcome this challenge in vaccine evaluation, SIV/HIV hybrids (SHIV) have been generated. These hybrids contain HIV envelope in an SIV backbone (39,40,41). Macaques are extremely difficult to infect by vaginal route with cell-associated SIV. Therefore, vaginal challenge inoculum is generally cell-free, which may have a different immune dynamic than vaginal infection with cell-associated virus (42). This is important because HIV is horizontally transmitted primarily by sexual contact (vaginal, anal) and contaminated blood or vertically from mother to child *in utero* or postnatally through breast milk (43,44). Transmission is therefore often at a mucosal surface and may be via cell-associated virus and cell-free virus.

Murine studies, while increasing our knowledge of basic immunology, fall short as HIV vaccine models, as mice cannot be infected with HIV, and there are no murine lentiviruses homologous to HIV. Murine retroviruses such as mouse mammary tumor virus and murine leukemia virus are genetically simple compared to HIV (45). Severe combined immunodeficiency mice (SCID) that have been repopulated with human lymphocytes (Hu-PBL-SCID) is a murine model that is capable of being infected with HIV but this is a highly contrived system of dubious value (46,47). With over five million new HIV cases a year worldwide, additional small animal challenge models need to be exploited to develop novel vaccines.

FIV as a Model for HIV Vaccine Design

Feline immunodeficiency virus (FIV), discovered in 1986 by Pedersen, is a lentivirus in the family *Retroviridae* (48). Like HIV infection in humans and SIV infection in macaques, FIV infection in the cat is characterized by loss of CD4 T cells, inverted CD4/CD8 ratio, altered cytokine profiles and loss of T cell proliferation in response to

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mitogen (49,50,51). FIV disease progression also follows a similar course to HIV. Upon infection, cats progress from an acute infection stage marked by fever, high plasma viremia and lymphadenopathy, to an aymptomatic stage that can last from months to years depending on pathogenicity of infecting viral strain and finally ending with terminal AIDS, characterized by both increased plasma viremia and susceptibility to opportunistic diseases (52,53,54).

Phylogenetically, SIV is more closely related to HIV than FIV is to HIV. FIV lacks several accessory proteins that are found in HIV, specifically Vpu, Vpr and Nef (55). FIV also lacks a distinct *tat* gene and TAR element (56,57). However, FIV contains Orf-2 that behaves like *tat* in that Orf-2 is essential for a replication efficient virus. However, Orf-2 requires additional LTR elements for activation (58).

Receptors and Co-receptors

The initial step in viral entry is dependent on virus and host cell receptor interactions. HIV and SIV utilize CD4 as the primary receptor and chemotactic cytokine (chemokine) receptors CXCR4 and/or CCR5 as the co-receptors (59,60). HIV isolates obtained during acute infection are predominately CCR5-tropic whereas during chronic infection isolates are a mixture of CCR5 and CXCR4, with CXCR4 predominant in terminal stages of HIV infection (61). FIV does not utilize CD4 as its primary receptor. Recently it has been discovered that CD134, a T cell activation and co-stimulatory molecule also known as OX-40, is the primary viral receptor and that CXCR4 can serve as the co-receptor (62). In cats, CD134 is primarily expressed on CD4 T cells, ensuring that FIV infection via CD134 will impact host ability to effect immune control through loss of T helper cells. CD134 is also expressed on CD8 T cells, B cells and macrophages, thus providing FIV with a broader cell

tropism than HIV or SIV. Some primary isolates and laboratory adapted strains of FIV act in a CD134-independent manner, increasing cellular targets to B cells and monocytes. While HIV and FIV utilize different receptors, both viruses target T helper subsets.

Correlates of Protection

In HIV infection, low plasma viremia has been correlated with virus-specific cytotoxic T lymphocytes (CTL). In rhesus macaque studies of SIV infection, depletion of CD8 T cells before challenge resulted in high levels of plasma viremia marked by inability to control viral replication (63,64). However, in highly exposed persistently seronegative (HEPS) cohorts, it has not been proven whether the CTL response observed is one of protection or indicative of non-productive HIV exposure (65,66). HIV specific CTL levels fall below limits of detection when subjects are on highly activated anti-retroviral therapy, leading to the conclusion that continuous or punctuated antigenic exposure might be necessary to maintain "protective" levels of CTL. This concept is reiterated by the seroconversion of HEPS sex workers when they are no longer being exposed to virus (66). Animal studies suggest that in order for anti-HIV CTL to be effective, they must be present at the site of infection. One of the methods of immune evasion utilized by HIV is the generation of viral variants with mutated CTL epitopes that are no longer recognized by T cells. The selective pressure applied by epitope-specific CTL clones as well as the rapid mutation rate of HIV drives the diversification of primary viral sequences (10,11,67). To combat this viral strategy of immune evasion, anti-SIV or HIV CTL must be generated against a broad spectrum of viral epitopes with emphasis on conserved epitopes.

Neutralizing HIV specific IgA antibodies found in saliva, serum and genital secretions of HEPS cohorts indicate that an effective vaccine might have to induce both arms

of the immune system (68,69). Recent studies performed by Mascola et al. show protection against vaginal SHIV challenge (89.6P) by passive immunization with neutralizing antibodies 2F5 and 2G12.2,70 Although serum antibody levels used to confer protection were substantially greater than those induced upon infection or vaccination, partial protection was afforded by neutralizing antibody levels that are more physiologically relevant. This suggests that a vaccine that could induce a strong CTL, CD4 T helper type-1 (Th1) and modest mucosal IgG or IgA response against the virus could interfere with early viral events and limit disease progression and transmission.

Vaccine Modalities

In the twenty years of HIV vaccine research, many vaccine candidates have been developed. Live-attenuated, heat-killed, subunit, recombinant virus, DNA and recombinant bacterial vectors either singly or in a combined prime/boost strategy have been or are currently being investigated through animal models. Cats are the natural host for FIV and can be infected vaginally with virus. Despite the phylogenetic distance between FIV and HIV, the route of transmission, disease progression and immune dysregulation are strikingly similar between these two viruses. On the other hand, SIV models rely on non-natural hosts with highly pathogenic strains that lead to rapid disease course. Nevertheless, SIV and FIV vaccine trials have provided valuable insight into disease immunopathogenesis and have generated potential vaccine candidates.

Traditionally, vaccine design has focused on generation of neutralizing antibodies rather than induction of CD8 and Th1 immune responses. While neutralizing antibodies have been shown to protect from disease, the generation of antibodies from vaccination that can neutralize diverse strains of HIV is proving difficult to achieve.

Live, Attenuated Vaccines

Live, attenuated viruses have been used successfully against polio, measles and chickenpox. The pathogenicity of the live virus is reduced either by extensive propagation *in vitro* or through deletion or mutation of viral genes. Early studies of immunization of macaques with a *nef*-deleted strain of SIV showed promise as the immunized macaques were protected from challenge with pathogenic SIV and SHIVs (71,72). However, due to the rapid mutation rate of HIV, researchers were cautious with this approach. An additional study with an SIV nef mutant (12 nucleotide deletion) underscored the need for caution as the SIV virus reverted to complete pathogenicity during the course of the infection (73). More recently it has been shown that SIV mutants can be pathogenic for neonatal macaques and older chronically infected macaques (74,75). Due to concerns about reversion back to wild-type HIV, this highly effective vaccine strategy cannot be seriously considered.

Killed Vaccines

Killed vaccines employ whole viruses that have been chemically inactivated so they cannot replicate. This strategy has been used successfully against polio and influenza virus. An early SIV macaque study with inactivated SIV Delta$_{B670}$ was extremely promising until it was discovered that the protective immune response generated by this vaccine was not against viral proteins, but rather against the human leukemic T cell line (H9) cells used in the virus preparation (76,77). It is hypothesized that the antibodies against human cell-surface molecules may have helped to prevent viral infection by interfering with the virus's ability to interact with the host cellular receptors. Additional evidence for the strength of the xenogeneic immune response elicited by the culture cells was seen as vaccination of macaques with uninfected culture cells elicited some protection upon SIV challenge while

virus grown in SIV cell line afforded no protection. As a safe vaccine cannot be based on antibodies to host cell surface molecules, this line of investigation has been abandoned.

In comparison, inactivated whole-virus vaccines have successfully protected cats against homologous and closely related heterologous strains of FIV (78,79,80). Protection from these vaccines was not due to anti-cellular immunity, as in the SIV model, but rather to the induction of anti-FIV immune responses. A dual-subtype FIV vaccine comprised of inactivated strains of subtype A (FIV$_{Pet}$) and subtype D (FIV$_{Shi}$) has been developed and tested against both homologous and heterologous FIV strains. Dual-subtype immunized cats developed virus neutralizing antibodies by the third immunization and were protected against low-dose challenge (81). While inactivated whole-virus vaccines may not be applicable for human vaccines, the immune mechanism of protection induced by these vaccines can be examined in the feline model.

DNA Vaccines

DNA vaccines are plasmids, preferably in supercoiled form, that contain viral genes of interest under control of a strong eukaryotic promoter that drives efficient transcription of the gene of interest (82). Furthermore, bacterial DNA contains unmethylated cytosine-guanine motifs (CpG) that can be highly immunostimulatory to B cells and plasmacytoid dendritic cells in humans (83,84). Bacterial CpG DNA has been shown to be a powerful T helper-1 (Th1) adjuvant similar to complete Freunds adjuvant (85). DNA vaccines are extremely interesting as they are a complete package containing the antigen of interest and adjuvant.

DNA vaccines are typically injected either intramuscularly or shot into the skin via as DNA-coated gold particles using a gene gun. The DNA-coated gold particles are propelled

through the skin and delivered into the cytoplasm of cells. By comparison, DNA injected intramuscularly is taken up by resident dendritic cells (Langerhan cells of skin) or other antigen presenting cells that then process and present antigen to T cells via the MHC Class II pathway. It has also been shown that plasmid DNA that contains open reading frames as well as eukaryotic transcription and translation signals allows *in vivo* synthesis of the proteins that are then processed as other endogenous proteins are, through the MHC Class I pathway (82).

Early DNA vaccines in mice were extremely promising in that they induced strong cell-mediated and humoral immune responses. However, DNA vaccines have not yet reached similar levels of immunogenicity in non-murine models and tend to require multiple rounds of immunization (7,86,87). DNA vaccines that are paired with live replicating vaccines have shown more potential. DNA prime followed by recombinant modified vaccinia virus (MVA) boost have been successful in the macaque model in inducing control against mucosal challenge as defined by prevention of progression to clinical AIDS. Multiple DNA prime/boost studies have been conducted and typically require a single prime followed by one or more boosts to elicit strong immune responses (8,88,89). This strategy needs further optimization for it to become a realistic vaccine candidate. As the HIV crisis is most severe in sub-Saharan African countries such as Botswana and Swaziland, a prime/boost vaccine strategy will require limited boosts to insure feasibility of delivery.

Subunit Vaccines

Subunit vaccines are comprised of highly purified viral proteins that have been expressed in tumor cell lines or yeast. This approach is currently being used against Hepatitis B virus. Studies in chimpanzees with recombinant monomeric HIV envelope proteins were successful against an intravenous cell-free homologous HIV challenge (90). However, the

HIV strain used (IIIB) was laboratory adapted and did not replicate well in chimpanzees (91). Initial human immunogenicity studies have shown that the subunit strategy does not elicit HIV-1 envelope-specific CTLs and that antibodies failed to neutralize primary isolates of HIV-1 (92). A major obstacle to this vaccine strategy is the inability of subunit vaccines to induce antibodies against conserved neutralizing epitopes of the HIV glycoprotein gp160. Since native HIV-1 envelope exists as a trimer, a subunit vaccine that can deliver trimeric gp120 in the appropriate conformation might be more effective at virus neutralization.

Live-Vector Vaccines

Live-vector vaccines are attractive as they combine the components of a subunit or DNA vaccine with the immunogenicity of a live, replicating organism. Viral genes can be inserted into viruses or bacteria that will express the foreign gene products during the course of infection. Infection with the recombinant vectors leads to an immune response to the vector and to the inserted viral gene. One drawback of this modality is that the strength of immune response is directly correlated to the pathogenicity and replicative capacity of the vector.

Poxviruses have been widely studied as HIV vaccine vectors. Studies in macaques using a recombinant vaccinia virus expressing HIV-1 envelope followed by a protein boost completely protected the macaques upon homologous challenge (SIVmne) (93,94). Additional studies using a heterologous challenge (SIVmac) showed partial protection, perhaps due to strain specific antibodies (95). Unfortunately, HIV-infected immunosuppressed people have developed life-threatening disseminated vaccinia infections when immunized with vaccinia virus, curbing the enthusiasm for that strategy (8,88,96). However, less replication efficient strains, such as modified vaccinia Ankara (MVA), a

vaccinia strain that has multiple deletions of pathogenicity genes, are under investigation (97). MVA-based SIV vaccines alone or in conjunction with protein or DNA boost have had some success in delaying disease progression and limiting viral load (8,88,98).

Another virus currently being studied is canarypox, an avian poxvirus that cannot complete a replication cycle in humans and consequently has a promising safety record (99,100). Human clinical trials with HIV-1 *gag* and *env* encoded canarypox vaccines have elicited production of low-titer Gag and Env-specific antibodies and to a lesser extent CTL response (101,102). Due to limited replication and inefficient production of exogenous proteins, this vaccine strategy would require multiple boosts. A novel approach to boosting the immunogenicity of canarypox is to load dendritic cells (DC) with recombinant canarypox and use the natural function of DC presentation of antigen to T cells to enhance immune responses (103). Proof of principle experiments have been completed and will be followed up with human clinical trials. There are many other virus-based vaccine vector strategies under investigation including a replication deficient Venezuelan equine encephalitis virus and adenovirus (104).

Bacterial Vectors

Salmonella and *Mycobacterium bovis* bacillus Calmette-Guerin (BCG) are intracellular bacteria that are currently being explored as vaccine vectors. These intracellular bacteria are processed and presented to the immune system in the same manner as viruses. Both *Salmonella* and BCG are primarily retained in the phagosome limiting presentation through MHC Class I pathway. BCG is attractive as it induces a chronic infection that can enhance foreign antigen presentation. While BCG has been engineered to express viral antigens and promote a CTL response, it has not been shown to protect against heterologous

SIV challenge (105,106). *Salmonella* can be manipulated to express the *Listeria monocytogenes* gene HLY that then enables *Salmonella* to escape the phagolysosome and induce a modest CD8 T cell response (107). Other strategies have been devised to enable *Salmonella* to enter the cytosol and present proteins via MHC Class I pathway. These modified *Salmonella* vaccines when used in conjunction with MVA or DNA boosts have had success in inducing SIV-specific Gag CTLs. Unfortunately, immunized macaques failed to control viral replication upon rectal challenge with SIVmac239 (108).

Listeria monocytogenes

Listeria monocytogenes (LM) is a food-borne gram-positive intracellular bacterium that infects humans and animals. For over 30 years, LM has been used to study cell-mediated immunity as control of LM infection is mediated by a strong CD8 CTL and CD4+ Th1 response (17,19). Less emphasis has been placed on the humoral responses induced by LM infection, however, several studies have shown the protective role of anti-listerial antibodies (21,22,23).

Natural infection by LM can occur upon ingestion of contaminated salads, soft cheeses, milk and corn. Symptoms of infection include gastroenteritis, diarrhea and abdominal pain. After ingestion of LM, bacteria survive the acidic environment of the stomach and enter the small intestine, and translocate through the intestinal mucosa via the Peyer's Patches (109,110). Bacteria then spread to the mesenteric lymph nodes (MLN), spleen and liver where they infect macrophages and hepatocytes as well as epithelial and dendritic cells (111). Two surface proteins, internalin A and B (InlA, InlB), permit LM to adhere to a variety of cells (112). The cellular receptor for InlA is E-cadherin, which is expressed on epithelial cells. InlA acts as an invasin and mediates the internalization of LM

by epithelial cells (112). The cellular receptor for InlB is gC1-q-R and is found on hepatocytes, epithelial and endothelial cells thereby enabling wide dissemination of infection (113). Upon internalization, LM escapes the phagosome by secreting a virulence factor, listeriolysin O (LLO). LLO is a crucial factor, as it disrupts the phagosomal membrane, allowing LM to survive and multiply in the safety of the cytoplasm (109). LM escapes immune surveillance by utilizing another factor called ActA. ActA mobilizes host actin to enable LM to infect neighboring cells without entering the extracellular environment (114). The sequestration of LM to intracellular compartments directs its secreted antigens to be presented through the MHC Class I pathway to CD 8 T cells (115,116). Early host immune response to LM infection is through recruitment of neutrophils and NK cells to the liver and spleen followed by activation and expansion of CD4 and CD8 T cells (117).

In addition to a strong CD8 T cell response, LM infection induces a CD4 Th-1 response (118). Not all bacteria exit the phagolysosome, so bacterial antigens are also processed and presented via the exogenous MHC class II pathway. The cytokines IL-12 and IFNγ produced by early responder cells, direct the CD4 response to a Th-1-type response rather than Th-2 (119).

Bacterial localization determined by route of infection.

Although LM is an enteric organism, most experiments have delivered LM intravenously (IV) or intraperitoneally (120,121,122). Y. Paterson showed that orally and IV delivered recombinant *Listeria monocytogenes* (rLM) expressing *Escherichia coli* ß-galactosidase generated ß-galactosidase specific CTL in murine splenocytes as determined by chromium release assay.15 T. Abo et al (1995) compared oral to IV immunization and

determined that oral immunization required a significantly greater number of organisms (1-5x10^9 cfu) compared to systemically delivered LM (10^4 cfu) to generate immunity (123).

The kinetics and localization of bacterial colonization are different based on route of infection. Oral inoculation of LM results in colonization of the intestinal lumen, spleen, liver and MLN with peak infection occurring on day three. MLN of orally infected mice had 10 to 1000-fold higher bacterial counts than IV infected mice (124,125,126). Investigations of the small intestinal epithelial lymphocytes (IEL) and lamina propria lymphocytes (LPL) showed no to low colonization of the IEL and protracted colonization of the LPL (viable bacteria until day 5) (124). Another study examined bacterial colonization of the cecum, small intestine (SI) and the large intestine (LI). Their results showed clearance in the SI by day eight and delayed clearance in the cecum and LI with few listeria remaining in the distal part of the LI on day thirteen (125). The progression of oral LM infection is severe intestinal infection followed by bacterial localization to the MLN within 5-6 hours post infection and then delivery to the spleen and liver with peak bacterial counts at day three (126).

Intravenous infection with LM results in high colonization in the spleen and liver one to three days post infection, with clearance by day ten. Surprisingly, modest numbers of viable bacteria were sporadically detected in the MLN and intestinal lumen (124).

Effect of Route of Infection on Generation of Antigen-specific CD8 T cell Response

Early studies utilizing orally delivered rLM showed that oral immunization led to systemic production of antigen-specific CTL but did not quantify antigen-specific CD8 T cell responses in the liver or intestinal tissues (15,127). In the past decade there have been several studies examining the potential of LM to deliver HIV-1 genes (126,128,129,130). As HIV is primarily transmitted mucosally, the ability of LM to induce mucosal immune responses

needs to be investigated. In 2001, L. Lefrancois used OVA-H-2K[b] tetramers to examine the CD8 T cell response induced by oral immunization of rLM-OVA (131). C57Bl/6 mice were orally immunized with $1x10^9$ cfu rLM-OVA and 9, 15 and 30 and 41 days later tissues were harvested, processed and stained for 3-color flow cytometry. At nine days post immunization, CD8+ OVA-specific tetramers were detected in spleen, liver, SI, IEL and LPL. Only small numbers of tetramer positive cells were found in MLN despite bacterial colonization of MLN. Fifteen days after infection, the splenic response dropped to memory levels whereas 13% of LPL cells were OVA-specific. The liver and LPL CD8 T cell responses did not reach memory levels until 30 days post infection when approximately 3% of CD8+ T cells were OVA-tetramer positive. These results are consistent with E. Pamer's study with LM-specific tetramers, which also characterized phenotypic differences in intestinal CD8 T cells after oral and IV inoculation (124). After both oral and IV infection, the proportion of IEL TCRαβ T cells increased. Upon oral inoculation, this increase was attributable to CD8αβ T cells. However, after IV inoculation there was expansion of both CD8αα and CD8αβ T cells. The increase in TCRαβCD8αβ LPL was significant over uninfected mice after both oral and IV inoculation. This study demonstrated that only oral inoculation resulted in isolation of viable bacteria from the intestinal lamina propria. It is interesting that mucosal immune responses induced by oral infection were similar to those induced by IV inoculation. The authors hypothesize that bacterial antigens or antigen-presenting cells migrate to the lamina propria after systemic infection, thereby facilitating lymphocyte expansion in the intestinal mucosa. It is also possible, but less likely, that T cells activated in the spleen migrated to the intestine (124). Finally, the cytolytic activity of the LLO-specific CD8 T cells from the spleen and lamina propria was measured by chromium

release assay and showed that LPL had greater cytolyic activity against target cells than splenocytes, regardless of the route of infection. However, LPL from orally infected mice had greater cytolytic activity than LPL from mice infected intravenously. Interestingly, splenocytes from IV inoculated mice had greater CTL activity than splenocytes of orally infected mice even though both groups had similar bacterial titers by day three post infection.

L. Lefrancois (2001) further expanded on the localization of effector memory cells into nonlymphoid tissue such as liver, lung and fat pads (132). Mice were orally gavaged with 1×10^9 cfu LM-OVA, and evaluated at 9, 20 and 59 days post rLM-OVA infection. Tissues evaluated included spleen, peripheral lymph nodes, MLN, Peyer's Patches, peripheral blood, bone marrow, lung, LPL, IEL, liver, kidney, fat pad and peritoneal lavage. Nine days post-infection tetramer positive cells were found in all tissues surveyed with the greatest percentage found in peripheral blood, lung, LPL, liver, kidney, fat pad and peritoneal lavage. By day 59, half the tissues had undetectable or less than 1% tetramer positive CD8 T cells. Sites with significant percentages of OVA-specific tetramers included peritoneal lavage, fat pad, kidney, liver, LPL, lung and bone marrow. Although these results don't address functionality, they demonstrate the tendency of LM-specific CD8 memory cells to migrate to nonlymphoid tissue.

Organ-Specific Requirements for Co-stimulation

It is generally accepted that primary CD8 T cell responses to non-inflammatory immunogens such as male-specific antigen and cell-based antigens require CD4 help, either by activation of antigen-presenting cells (APC) or through CD40-CD40L interaction between CD4 and CD8 T cells (133). In contrast, the primary CD8 T cell response to inflammatory agents can occur in a CD4-independent manner through TLR recognition of microbial

products (134). This can activate APC and bypass the need for direct CD4-CD8 contact through CD40-CD40L interaction. Multiple studies have been conducted with LM to address questions associated with memory response such as the requirement for CD4 help, organ-specific need for CD40-CD40L interaction, and the requirement for CD28 interaction as well as the effect of CD4 help on development and maintenance of CD8 memory (125,135,136,137).

Leo Lefrancois has shown that while the primary mucosal CD8 T cell response to systemic vesicular stomatitus virus was dependent on CD40/CD40L interactions, it was less important for splenic response (138). To determine if this finding was pathogen specific or applicable to all mucosal immune responses, CD40-deficient mice were orally immunized with rLM-OVA and OVA-specific responses were measured by tetramers (137).The splenic and liver CD8 T cell responses were partially inhibited (35% and 50% respectively), while responses in IEL and LPL were reduced 75-90%. Tissue specific responses to rLM-OVA infection of MHC class II-deficient mice resulted in the same marked OVA-specific CD8 T cell reduction (60-75%) in LPL and IEL. However, the splenic response was normal and the liver OVA-specific CD8 T cell population was reduced 20% compared to wild-type mice. These results show differential requirements for CD40 costimulation in different tissues. Further studies by Shen and Bevan (2003) revealed that while the primary systemic immune responses can be generated without CD4 help, the generation, maintenance and quality of antigen-specific memory CD8 T cells was diminished (135,139,140). Their studies were performed using CD8 T cells specific for OVA or LCMV glycoprotein 33 (gp33) that were generated in CD4+/+ and CD4-/- mice and then transferred into naïve mice before challenge with rLM-OVA or rLMgp33. Both studies report that adoptively transferred CD8 T cells

from CD4+/+ mice were protective while CD8 T cells from CD4-/- mice mounted a defective response. Further investigation revealed that while CD4 T cells may not be required during initial priming of CD8 T cell response, they were necessary for generation of optimal levels and perhaps enhanced proliferation of CD8 memory cells.

CD8 T cells need for B cell help is similar to that observed for CD4 T cells, in that the primary systemic response to infection is not affected by the lack of B cells (141). The absence of B cells does not inhibit CD8 T cell activation and expansion. However, during the contraction phase, the absence of B cells results in enhanced death of activated CD8 T cell populations leading to a reduction of antigen-specific memory cells.

These LM studies have enhanced our knowledge of the effects of immunization route and the induction of appropriate CD8 T cell response, CD8 T cell requirements for appropriate co-stimulation, and requirements for CD4 T cell and B cells to generate long-term functional CD8 memory cells.

LM as a Vaccine Vector

Listeria monocytogenes may be a useful vaccine vector against lentiviruses. LM induces a strong cell mediated immune response, can be delivered by mucosal routes, is amenable to genetic manipulation to express viral antigens, can carry plasmids to enhance exogenous antigen exposure and is easy and inexpensive to produce (12,13,14,16). Clearance of LM involves a concerted effort between CD8^{+} CTLs and CD4^{+} Th1 cells, making LM infection a paradigm of cell-mediated immunity. While LM has been investigated for over thirty years with emphasis on its pathogenicity, host immune responses and intracytoplasmic existence, only recently has it been evaluated as a vaccine vector. In 1994, LM was engineered by Portnoy et al to secrete a fusion protein consisting of LLO and murine

influenza nucleoprotein (142). This experiment showed that the fusion protein was processed and presented via the MHC Class I pathway and mice immunized with the rLM construct mounted potent CTL responses against the viral protein. In 1995, LM constructs were generated to secrete either the entire lymphochoriomeningitis virus (LCMV) nucleoprotein or an immunodominant H-2Ld-restricted CTL epitope (143). Mice were immunized and boosted IV then challenged with LCMV clone 28b. All immunized mice remained healthy and controlled viral replication in comparison to wild-type LM (LM-wt) immunized control mice. Additional studies utilizing a more virulent LCMV strain, clone 13, demonstrated high host viral titers in all tissues sampled from LM-wt immunized mice whereas rLM immunized mice had undetectable virus levels. This experiment suggested that LM can be used as a vaccine vector to confer protection against lethal LCMV challenge. LM has subsequently been used as a vaccine vector against cotton-tail rabbit papillomaviruses and murine neoplasms as well as HIV and FIV (144,145). LM constructs containing HIV *gag* have induced strong anti-Gag CTL responses in mice (126,127,129,130,146). Since mice are not susceptible to HIV infection, pseudo-challenge systems comprised of a recombinant vaccinia construct containing HIV-1 *gag* (vacc-*gag*) have been used to determine effectiveness of the LM*gag* constructs (25,146). To investigate the mechanism of protection against vacc-*gag* challenge, mice were depleted of CD4 and CD8 T cells or just CD8 T cells. Mice that were depleted of both CD4 and CD8 T cells were completely unprotected whereas most mice depleted of CD8 T cells were protected. This is consistent with studies that show CD8 T cells are not essential for clearance of vaccinia virus. This vacc-*gag* study suggests that LM immunization induces strong CD4 and CD8 T cell responses.

Most recently, Patterson et al, examined the effect of route of immunization with their recombinant LM construct that expresses HIV Gag (LMgag) (126). Mice generated Gag-specific CD8 T cell responses in spleen, MLN, Peyer's Patches and LPL. Mice were immunized orally, intravenously or rectally and their response to primary and secondary immunization was measured by intracellular IFNγ staining and H-2K^d Gag peptide tetramer staining. Oral immunization generated the most significant immune response, with 35% of activated CD8 T cells in the LPL specific for HIV Gag. However, rectal and IV immunization generated anti-Gag CD8 T cell responses in the spleen and MLN but were undetectable in LPL.

Our studies have shown the efficacy of oral immunization with our construct, LM*gag*/pND14-Lc-*env* to: 1) Control viral load upon vaginal challenge with FIV through the generation of FIV-specific antibodies and CD8 T cells and 2) Generate FIV-specific antibodies and CD8 T cells in the face of pre-existing LM immunity (23).

The studies described above demonstrate that proteins expressed from exogenous genes stably integrated into the LM genome are immunogenic. LM can also be exploited as a plasmid DNA vaccine delivery vehicle. Plasmid delivery to eukaryotic host cells has been demonstrated with *Shigella flexneri*, *Salmonella typhi* and *S. typhimurim* as well as *Listeria monocytogenes* (12). Applications of bacteria-mediated gene transfer include vaccination, gene therapy and production of therapeutic proteins. Utilization of a live vector provides adjuvant effect and targets plasmids to APC in inductive sites of the immune system (147). *Listeria monocytogenes* is an enteric vector that can target the plasmid to mucosal inductive sites (148). Its intracellular life cycle and ability to escape the phagolysosome enable the plasmid to enter the cytosol to present antigen via the MHC class I pathway and its target cell

range includes dendritic cells and macrophages (149,150,151). The immune responses induced by LM infection including CD8 CTL, CD4 Th-1 and humoral responses, may prevent or control HIV infection.

Safety of LM Vaccination

The use of LM as a vaccine vector has raised serious safety concerns. LM infection can cause life-threatening disease in immunocompromised individuals, pregnant women and neonates. One of the requirements of a vaccine is that it be universally safe, therefore attenuated but immunogenic strains of LM need to be evaluated. A mutated LM strain has been generated that lacks virulence factors actA and phospholipase B (plcB) LMΔactAΔplcB (152). In comparison with LM-wt, this construct was cleared from the spleen and liver more rapidly and caused less morphological damage to the spleen. Two months after IV infection with LMΔactAΔplcB or LM-wt, mice were challenged with a lethal dose of LM-wt. All immunized mice survived the challenge while control mice succumbed. Both groups mounted similar LLO-specific CD8 T cell responses as determined by IFNγ ELISPOT.

Another attenuated LM construct, LM*daldat,* has been generated and extensively tested by Frankel et al. (25,153). The LM*daldat* mutant is a double deletion mutant in which large parts of the alanine racemase gene (dal) and D-amino acid aminotransferase gene (dat) have been removed. For this construct to replicate, D-alanine must be given along with the mutant LM. As D-alanine cannot be synthesized by eukaryotes, bacterial replication and transmission can be tightly controlled. Experiments with *daldat* mutants expressing HIV-1Gag (LM*daldat-gag*) have been examined in adult and neonatal mice. Two oral immunizations of adult mice with LM*daldat-gag* led to CD8 Gag-specific CTLs in the spleen, MLN and Peyer's Patches that were significantly increased compared to single

immunization. Unfortunately, LPL were not evaluated in this study. However, the anti-Gag CTL were greatly diminished two weeks post infection. Importantly, all immunized mice survived a vacc-*gag* challenge.

The safety and immunogenicity of the LM*daldat-gag* construct was investigated in neonatal mice that are extremely vulnerable to LM infection. Neonatal mice survived injection of 10^6 cfu of the mutated construct whereas the LD_{50} of LM-wt for neonatal mice is ten bacteria (153). They investigated whether adult mice who were immunized as neonates retained Gag-specific CD8 T cells. Splenocytes derived from these mice as adults retained Gag-specific cytolytic activity. Additionally, adult mice were boosted and then assayed three days later by chromium release assay for CTL activity. Boosted mice had five times the cytolytic ability versus naïve mice initially infected three days earlier. Mice immunized as neonates were also able to survive a vacc-*gag* challenge. This construct shows safety in immunocompromised animals and effectiveness in generating antigen-specific CTLs that were greatly enhanced upon boost.

The LMΔactAΔplcB construct has been examined in a human safety trial that measured clinical response to escalating doses of LM ($1x10^6$-$1x10^9$) (154). While limited data were collected, the subjects shed LM in their feces for typically no more than three days, while more frequently only a single day of bacterial colonization in the feces was observed. All subjects tolerated the bacteria well with no fever, diarrhea or need for antibiotic therapy. This study used low oral doses of attenuated LM in light of 1-$5x10^9$ dose of rLM required to generate immunity in mice. However, it was a good first step in the effort to prove the utility and safety of attenuated LM constructs.

In summary, HIV infection leads to loss of CD4 T cells and eventual immune failure to control infection. A successful vaccine must be able to induce both CD8 and CD4 Th1 immune responses. As HIV is primarily a mucosal disease, the vaccine must target the mucosal sites of early virus entry and replication. Modest amounts of mucosal anti-virus antibodies may be sufficient in conjunction with CTL responses to limit viral entry and protect against disease progression. LM is amenable to genetic manipulation and can induce protective CD8 and CD4 T cell responses as well humoral immunity. LM is an enteric organism that homes to liver, spleen and the intestinal mucosa regardless of method of delivery although oral delivery induces a more significant mucosal immune response compared to systemic inoculation. While attenuated strains of LM are being investigated as vaccine vectors, LM infection is extremely responsive to antibiotic treatment and induces expected immune responses within 24 hours of infection. Orally delivered LM constructs are easy to prepare and mixtures of rLM constructs can be given to enhance foreign antigen exposure. Previous exposure to LM does not appear to prevent induction of immune response against exogenous antigens (155,156). Thus, LM appears to be ideally suited for use as a vaccine vector against HIV.

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

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ABSTRACT

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 (LM*gag*/pND14-Lc-*env*). Control cats were either sham immunized or immunized with wild-type *L. monocytogenes* (LM-wt). One 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 eight of ten control animals. Virus was isolated from bone marrow of 4/5 LM*gag*/pND14-Lc-*env* immunized cats using a stringent co-culture system but required CD8+ T cell depletion, indicating CD8+ T cell suppression of virus replication. Control animals had an inverted CD4:CD8 ratio in mesenteric lymph node and were depleted of both CD4+ and CD8+ intestinal epithelial T cells while LM*gag*/pND14-Lc-*env* immunized animals showed no such abnormalities. Vaginal FIV-specific IgA was present at high titer in three LM*gag*/pND14-Lc-*env* immunized cats before challenge and in all five at one year post challenge. This study demonstrates recombinant *L. monocytogenes* conferred some control of viral load after vaginal challenge with FIV.

INTRODUCTION

Because natural transmission of human immunodeficiency virus (HIV) occurs at the mucosa and 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 compared to killed or subunit vaccines and are generally more effective because of the antigen load amplification by the replicating agent. Given the safety concerns of 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.

Listeria 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 MHC I presentation pathway (54, 59, 67). The host response against *L. monocytogenes* has been studied for more than 40 years and is considered the paradigm for cell-mediated immune 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 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 MHC I pathway (27). *L. monocytogenes* is also an effective delivery vehicle for plasmids designed for eukaryotic expression of immunogens (21, 32). With regards 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 APC, 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* have been successfully used as biologic vaccine vectors against lymphocytic choriomeningitis virus (LCMV), cottontail rabbit papillomavirus, murine influenza and murine neoplasms (40, 68, 77). Several studies using the mouse model have demonstrated the potential of *L. 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 against 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 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 HIV infection in humans (78). Like HIV, FIV infection leads to chronic immune dysfunctions including depletion of CD4+ 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 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* conferred some control of viral load after vaginal challenge with FIV.

MATERIALS AND METHODS

Animals, bacterial stock and challenge inoculum. Fifteen, 16 week old, female specific pathogen free (SPF) cats were randomly divided into three groups of five 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, manuscript in preparation). 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* was grown in brain/heart infusion media (Difco, Detroit, MI). LM*gag*/pND14-Lc-*env* is the recombinant strain of *L. monocytogenes* that carries a full-

length copy of the FIV molecular clone $NCSU_1$ *gag* gene stably integrated into the *L. monocytogenes* genome. The LM*gag*/pND14-Lc-*env* also carries a DNA vaccine plasmid with the $NCSU_1$ FIV envelope (SU and ectodomain of TM) cloned into pND14-Lc for eukaryotic expression of the FIV Env under the control of a CMV promoter. The construction and validation of LM*gag*/pND14-Lc-*env* have been previously described (G. A. Dean, A. LaVoy, R. Stevens, S. Nordone, M.J. Burkhard, submitted for publication). Cats were either sham-immunized with PBS or given a single oral immunization consisting of $5x10^6$ colony-forming-units (cfu) of LM-wt or LM*gag*/pND14-Lc-*env*. 12 weeks post immunization, all 15 cats were challenged by vaginal inoculation with 7.5 $x10^4$ FIV infected feline PBMC and $7.5x10^4$ $TCID_{50}$ cell-free virus. The challenge virus was FIV-$NCSU_1$, a pathogenic sub-group A molecular clone. Animals were sacrificed 52 weeks post challenge.

Sample Collection and Processing Serum, saliva and vaginal wash fluids for ELISA were collected at 0, 4, 8, 16, 20, 40 and 60 weeks post immunization and processed as previously described (10). PBMC prepared by centrifugation (600xg, 30 minutes) over histopaque (Sigma, St Louis, MO) were washed in LBT media (RPMI supplemented with 10% FBS, 15mM HEPES, 1mM sodium pyruvate, 4mM L-glutamine, 10 IU/ml penicillin and 10μg/ml streptomycin) and cryopreserved in 90% FBS with 10% DMSO. 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 hours at 37°C and 5% CO_2. Non-adherent cells were washed off and macrophages were gently

removed with a 1:3 solution of lidocaine, washed, and pelleted for DNA extraction. Intraepithelial lymphocytes (IEL) were isolated from a 20cm section of distal jejunum. The intestine was flushed with wash medium (PBS supplemented with 20% FBS, 4mM L-glutamine, 2mM penicillin/streptomycin, 1.25% fungizone (BioWhittaker, Walkersville, MD) and 10μg/ml gentamicin) and cut into 0.5cm strips following excision of Peyer's patches and lymphoid follicles. Cut intestinal sections were stirred vigorously for 30 minutes at 37°C in spin medium (Hanks balanced salt solution without Ca^{2+} or Mg^{2+} supplemented with 20% FBS, 4mM L-glutamine, 2mM penicillin/streptomycin, 1.25% fungizone (BioWhittaker, Walkersville, MD), 10μg/ml gentamicin, 2mM EDTA and 2mM dithiothreitol). The supernatant was collected then centrifuged at 1000xg for 15 minutes at 16°C. The supernatant was removed, the pellet resuspended in 30% Percoll (Amersham Biosciences, Piscataway, NJ), then underlaid with 70% Percoll and centrifuged at 400xg for 30 minutes at 25°C. Following centrifugation, the 30/70 interface layer was collected, washed twice in wash medium and counted.

Flow Cytometric Analysis. PBMC, IEL and mesenteric lymph node samples ($1x10^6$ cells) were stained with FITC- or PE-conjugated mouse monoclonal antibodies against feline CD8α (71) or CD4 (3-F4, Southern Biotechnology, Birmingham, AL.) analyzed with a FACScan flow cytometer (Becton Dickenson, San Jose, CA) using CellQuest software (Becton Dickenson, San Jose, CA). Data was compared using ANOVA with Bonferoni post test (GraphPad Prism, GraphPad Software, San Diego, CA).

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/ml p24 -GST fusion protein (65). Antibody was detected

with goat anti-cat IgG (Bethyl Labs, Montgomery, TX) or IgA (Serotec, Raleigh, NC) diluted 1:16,000 and 1:250 respectively and incubated for one hour at room temperature. Plates were developed with tetramethylbenzidine (TMB) (Kirkegaard and Perry, Gaithersburg, MD) for ten minutes and stopped with 1N H_2SO_4 before reading the optical density at 450nm. Titers of antibody were considered positive if they were two-fold greater than preimmune values for washes and three-fold greater than preimmune values for serum. Statistical differences in antibody endpoint titers between immunization groups were determined by analysis of variance and Duncan's multiple range test using the ANOVA and DUNCAN procedure of SAS (version 8.02, SAS Institute, Inc., Cary, NC). Kinetics of antibody responses were analyzed using regression analysis, including group and time interactions. Significance was defined as $p \leq 0.05$.

Virus Isolation. Virus isolation and CD8+ T cell depletion were performed as previously described (17) with minor modifications. Bone marrow mononuclear cells were stained with a fluorescein isothiocyanate conjugated anti-CD8 antibody cocktail consisting of monoclonal antibodies 357 (71) and fCD8 (Southern Biotechnology, Birmingham, AL) for 20 minutes. 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, CA) for 20 minutes, washed again and then run on an AutoMACS separation unit (Miltenyi Biotec, Auburn, CA) per manufacturers instructions. Sorting purity was greater than 98% as determined by flow cytometric analysis (FACScan, Becton Dickenson, San Jose, CA). Unfractionated or CD8+ depleted bone marrow cells (2.5×10^6) were washed in LBT before co-culturing in triplicate with 1×10^6 FCD4E cells (a feline CD4+, T cell line, (9)) in the presence of IL-2 (NIH AIDS

Research and Reference Reagent Program). Supernatants were analyzed for FIV p24 by antigen capture ELISA every 2-3 days over the 21 day culture period.

FIV p24 Antigen Capture ELISA. Maxisorp plates (Fisher Scientific, Houston, TX) were coated with the anti p24 monoclonal antibody 51G11.1, (a generous gift from Dr. Edward A. Hoover, Colorado State University) at (0.45 µg/ml) and incubated overnight at 4°C. Plates were washed and blocked with TEN/2% BSA overnight at 4°C. Plates were washed and 100µl of co-culture supernatants were added to the plate. Positive and negative controls were run on each plate and consisted of supernatants collected from persistently FIV-NCSU$_1$ infected Crandell feline kidney (CrFK) cells or uninfected CrFK cells, respectively. The plates were incubated for 2 hours at 37°C. After washing, 100µl of CG5 antibody (DEAE-purified, S-300 sized IgG from FIV+ cat plasma, Custom Monoclonals International, Sacramento, CA) (2µg/ml) was added to each well and the plate was incubated at 37°C for one hour. After washing, 100µl of KPL goat anti-cat IgG-HRP diluted 1:1000 in buffer containing 5% mouse serum and 2% goat serum was added to each well and incubated at room temperature for 45 minutes. Plates were developed with TMB (Kirkegaard and Perry, Gaithersburg, MD) for ten minutes and stopped with 1N H_2SO_4 before reading the optical density at 450nm.

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 using DNeasy Tissue Kit as directed by the manufacturer (Qiagen, Valencia, CA). Each amplification reaction contained 0.5µg DNA. PCR primers and probe specific for the NCSU$_1$ *gag* gene were designed using Primer Express (PE Applied Biosystems, Foster City, CA). The primers (FIVNC.491f, 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 FIV copy number. The CCR5 primers and probe (UC Davis Taqman Service, Davis, CA) for quantification of the feline CCR5 gene were used in the PCR reaction at 400nM and 80nM, 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 $1\mu g$ DNA.

RESULTS

Immunization and challenge strategy. Fifteen, 16-week old female cats were either sham immunized or orally inoculated with 5×10^6 cfu wt-LM or LM*gag*/pND14-Lc-*env*. Three months post vaccination, all cats were vaginally challenged with FIV-NCSU$_1$, a pathogenic sub-group A molecular clone. Fifty-two weeks post challenge, 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 LM*gag*/pND14-Lc-*env* immunization on proviral burden after vaginal FIV challenge, we performed quantitative real-time PCR (sensitivity ≤ 10 provirus copies) on DNA extracted from PBMC at 4, 32 and 52 weeks post challenge (Table 1). At 4 weeks post challenge 2 sham and 1 LM-wt cat were PCR positive while at 32 weeks, 2/5 sham and 5/5 LM-wt cats were positive. Intestinal intraepithelial lymphocytes, 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, one year post challenge. By 52 weeks post challenge, provirus was detected in the tissues of 8/10 control cats (3/5 sham and 5/5 LM-wt immunized). Notably, provirus was not detected in any tissue evaluated from the LM*gag*/pND14-Lc-*env* immunized cats.

Virus isolation. A maximum of 0.5 μg DNA, representing approximated $5x10^5$ 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 $2.5x10^6$ unfractionated or CD8+ T-cell depleted bone marrow cells. Bone marrow mononuclear cells were co-cultured with highly permissive FCD4E cells and FIVp24 was measured in culture supernatants by antigen capture ELISA every 2-3 days. Cells were co-cultured 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 was positive for 4/5 sham cats by co-culture day 14 in both unfractionated and CD8+ depleted cultures, while cat IIU3 was positive only when CD8+ T cells were depleted. Of the LM-wt immunized cats, 3/5 were positive in unfractionated cultures and 4/5 were positive in CD8+ T cell depleted cultures. Thus, all 10 sham and LM-wt immunized cats were FIV positive by PCR and/or virus isolation. Only 1/5 LM*gag*/pND14-Lc-*env* immunized cat was positive by virus isolation in unfractionated bone marrow cells. However, virus was isolated from all five LM*gag*/pND14-Lc-*env* immunized cats when CD8+ T cells were depleted (Table 2). These results are consistent with suppression of virus replication by CD8+ 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 LM*gag*/pND14-Lc-*env* immunized cats at 52 weeks post vaginal FIV challenge was 1.55±0.45. In contrast, sham and LM-wt immunized groups had inverted mean CD4:CD8 T cell ratios of 0.89±0.23 and 0.96±0.25, respectively (Figure 1A). These latter ratios were significantly lower than the LM*gag*/pND14-Lc-*env* immunized group (p=0.04, ANOVA). The inverted ratios were the result of both CD4+ T cell loss and increased CD8+ T-cell percentages (Figures 1B and 1C).

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

Mucosal and systemic induction of Gag specific IgG and IgA. FIVp24-specific IgG and IgA in vaginal washes, saliva and serum were measured by ELISA. Table 3 lists titers for each cat at 8 weeks post immunization and at study termination (52 weeks post challenge, 60 weeks post immunization). Immunization with LM*gag*/pND14-Lc-*env* induced vaginal anti-p24 IgA responses in 3/5 cats. Vaginal IgA was found in all five immunized cats after challenge, while only 2/10 control cats mounted a minimal anti-p24 vaginal IgA antibody response upon vaginal FIV challenge (Figure 3). Interestingly, 4/5 cats immunized with LM*gag*/pND14-Lc-*env* mounted a post-challenge anti-p24 IgG vaginal antibody response and by 52 weeks post challenge there was a strong statistical difference between LM*gag*/pND14-Lc-*env* immunized cats and sham immunized cats (p<0.009, ANOVA, Duncan multiple comparison test.). Two LM*gag*/pND14-Lc-*env* immunized cats mounted anti-p24 IgG and IgA antibody responses in saliva, however post-challenge antibody titers were not significantly different among the three groups. LM*gag*/pND14-Lc-*env* immunized cats did not mount a pre-challenge serum p24 IgG antibody response. However by 52 weeks post challenge, the mean p24-specific serum IgG titer for LM*gag*/pND14-Lc-*env* immunized cats was 3270±3635 versus 716 ± 857 and 1024 ±1024 (Microsoft Excel, Microsoft Corporation) for sham- and LM-wt immunized cats, respectively. Although the mean titer of LM*gag*/pND14-Lc-*env* immunized group was 3-5 times greater versus control groups, this was not statistically significant due to variations among individual 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 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 occurs 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* showed some control of virus burden after vaginal challenge with pathogenic FIV. One year after vaginal FIV challenge, immunized cats had undetectable provirus 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 co-culture with large numbers of lymphocytes was required to reveal the presence of virus in the LM*gag*/pND14-Lc-*env* immunized cats. Furthermore, CD8+ T cell depletion was necessary in four of five of the cats before virus replication was detected. This result emphasizes the importance of CD8+ T cells in the control of viral

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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 analysis of lymph nodes and mucosa-associated lymphocytes is a more sensitive approach to detecting alterations in lymphocyte subpopulations due to HIV or SIV infection (37, 47, 69, 73, 75). This was apparent in the present study as no lymphocyte subset alterations were seen in peripheral blood of any FIV challenged animals. However, an inversion of the CD4:CD8 ratio in the mesenteric lymph node occurred in sham and LM-wt immunized but not in LM*gag*/pND14-Lc-*env* immunized cats. The loss of CD4+ T cells and increase of CD8+ T cells observed is characteristic of FIV infection (78). Loss of CD4+ T cells from the intestinal immune system occurs very early in infection and is known to precede losses in lymph nodes of SIV 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+ T cell depletion (28, 37, 47, 73, 75). We observed a loss of both CD4+ and CD8+ T cells in sham and LM-wt immunized cats while LM*gag*/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+ 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+ 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 against FIV Gag. Vaginal IgA has been well studied in exposed, uninfected individuals and 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 of the IgA (14, 48). Mapping of IgA neutralizing activity has identified the coiled coil pocket in the alpha-helical region of the transmembrane protein, gp41, 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 TM or SU 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 stimulate a useful antibody response at the vaginal mucosa.

Demonstration of antigen-specific IFN-γ production by ELISPOT 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 constitutive IFN-γ production by PBMC that persists 30-60 days post immunization with *L. monocytogenes* (G. A. Dean, A. LaVoy, R. Stevens, S. Nordone, M. Burkhard, submitted for publication). 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-γ 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 to study IFN-γ 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+ T-cell suppression of virus replication in the virus isolation assay provided the some evidence for cell mediated immunity in this study.

Bacteria have received much less attention than viruses as 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 APCs are target cells. Furthermore, it has been proven *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 (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

TABLES

Table 1. Real-time PCR detection of proviral DNA in tissues

Group	Cat	PBMC[a] 4 WPC[b]	PBMC 32 WPC	PBMC 52 WPC	Retro [c] 52 WPC	IEL [d] 52 WPC	Bal [e] 52 WPC
Sham	IIU3	8.20×10^1	8.00×10^2	5.48×10^2	6.63	1.07	2.78×10^1
	IIS2	ND	ND	ND	ND	ND	ND
	IIH4	2.42×10^3	5.75×10^4	2.07×10^4	1.20×10^3	5.70×10^4	1.39×10^9
	IIG5	ND	ND	ND	ND	ND	ND
	IIM3	ND	ND	ND	ND	ND	ND
Wt-LM	IIG4	ND	2.07×10^2	ND	9.37	5.42×10^2	3.28×10^3
	IIS1	ND	3.88×10^2	ND	3.46×10^1	1.03×10^3	5.36×10^3
	IIH5	ND	5.03×10^2	3.60×10^2	5.60×10^2	5.28×10^3	3.14×10^2
	IIP4	1.40×10^1	5.75×10^2	1.85×10^2	3.55	2.63	2.42×10^1
	IIM2	ND	1.88×10^2	ND	1.06×10^2	ND	ND
LMgag/env	IIH2	ND	ND	ND	ND	ND	ND
	IIH3	ND	ND	ND	ND	ND	ND
	IIO5	ND	ND	ND	ND	ND	ND
	II06	8.0	ND	ND	ND	ND	ND
	IIO7	ND	ND	ND	ND	ND	ND

[a] peripheral blood mononuclear cells, [b] weeks post challenge. [c] lymph node, [d]intra-epithelial lymphocytes, [e]bronchoalveolar lavage mononuclear cells, ND= not detected.

Table 1. Real-time PCR detection of proviral DNA in tissues

Group	Cat	Thymus 52 WPC	BM [f] 52 WPC	MLN [g] 52 WPC	Spleen 52 WPC	Iliac [h] 52 WPC
Sham	IIU3	5.48	1.29×10^4	4.03×10^4	1.32×10^2	1.70×10^3
	IIS2	ND	ND	ND	ND	ND
	IIH4	2.24×10^9	5.74×10^9	7.22×10^6	7.24×10^5	1.16×10^6
	IIG5	ND	1.35×10^2	ND	ND	ND
	IIM3	ND	ND	ND	ND	ND
Wt-LM	IIG4	7.11×10^4	5.29×10^1	ND	6.29×10^2	8.64×10^4
	IIS1	1.24×10^3	1.14×10^2	4.23×10^5	7.69×10^3	6.38×10^2
	IIH5	1.20×10^5	1.30×10^3	3.93×10^2	ND	9.42×10^3
	IIP4	6.38×10^2	7.95×10^1	6.11×10^1	9.40×10^1	2.92×10^1
	IIM2	ND	ND	ND	ND	ND
LMgag/env	IIH2	ND	ND	ND	ND	ND
	IIH3	ND	ND	ND	ND	ND
	IIO5	ND	ND	ND	ND	ND
	II06	ND	ND	ND	ND	ND
	IIO7	ND	ND	ND	ND	ND

[f]bone marrow, [g]mesenteric lymph node, [h]medial iliac lymph node,

ND= not detected.

Table 2. Virus isolation from unfractionated and CD8+ T cell depleted bone marrow 52 weeks after vaginal challenge with FIV.

Group	Cat	Unfractionated	CD8 Depleted
Sham	IIU3	Neg[b]	Day 14 [c]
	IIG5	Day 14	Day 14
	IIM3	Neg	Day 9
	IIS2	Day 14	Day 14
	IIH4	Day 7	Day 7
LM-wt	IIG4	Neg	Day 21
	IIH5	Neg	Neg
	IIS1	Day 19	Day 19
	IIP4	Day 8	Day 8
	IIM2	Day 19	Day 19
LM-gag/env	IIH2	Neg	Day 14
	IIH3	Neg	Day 14
	IIO5	Neg	Day 19
	IIO6	Day 7	Day 7
	IIO7	Neg	Day 21

[a] For cat IIM3 mesenteric lymph node was evaluated.

[b] Virus isolation negative after 21 days of coculture.

[c] Day culture was FIVp24positive by ELISA.

Table 3. Anti p24 IgG and IgA antibody response[a].

Group	Cat	Vaginal IgA		Vaginal IgG		Saliva IgA	
		8wpi[a]	52wpc[b]	8wpi	52wpc	8wpi	52wpc
Sham	IIU3	0	0	0	32	0	0
	IIG5	0	0	0	128	0	4096
	IIM3	0	64	0	64	0	128
	IIS2	0	0	32	128	64	0
	IIH4	0	0	0	0	0	0
LM-Wt	IIG4	0	0	0	0	0	0
	IIH5	0	0	0	0	0	0
	IIS1	0	0	0	0	0	0
	IIP4	0	32	0	0	0	4096
	IIM2	0	0	0	0	0	1024
LM-gag/env	IIH2	0	1024	0	128	0	64
	IIH3	0	1024	0	0	512	1024
	IIO5	32	2048	256	32	256	0
	IIO6	256	2048	0	256	0	128
	IIO7	512	256	0	256	0	0

[a] Data shown is endpoint antibody titers

[b] wpi, weeks post immunization

[c] wpc, weeks post challenge

Table 3. Anti p24 IgG and IgA antibody response[a]

Group	Cat	Saliva IgG		Serum IgG	
		8wpi	52wpc	8wpi	52wpc
Sham	IIU3	0	64	0	2048
	IIG5	0	512	0	0
	IIM3	0	64	0	512
	IIS2	0	0	0	1024
	IIH4	0	0	0	0
LM-Wt	IIG4	0	512	0	1024
	IIH5	0	0	0	2048
	IIS1	128	64	0	0
	IIP4	0	128	0	2048
	IIM2	0	512	0	0
LM-gag/env	IIH2	64	1024	0	8192
	IIH3	4096	128	0	0
	IIO5	0	0	0	1024
	IIO6	0	128	0	2048
	IIO7	0	0	0	4096

[a] Data shown is endpoint antibody titers

[b] wpi, weeks post immunization

[c] wpc, weeks post challenge
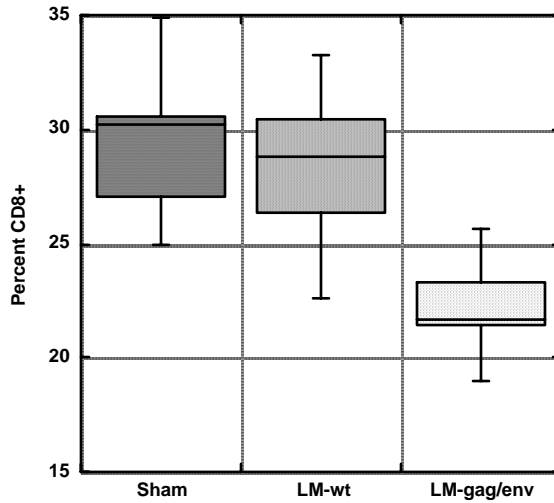
Figure 1.

**A.**
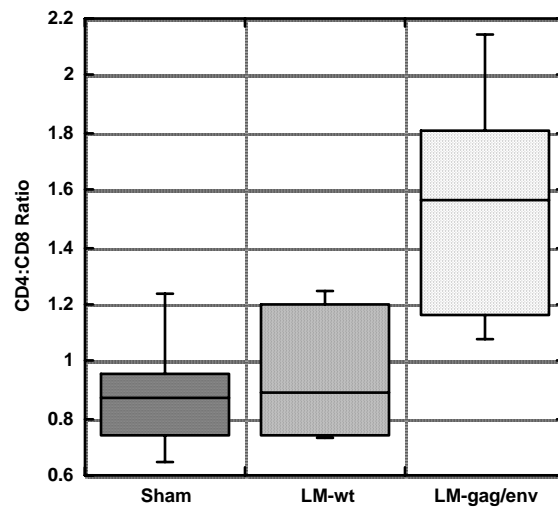
B.
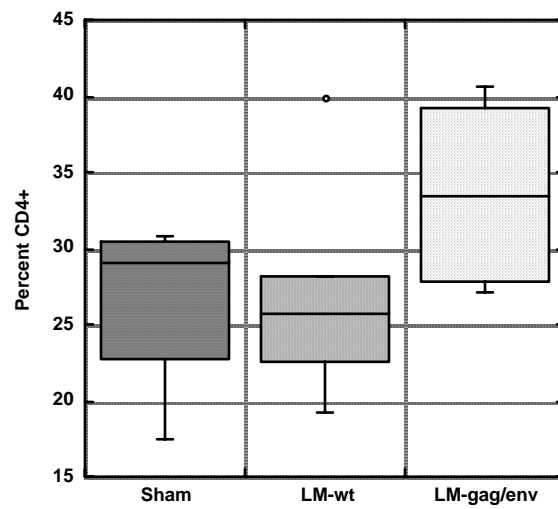
Figure 1.

C.

Figure 1. Lymphocyte subsets in mesenteric lymph node. CD4:CD8 ratios (A), %CD4+ (B), and %CD8+ (C) are shown in a box (encloses 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. Outlier, is displayed as an individual point. Mean CD4:CD8 ratio for LM*gag*/pND14-Lc-*env* (LM-gag/env) group is significantly greater than LM-wt or sham groups (p=0.04, ANOVA).
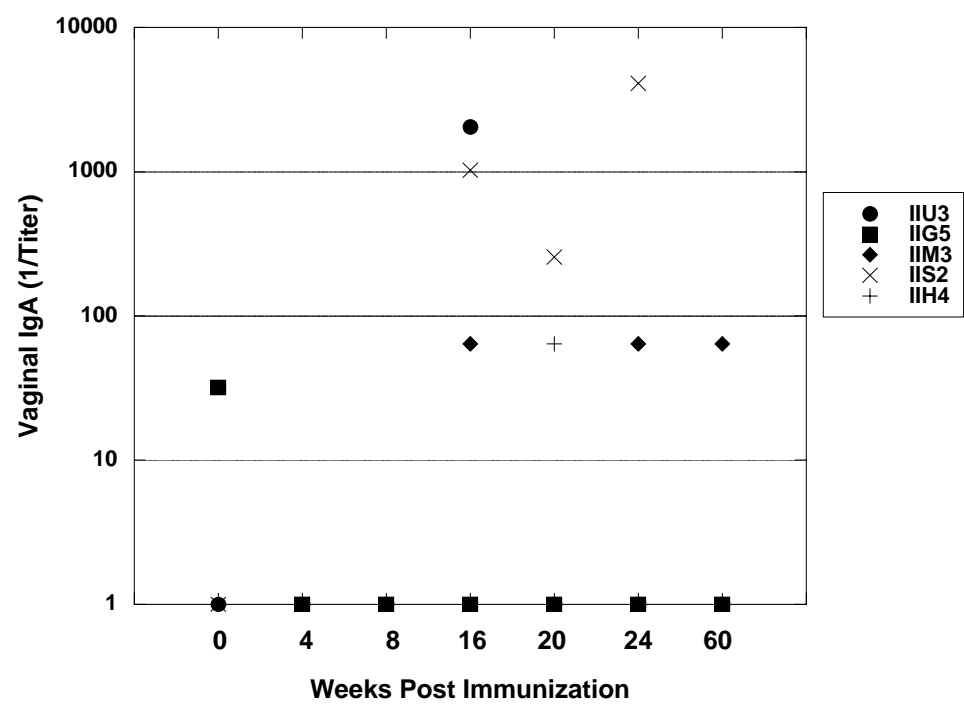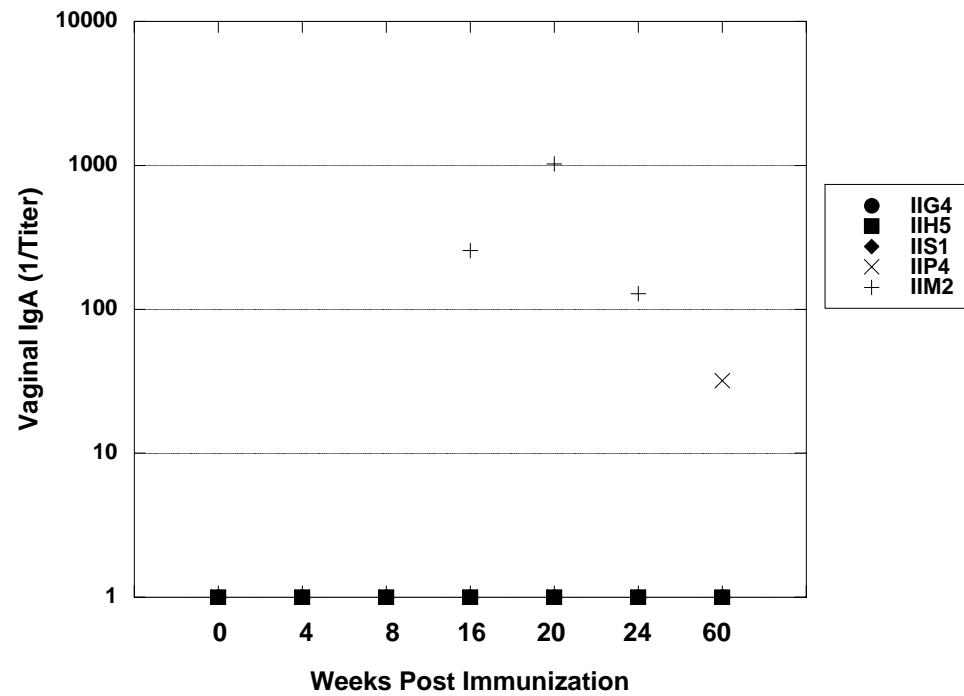
Figure 2.

A.
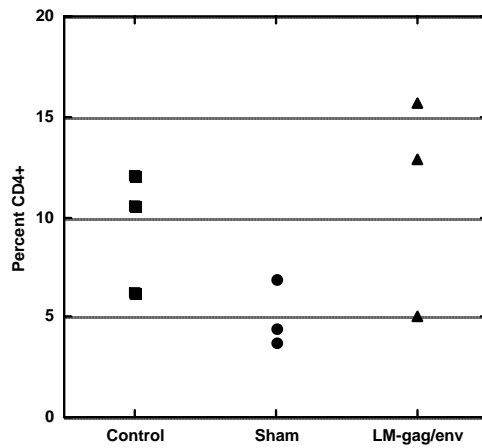
B.
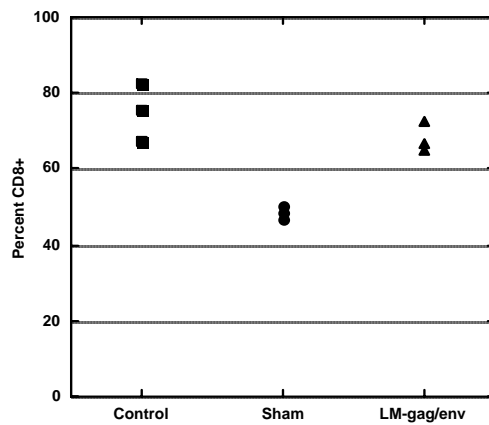
Figure 2. Lymphocyte subsets in intestinal epithelium. Percentages of CD4+ (A) and CD8+ (B) are shown for three FIV-naïve, SPF control cats, three sham immunized cats, and three LM*gag*/pND14-Lc-*env* (LM-gag/env) immunized cats.
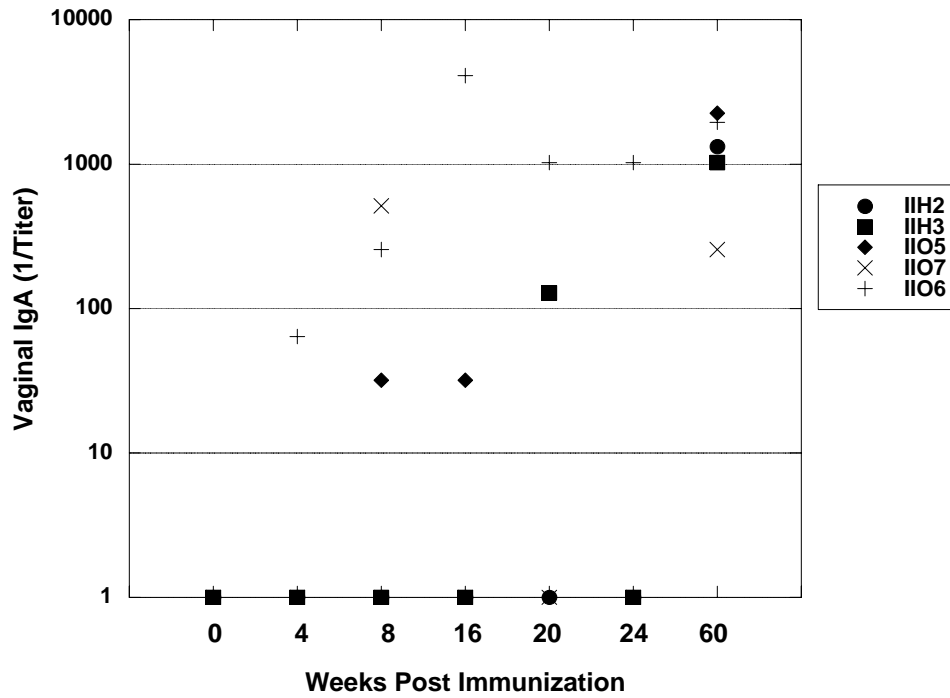
Figure 3.

A.

B.

C.

Figure 3**. Induction of FIV anti-p24 IgA in vaginal secretions. FIV Gag-specific IgA was measured in vaginal washes by ELISA at 0, 4, 8, 16, 20 and 60 weeks post immunization. Cats were vaginally challenged with FIV at week 12. Titers were determined by end-point dilution.**

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Pre-existing immunity to pathogenic Listeria monocytogenes does not prevent induction of immune responses to feline immunodeficiency virus by a novel recombinant Listeria monocytogenes vaccine

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Abstract

Listeria monocytogenes is an attractive biologic vaccine vector against HIV because it induces a strong cell mediated immune response, can be delivered by mucosal routes, can be readily manipulated to express viral antigens, and is easy and inexpensive to produce. Proof of concept studies have been performed using HIV Gag expressing recombinant *L. monocytogenes* in the mouse. Here we report the development and validation of recombinant *L. monocytogenes* to be evaluated in the FIV/cat model of HIV. Using a simplified approach to introduce individual and polyprotein FIV *gag* genes, we show recombinant *L. monocytogenes* containing the entire *gag* expresses the full-length Gag polyprotein in a soluble secreted form. A DNA vaccine plasmid (pND14-Lc-*env*) that replicates in Gram positive bacteria and contains the FIV SU (gp100) and the ectodomain of TM (gp40) in a eukaryotic expression cassette was transfected into LM-*gag* to create LM-*gag*/pND14-Lc-*env*. After infection of target cells with LM-*gag*/pND14-Lc-*env in vitro*, both FIV Gag and Env proteins were detected in soluble cell lysates. Whether previous exposure to *L. monocytogenes* affects the immunogenicity of LM-*gag*/pND14-Lc-*env* was determined in cats infected with wild-type *L. monocytogenes* orally and/or subcutaneously. After a single oral dose of LM-*gag*/pND14-Lc-*env*, cats with existing anti-*L. monocytogenes* immune responses developed anti-FIV Gag IgA titers in vaginal secretions, saliva, and feces. Similarly, FIV Gag and Env specific IFN-γ ELISPOT responses were measurable in spleen and lymph node but at a statistically higher frequency in cats exposed to a single subcutaneous dose of wild-type *L. monocytogenes* versus cats exposed both subcutaneously and orally. The FIV/cat model will provide a useful challenge system to determine whether recombinant *L. monocytogenes* can protect against a lentivirus in its natural host after challenge by the routes common to HIV transmission.

1.   **Introduction**

Recombinant *Listeria monocytogenes* has encouraging potential as a biologic vaccine vector against human immunodeficiency virus (HIV). The value of *L. monocytogenes* as a vaccine vector is a result of its life cycle and associated immune system activation. *Listeria monocytogenes* is a Gram positive bacterium that targets

antigen presenting cells, including dendritic cells and macrophages, as well as other epithelial cell types [1-4]. Once *L. monocytogenes* is phagocytosed by target cells, it escapes the phagolysosome and replicates in the cytosol. The cytoplasmic localization of *L. monocytogenes* results in direct access of secreted bacterial and recombinant proteins to the MHC I antigen presentation pathway. *Listeria monocytogenes* induces a multifactorial immune response involving neutrophils, macrophages, natural killer cells, CD4+ T-cells, CD8+ T-cells (cytotoxic T lymphocytes, CTLs), and numerous cytokines [5]. The strong cell-mediated response to *L. monocytogenes* has been shown to involve both CD4+ and CD8+ T-cells that act through cytokine production (IFÑγ) and cytolysis [6-13]. Importantly, natural infection by *L. monocytogenes* occurs by the oral route and leads to stimulation of a mucosal immune response. Thus, *L. monocytogenes* has many attributes considered important in designing an effective HIV vaccine.

Critical to its potential as a vaccine vector, *L. monocytogenes* can be engineered to express exogenous proteins [14]. Integration of foreign proteins into the genome of *L. monocytogenes* by sequential homologous recombination results in a stable recombinant bacterium that can produce and secrete the protein of interest [15]. To efficiently enter the MHC I antigen presentation pathway, the recombinant protein must be secreted in a soluble form by the bacterium into the cytoplasm of the host cell [16]. A CTL response against recombinant protein expressed by *L. monocytogenes* has been identified in several systems and has been demonstrated when *L. monocytogenes* is administered by oral, nasal, intraperitoneal or intravenous routes [17]. Protective immunity has been achieved against lymphocytic choriomeningitis virus, murine influenza, and cottontail rabbit papillomavirus, as well as some murine neoplasms [15,17-21]. Furthermore, recombinant *L. monocytogenes* containing HIV-1 *gag* has been shown to induce a strong, specific, long-lasting CTL response against the HIV-1 Gag protein in the mouse [22] [23]. These encouraging results merit further study in a challenge model system.

The majority of work with *L. monocytogenes* as an HIV vaccine has been performed in mice. This has been useful to develop recombinant *L. monocytogenes* constructs and demonstrate immunogenicity, but since HIV does not infect mice, this model cannot be used for a true virus challenge. Feline immunodeficiency virus (FIV) is a lentivirus of cats that induces a disease syndrome similar to HIV infection of humans.

Because the viruses and disease pathogenesis of FIV and HIV are similar, FIV infection of cats provides a valuable model for HIV infection of humans [24-28]. The FIV/cat model of HIV is particularly useful for vaccine development because statistically significant numbers of out-bred, specific-pathogen-free animals can be evaluated in a highly controlled system that culminates with a virus challenge [29-33].

The objectives of the present studies were to construct an FIV Gag expressing recombinant *L. monocytogenes*, determine whether recombinant *L. monocytogenes* could be used to deliver an Env-expressing DNA vaccine, and investigate the effect of previous *L. monocytogenes* exposure on immunogenicity of recombinant *L. monocytogenes in vivo*. Here we describe the construction of LM-*gag*/pND14-Lc-*env* and validate *in vitro* expression of FIV Gag and Env. We also show that cats with prior exposure to *L. monocytogenes* develop humoral and cellular immune responses against FIV Gag and Env after a single, relatively low dose, single oral immunization with LM-*gag*/pND14-Lc-*env*.

2. Methods

2.1 Construction of recombinant Listeria monocytogenes

Wild-type *L. monocytogenes* (LM-wt) isolate 10403S, serotype 1, was used in these studies. All *L. monocytogenes* strains were grown using brain-heart infusion (BHI) broth or agar plates (Difco Labs, Detroit, MI). The empty antigen cassette containing a multiple cloning site and kanamycin resistence gene were excised from pEJ140 by NotI digestion and ligated into the unique NotI site in pHS-LV [21], creating the plasmid pHS-LV2. Four regions of the *gag* gene were amplified from the molecularly cloned FIV NCSU$_1$ [34] representing different proteins or polyproteins of the Gag polyprotein as shown below.
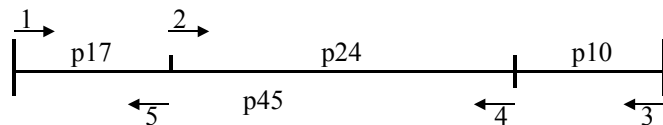
The full-length *gag* was amplified using primers 1 and 3, p45 using primers 1 and 4, p24 using primers 2 and 4 and p17 using primers 1 and 5. Each 5' primer added a BamHI site and each 3' primer added a SalI site.   The sequences for the five primers are as follows:

Primer 1: 5'GCGGGATCCGATGGGGAACGGACAGGGGCG3'

Primer 2: 5'GCGGAATCCGCCTATTCAAACAGCAAATGG3'

Primer 3: 5'GCGGTCGACTTATAAATCCAATAGTCTCTCC3'

Primer 4: 5'GCGGTCGACCAAGAGTTGCATTTTATATCCTGG3'

Primer 5: 5'GCGGTCGACAGATGCCTGTGGAGGGCTTTCC3'

The PCR products were gel purified, digested using BamHI and SalI restriction enzymes, then ligated into similarly digested pHS-LV2. The plasmids were sequenced to ensure fidelity of the PCR amplification of the FIV *gag* sequence. Each of the four plasmids containing different *gag* sequences was electroporated into penicillin-treated *L. monocytogenes* [35]. Electrotransformants were grown in BHI with erythromycin at 30°C, then at 42°C to select transformants that integrated the plasmid into the genome. Subclones were then cultured again at 30°C without erythromycin to force excision of extraneous vector sequences.   Subclones that had undergone the two-step allelic exchange were identified as kanamycin resistant and erythromycin sensitive [21].   The recombinant *L. monocytogenes* were sequenced to ensure the bona fide *gag* sequence was inserted in-frame into the correct region of the *L. monocytogenes* genome.

2.2 Construction of pND14-Lc-env

The Gram positive origin of replication and erythromycin resistance gene (2815 bp) were amplified by PCR from pBD9 [36] [37]. The primers also added 20-base 5' and 3' tails that were homologous to a linear 40bp region in pND14.   The forward primer was 5'GCGGTGGGCTCTATGGCTTCGGTCGATAGAAAGCGTGAG3'   and   reverse primer was 5'GCACCATAT GTTCCGCCTCACCGTCCACATGTCGAATTGC3'. The amplicon was gel purified and then inserted into pND14 by mutagenesis (QuikChange Site Directed Mutagenesis kit, Stratagene, La Jolla, CA) [38].   The resulting plasmid, pND14-Lc, was transfected into *L. monocytogenes* to ensure function of the Gram positive bacterial origin of replication and antibiotic resistance gene.   The *env* from

NCSU$_1$ was then amplified with forward primer 5'GCGGGATCCAGACTTCCCCCTTTAGTAGTTCC3' and reverse primer 5'CGGGGTACCTCACTTTAAGTATTGTGG3'. These primers were designed to amplify from the beginning of the SU mature peptide to the beginning of the membrane-spanning region of the TM and add a 5' BamH1 site and 3' Kpn1 site. The amplicon and the pND14-Lc were digested with BamHI and KpnI then ligated together. The resulting plasmid was called pND14-Lc-*env*. The integrity and frame of the FIV *env* within the plasmid were verified by sequencing.

2.3 Immunocytochemistry

P388D1 cells (ATCC, Manassas, VA) grown in a chamber slide were infected with LM-*gag* at an MOI of 50 for 1 hour at 37°C, 5% CO_2, then washed with PBS, incubated with medium including gentamicin (10µg/ml) for 3 hours at 37°C 5% CO_2. The cells were fixed in methanol for 2 min., blocked with goat serum, and probed with anti-FIVp24 mAb 43-1B9-FITC (a generous gift from Dr. Niels C. Pedersen, University of California, Davis).

COS-7 cells (ATCC, Manassas, VA) were transfected with pND14-Lc-*env* using FuGENE 6 (Roche Applied Science, Indianapolis, IN) according to manufacturer's instructions. After 40 hours, transfected cells were treated with brefeldin A (10µg/ml) for 4 hours at 37°C 5% CO_2 then fixed in methanol for 2 min. Fixed cells were blocked with goat serum, probed with anti-FIV Env mAb SUI-30 (Custom Monoclonals Inc., West Sacramento, CA), then incubated with goat anti-mouse-FITC (Jackson ImmunoResearch Laboratories, West Grove, PA).

2.4 Detection of recombinant proteins

For detection of recombinant protein in bacterial cultures, *L. monocytogenes* and recombinant *L. monocytogenes* were grown to mid-log phase in BHI broth buffered to pH 7.5 with 100mM MOPS, then the bacteria were pelleted. The culture supernatant was filtered, protein was precipitated with trichloroacetic acid, and the pellet was resuspended in 5X reducing sample buffer (Pierce Biotechnology, Rockford, IL) and heated to 95°C for 5 min. The bacterial pellet was washed with ice-cold PBS, pelleted, and resuspended

in 20μl of Tris/EDTA with 0.2% lysozyme per OD_{600} to lyse the bacteria. After incubation for 1h at 37°C, 0.4 volume of 5X reducing sample buffer was added and the lysate was heated to 95°C for 5 min. The culture supernatant protein and bacterial lysate were electrophoresed on 12% SDS-PAGE and transferred to nitrocellulose. The nitrocellulose membrane was blocked with 5% non-fat dry milk in PBS with 0.2% Tween-20, then probed with mAb 5G11.1 to detect FIV p24, p45 and Gag or mab 16A7.1 to detect p17 (antibodies were a generous gift from Dr. Edward Hoover, Colorado State University), incubated with goat anti-mouse-HRP (Pierce Biotechnology, Rockford, IL) and developed with DAB (Sigma, St. Louis, MO).

The human myeloid cell line, THP-1, was used for detection of recombinant proteins in eukaryotic target cells. For FIV Gag detection, THP-1 cells were activated with PMA (50 ng/ml) and then infected at 50 MOI *L. monocytogenes* or recombinant *L. monocytogenes* for 30 minutes at 37°C 5% CO_2. Infected cells were washed three times with sterile PBS, incubated for 3.5 hours in media containing 10 ug/ml gentamicin, then lysed as described below. To detect FIV Env, THP-1 cells were activated with PMA (50 ng/ml) and ionomycin (300 ng/ml) then infected at 50 MOI *L. monocytogenes* for 30 minutes at 37°C 5% CO_2. Infected cells were washed three times with sterile PBS and incubated for 3.5 hours in media devoid of antibiotics, then penicillin and streptomycin were added and cells were incubated for an additional 24 hours [39]. After incubation, adherent cells were removed from culture flasks by incubation with lidocaine, diluted 1:3 in PBS, for 10 minutes at 37°C 5% CO_2. Cells were lysed with M-PER mammalian protein extraction buffer (Pierce Biochemical, Rockford, IL), and protein was precipitated from cell-free soluble extract with 5% trichloroacetic acid. 40 μl of precipitated whole cell lysate material was added to 10 μl 2X Laemmli sample buffer, boiled for 3 minutes and run under reducing conditions on 10% tris-glycine SDS-PAGE (Novex, San Diego, CA). Proteins were transferred to an Immobilon-P PVDF membrane (Millipore, Billerica, MA) and the membrane was incubated overnight in blocking buffer containing 50 mM Tris, 0.5 M NaCl, 0.1% Tween 20 and 5% non-fat dry milk. For detection of Gag, the membrane was incubated with mAb 5G11.1 (specific for epitopes in the p24 region of Gag) diluted to 10 μg/ml in 50mM Tris, 0.5 M NaCl, 0.1% Tween-

20, 1% non-fat dry milk, at room temperature for 4 hours. The blot was then incubated in 80 ng/ml HRP-linked goat anti-mouse antibody (Kirkegaard and Perry Laboratories Inc., Gaithersburg, MD) for 1 hour at room temperature. For detection of Env, the membrane was incubated with 10 µg/ml biotinylated mAb SU1-30 (Custom Monoclonals Inc., West Sacramento, CA), followed by a 1 hour incubation with 1 µg/ml streptavidin-HRP (Pierce Biochemical, Rockford, IL). The SuperSignal West Pico chemiluminescent detection system (Pierce Biochemical, Rockford, IL) was used to visualize proteins according to manufacturer's directions.

2.5 Animal studies

Eight cats were divided into two groups consisting of 2 males and 2 females. All cats were exposed to 4×10^6 cfu wild-type *L. monocytogenes* (strain 10403S) by subcutaneous route. Four months later, four cats were given 5×10^6 cfu wild-type *L. monocytogenes* orally, followed 2 months later by a single oral dose of 1×10^8 cfu LM*gag*/pND14-Lc-*env*. This group was termed SQ/Oral pre-exposure (SQ/Oral-PE). The remaining four cats received 1×10^8 cfu LM*gag*/pND14-Lc-*env* orally six months after the subcutaneous exposure to wild-type *L. monocytogenes* and were termed SQ pre-exposure (SQ-PE). All cats were euthanatized 2 months after receiving LM-*gag*/pND14-Lc-*env*. Blood, saliva and vaginal wash fluids were collected monthly and processed as previously described [40]. Spleen, mesenteric lymph nodes and medial iliac lymph nodes were collected at the time of necropsy and processed as previously described [41]. All animals were housed and cared for in accordance with the standards of the Association for Assessment and Accreditation of Laboratory Animal Care and the guidelines of the Institutional Animal Care and Use Committee.

2.6 Soluble Listeria antigen

Soluble Listeria antigen (SLA) was prepared for use in antibody ELISA and IFN-γ ELISPOT. An overnight culture of *L. monocytogenes* (strain 10403S) was centrifuged at 1700 x *g* for 15 minutes to pellet the bacteria. The pellet was washed 3x with sterile PBS then resuspended in sterile PBS. The bacterial suspension was sonicated 5x for 1 minute with 20 second cooling on ice between each round of sonication. The sonicate was

clarified by centrifugation for 50 minutes at 39,000 x g. The supernatant was then passed through a 0.45μM filter and protein concentration of the SLA was determined.

2.7 Fecal Listeria quantification

Fecal samples were collected daily from cats for five days after the single oral immunization with LM-*gag*/pND14-Lc-*env*. Samples were homogenized and serial dilutions were plated on BHI agar containing 40μg/ml nalidixic acid and 50μg/ml streptomycin to prevent the growth of other enteric bacteria. The plates were incubated overnight at 37°C and bacterial colonies were counted. The number of cfu per gram of dried feces was calculated.

2.8 IFN-γ ELISPOT

Peptide pools representing FIV NCSU$_1$ Gag and Env were used at 1μM as previously described [42]. SLA was used to stimulate cells at 1μg/ml. The Feline IFN-γ Detection Module consisting of capture and detection antibodies was purchased from R&D Systems (Minneapolis, MN) and used according to manufacturer's instructions. Frozen mononuclear cells from spleen and lymph node were thawed in a 37°C water bath, washed with LBT medium (RPMI 1640 supplemented with 10% fetal calf serum, 15mM HEPES, 1mM sodium pyruvate, 2mM L-glutamine, 10 IU/ml penicillin and 10μg/ml streptomycin) and counted. Cells were allowed to rest overnight in a 37°C, 5% CO_2 incubator before stimulation with antigen (peptides or SLA as described above). Cells were cultured with antigen for 4 days in 96 well culture plates before being counted and transferred to ELISPOT plates. Multiscreen HTS IP sterile plates (Millipore, Billerica, MA) were pre-wet with 70% methanol, washed with PBS and then coated with 100μl capture antibody overnight at 4°C. The plates were washed 3x with PBS and then blocked with 200μl LBT for 1 hour at 37°C. Control wells consisted of cells stimulated with PMA (50ng/ml) and ionomycin (300ng/ml) or left unstimulated. Cells were added at a concentration of 3.5×10^5 per well and incubated for 16-20 hours at 37°C, 5% CO_2. The following day, plates were washed with PBS containing 0.05% Tween-20 (PBST) and incubated with detection antibody overnight at 4°C. Plates were washed and 100μl/well streptavidin-HRP (1μg/ml) (Pierce Biochemical, Rockford, IL) was added for 1 hour at

room temperature. Plates were washed with PBST and PBS, then developed with 3-amino-9-ethylcarbazole substrate (Sigma, St. Louis, MO). The reaction was stopped after 10 minutes by rinsing the plate with deionized water. Spot-forming cells (SFC) were counted and analyzed on an Immunospot Series 1 Analyzer (Cellular Technologies Ltd., Cleveland, OH). Samples were run in triplicate. To determine the number of SFC, triplicate wells were averaged, background counts from unstimulated wells were subtracted and the total was normalized to $1x10^6$ cells. The mean background SFC was 4.9+/-2.8.

2.9 Enzyme-linked Immunosorbent Assay

Serum, saliva, vaginal and fecal washes were collected throughout the study. Serum, saliva and vaginal washes were processed as previously described [40]. Fecal samples were collected, weighed and homogenized in fecal extraction buffer (PBS supplemented with 10% goat serum) by vigorous vortexing for 20 minutes. The extract was centrifuged at 20,000 x g for 10 minutes. The supernatant was removed and stored at -80°C. Enzyme-linked immunosorbent assay (ELISA) was used to determine the presence of anti-SLA and anti FIV-p24 antibodies as previously reported [41].

Total IgA concentrations in vaginal secretions, saliva, and feces were determined using a feline IgA ELISA (Bethyl Lab, Montgomery, TX) performed according to manufacturer instructions. SLA and FIV-p24 endpoint titers were considered positive if they were 3-fold greater than endpoint titers of samples from naïve cats. Naïve animals were age-matched and for serum, samples were from cats used in this study (taken prior to exposure to *L. monocytogenes*). All sample titers from naïve cats were less than 1:64. Normalized data are presented as highest positive endpoint titer divided by micrograms of total IgA to yield endpoint titer per microgram of IgA.

2.10 Statistics

Two-tailed student *t*-test was performed using GraphPad Prism version 3.0 for Windows to determine the significance of differences between the two study groups (GraphPad Software, San Diego, CA). Significance was defined as $p \leq 0.05$.

3. Results

3.1   Generation of stable *Listeria* constructs containing FIV *gag* genes.

Using plasmids and *Listeria* strains generously provided by Dr. Jeff Miller at UCLA, we generated several recombinant *L. monocytogenes* for use as biologic vaccine vectors. We employed methods previously described by Shen et al. with minor modification (see Figure 1) [21]. Briefly, pHS-LV was constructed with a region of homology to the *Listeria* genome, a temperature sensitive ori (E194ts) for replication in *Listeria*, and the ColE1 ori for replication in *E. coli*. A second plamid (pEJ140) contained the antigen expression cassette which consisted of a strong *Listeria* promotor (*Phly*), translation initiation and secretion signals (SS) capable of directing export of fused polypeptide sequences, a multiple cloning site (MCS), and a kanamycin resistance gene. The plasmids and techniques have previously been described in detail [15,21]. We excised the antigen expression cassette from pEJ140 and inserted it into pHS-LV to create pHS-LV2 as shown in Figure 1. This streamlined the cloning process by allowing direct insertion of FIV genes into the plasmid to be used in the recombination with the *Listeria* genome. Four pHS-LV2 constructs were generated with all or part of the FIV *gag* gene inserted into the MCS. The four *gag* genes used were the full-length *gag*, p45 (MA+CA), p24 (CA), and p17 (MA) (see Figure 1). After transfecting *L. monocytogenes* strain 10403S with the pHS-LV2-FIV constructs, a process of selective antibiotic and temperature culturing was used to force a double crossover event resulting in stable integration of the antigen cassette into the *Listeria* genome.

3.2   *In vitro* validation of FIV Gag-expressing recombinant *L. monocytogenes* constructs.

Next we sought to determine whether recombinant *L. monocytogenes* expressing different regions of FIV *gag* would produce protein, whether the protein was soluble and secreted, and whether the recombinant protein was produced at readily detectible levels after recombinant *L. monocytogenes* infection of macrophages. To this end, the four recombinant *L. monocytogenes* expressing various portions of the FIV *gag* (designated LM-*gag*, LM-p45, LM-p24, and LM-p17) were thoroughly characterized. Each recombinant *L. monocytogenes* was verified by polymerase chain reaction to contain the

FIV sequence of interest, and the recombinant *L. monocytogenes* were sequenced to ensure the gene was inserted in the correct transcriptional frame within the anticipated region of the *L. monocytogenes* genome. Western blot of recombinant *L. monocytogenes* cultures was used to determine if the FIV protein was expressed in a soluble form. Expression of FIV proteins is driven by a strong promotor, P*hly*, that is upregulated after the bacterium is phagocytosed by its target cell, the macrophage/dendritic cell [43]. Furthermore, the secretion signal should enhance the likelihood that the inserted FIV genes will be secreted by the bacterium into the cytosol of the eukaryotic target cell [21]. Whether FIV proteins are indeed produced as secreted soluble proteins can be determined by blotting bacterial culture supernatants and bacterial lysates. Three of the four recombinant *L. monocytogenes* expressed secreted, soluble protein (Gag and p24 are shown in Figure 2A). The LM-p17 did not express detectable protein, and mRNA could not be demonstrated (data not shown). A second LM-p17 was created, and it also failed to produce protein. The reasons for this are not clear, but this result emphasizes the need for thorough *in vitro* recombinant *L. monocytogenes* characterization before *in vivo* studies. The infectivity of each recombinant *L. monocytogenes* was tested by exposing macrophages to the recombinant *L. monocytogenes* and then immunostaining to determine if FIV protein was produced by the bacteria. All four recombinant *L. monocytogenes* readily infected macrophages *in vitro*, and LM-*gag*, LM-p24, and LM-p45 produced the anticipated recombinant FIV protein (Figure 2B).

3.3   Generation and *in vitro* validation of LM-*gag* containing pND14-Lc-*env*

Because the FIV Env is glycosylated and this may be important to induction of an efficacious immune response, we sought to utilize LM-*gag* to deliver a DNA vaccine plasmid containing the FIV *env*. The backbone plasmid, pND14, was generously provided by Dr. Gary Rhodes, University of California at Davis. The pND14 has an *E. coli* ori, ampicillin resistance gene and an eukaryotic expression cassette consisting of a human CMV immediate early promotor/enhancer/intronA, TPA signal peptide and start site, SRV1 (simian retrovirus) constitutive transport element (making the *env* gene *rev*-independent), and bovine growth hormone poly A. We first inserted a Gram+ origin of replication and erythromycin resistance gene into the pND14 using a modified site

directed mutagenesis method. The *Listeria* competent plasmid was named pND14-Lc. We then inserted the FIV NCSU$_1$ *env* coding for the entire extracellular region of gp100 (SU) and gp41 (TM) into the TPA *orf*. Immunocytochemistry of COS-7 cells transfected with this plasmid, pND14-Lc-*env* (Figure 3A), revealed that productive expression of FIV Env protein was achieved (Figure 3B). The pND14-Lc-*env* was electoporated into LM-*gag* and erythromycin resistant colonies were subcloned. The transfected LM-*gag* was named LM-*gag*/pND14-Lc-*env*. Finally, to fully validate LM-*gag*/pND14-Lc-*env*, the human myeloid cell line, THP-1, was infected, cultured and lysed, then evaluated by western blot. Soluble FIV Gag and Env proteins were produced in the THP-1 cells infected with LM-*gag*/pND14-Lc-*env* (Figure 4).

3.4   Effect of pre-exposure to *L. monocytogenes* on LM-*gag*/pND14-Lc-*env* immunogenicity.

Cats immunized with a single dose of LM-*gag*/pND14-Lc-*env* control viral load one year after challenge with FIV but sterilizing immunity is not achieved [41]. Protection against challenge may be enhanced by repeated immunization with LM-*gag*/pND14-Lc-*env*. A central question with our vector, and indeed with all vectored vaccines, is whether previous exposure to the wild type vector or multiple exposures to the vectored vaccine reduces the ability to induce an antigen-specific primary or secondary immune response   To address the question of primary responses, eight cats were exposed to wild-type *L. monocytogenes* by subcutaneous route and then were divided into two groups. Four months later one group was given oral wild-type *L. monocytogenes* followed two months later by oral LM-*gag*/pND14-Lc-*env* (SQ/Oral-PE). The other group received only oral LM-*gag*/pND14-Lc-*env* (SQ-PE) six months after the subcutaneous exposure. All cats had measurable, albeit low, anti-SLA IgA in vaginal secretions, saliva and feces four months after subcutaneous exposure to *L. monocytogenes* (Figure 5). At that time point, mean anti-SLA IgA titers in vaginal secretions were greater than mean titers in saliva or feces and remained unchanged upon subsequent oral exposure to *L. monocytogenes* or LM-*gag*/pND14-Lc-*env*. In SQ/Oral-PE cats, mean anti-SLA IgA titers in saliva and feces were low after subcutaneous exposure to *L. monocytogenes* (2.2+/-1.4, 5.4+/-2.4, respectively) but increased

significantly after oral exposure (92.9+/-94.2, 55.4+/-55.3, p=0.05 and p=0.03, respectively). Titers did not change significantly following oral exposure to LM-*gag*/pND14-Lc-*env*. The SQ-PE cats had increased mean anti-listeria IgA titers in saliva and feces following oral immunization with LM-*gag*/pND14-Lc-*env* (172+/-57.8, 158.2+/-81.3, respectively). Serum anti-SLA IgG titers were present after subcutaneous exposure to *L. monocytogenes* and tended to increase after oral exposure but did not reach statistical significance due to individual variation (Figure 5). Only fecal anti-SLA titers were significantly different between SQ/Oral-PE and SQ-PE cats at the time of administration of LM-*gag*/pND14-Lc-*env* (141+/-93 versus 11.3+/-7, p=0.03).

Fecal shedding of LM-*gag*/pND14-Lc-*env* was measured for 5 days after oral administration for each animal in both treatment groups. Shedding of LM-*gag*/pND14-Lc-*env* occurred at a higher level and for a longer period for SQ-PE cats as compared to SQ/Oral-PE cats (Table 1). These data suggest the higher anti-SLA fecal IgA in SQ/Oral-PE animals limited but did not prevent colonization of the intestine by LM-*gag*/pND14-Lc-*env*.

One month after oral administration of LM-*gag*/pND14-Lc-*env*, anti-FIV Gag IgA responses were present in vaginal secretions, saliva, and feces of cats from both groups (Figure 6) and were statistically greater in the saliva and feces of SQ-PE cats (6.1+/-1.9 and 18.3+/-3.7, respectively) as compared to SQ/Oral-PE cats (3.4+/-1.5 and 11.9+/-2.8; p=0.04 and p=0.02, respectively). All cats mounted a serum anti-FIV Gag IgG response (Figure 6) with no statistical difference in mean serum IgG titers between the groups (16384+/-11585 for SQ/Oral-PE cats and 25600+/-27247 for SQ-PE cats).

Two months after oral administration of LM-*gag*/pND14-Lc-*env*, cell-mediated immune responses against FIV Env, FIV Gag, and SLA were measured in spleen, medial iliac lymph node and mesenteric lymph node using the IFN-γ ELISPOT assay. Splenic anti-Env responses were greatest in SQ-PE cats (mean SFC=133, p=0.02) while no differences were apparent in responses to FIV Gag peptides or SLA between the groups (Figure 7). Local draining lymph nodes for the gastrointestinal and reproductive tracts were also assessed for antigen-specific IFN-γ production. Responses against all antigens were greatest in SQ-PE cats. Mean anti-Gag and Env IFN-γ SFC in the medial iliac lymph node of SQ-PE cats were 127 and 97, compared with 16 and 12 in SQ/Oral-PE

cats (p=0.04 and p=0.01, respectively). Responses were similar in the mesenteric lymph node, with the anti-Gag and anti-Env SFC responses of 164 and 129 for SQ-PE cats versus 3 and 0 for SQ/Oral-PE cats (p=0.01 and p=0.02, respectively).

4.   Discussion

Listeria monocytogenes appears particularly well-suited as a biologic vaccine vector against HIV because it induces a strong cell mediated immune response, infects APCs, enters the host by mucosal route, and can be readily manipulated to express foreign proteins [14,15,17,21,44]. Challenge with virus is the ultimate test of efficacy in any HIV vaccine strategy. However, the majority of studies evaluating recombinant *L. monocytogenes* as a vaccine vector for HIV have been performed in mice, thus no virus challenge could be performed. The FIV/cat model of HIV provides a well-characterized and accepted system to determine whether recombinant *L. monocytogenes* can protect against an immunodeficiency inducing lentivirus in a natural host. Importantly, cats can be infected with FIV by parenteral and mucosal routes (oral, nasal, vaginal, rectal) with either cell-free or cell associated virus [45-54]. Therefore, challenge studies can be performed to closely model the major routes of infection known to be common in human transmission of HIV.

For our purposes, we modified existing methodologies to facilitate the introduction of FIV genes into the *L. monocytogenes* genome. Our objective was to ensure the recombinant *L. monocytogenes* to be carried into *in vivo* studies was the optimal construct for delivering FIV Gag proteins. It is essential that the FIV proteins are produced and secreted in soluble form by the recombinant *L. monocytogenes* for maximal immunogenicity. Fortuitously, recombinant *L. monocytogenes* containing the full-length FIV *gag* sequence met these criteria. This has previously been shown to be the case with the HIV Gag protein, but as demonstrated by the lack of expression by LM-p17, it is critical to thoroughly characterize each recombinant *L. monocytogenes* individually.

A key objective of these studies was to determine whether recombinant *L. monocytogenes* deliver an FIV Env DNA vaccine. Evidence suggests the native structure of Env may be critical to induce an effective (neutralizing) humoral immune response [55-61]. The need for glycosylation precluded using the same strategy employed for the

gag gene since the Gag protein is a bacterial product from an integrated genomic sequence. Thus we chose to explore plasmid delivery of *env* by recombinant *L. monocytogenes*. The delivery of plasmids by *L. monocytogenes* has been previously demonstrated [39,62]. The plasmid we employed incorporated several strategies to improve Env expression and indeed robust expression was observed when the pND14-Lc-*env* was transfected into mammalian cells. Expression of the HIV SU and ectodomain of TM from plasmid has been shown to form native trimers in eukaryotic cells, and to induce neutralizing antibody in immunized rhesus macaques [63] [64]. Homologous regions of the FIV *env* were inserted into pND14-Lc-*env* and would be anticipated to trimerize, although this was not proven here. A Gram positive origin of replication was needed so the plasmid could replicate in *L. monocytogenes*. This was problematic in that no convenient restriction sites existed in the plasmid backbone. To overcome this we employed a previously described modified site-directed mutation strategy to insert the 2815bp fragment needed for replication and selection in *L. monocytogenes* [38]. This proved to be a simple and effective means to introduce a large DNA fragment without restriction enzyme sites. To fully validate the LM-*gag*/pND14-Lc-*env in vitro*, we showed that both FIV Gag and Env were detectable in the cell lysates of infected THP-1 cells.

Two important issues that must be addressed regarding *L. monocytogenes* as a vaccine vector are safety and anti-vector (anti-Listeria) immunity. Because wild-type *L. monocytogenes* represents a serious pathogenic risk to immunocompromised individuals, neonates, and fetuses, highly attenuated strains of *L. monocytogenes* are required. Some approaches to attenuation have also severely reduced immunogenicity, and the immunogenicity of a recently developed D-alanine dependent strain *in vivo* has not been reported [65]. Whether recombinant *L. monocytogenes* is immunogenic in the face of pre-existing immunity must also be considered in the event repeated doses of recombinant *L. monocytogenes* are necessary to provide protection and given that some individuals in the general population are likely to have had previous exposure to wild-type *L. monocytogenes*. It has been shown that mice with pre-existing immunity against recombinant *L. monocytogenes* devoid of two key CTL epitopes were able to generate a primary CTL response against those epitopes after immunization with wild-type *L.*

monocytogenes [66]. In the present study, we show this is also true for non-*Listeria* antigens such as FIV Gag and Env.

Cats exposed to wild-type *L. monocytogenes* subcutaneously or subcutaneously and orally mounted a humoral and cell mediated response against FIV antigens delivered by LM-*gag*/pND14-Lc-*env*. All cats had anti-listerial IgG in serum and IgA in mucosal secretions after subcutaneous exposure. Oral exposure to *L. monocytogenes* significantly boosted the anti-SLA IgA titers in saliva and feces of SQ/Oral-PE cats. This may account for the shorter duration and lower level of bacterial shedding after immunization with LM-*gag*/pND14-Lc-*env* observed in those animals. Overall, SQ/Oral-PE animals with two previous exposures to *L. monocytogenes* had lower antibody and IFN-γ ELISPOT responses to FIV antigen. This is likely due to lower colonization in the intestine and thus, a lower dose of FIV antigen. In this study, the dose of LM-*gag*/pND14-Lc-*env* was greater than the pre-exposure dose of wild-type *L. monocytogenes* in hopes that the larger dose might over-ride the anti-Listeria response. At 1×10^8, the immunizing dose of LM-*gag*/pND14-Lc-*env* is modest and a higher dose may lead to greater colonization of the intestine, increased antigen delivery and improved immunogenicity of FIV antigens. These results support the possibility that recombinant *L. monocytogenes* could be given more than once or in the face of anti-listerial immunity and still provide an immunogenic dose of FIV antigen.

We have previously shown that cats immunized with a single dose of LM-*gag*/pND14-Lc-*env* have a very low viral load one year after vaginal challenge with FIV as compared to sham-immunized cats or cats immunized with wild-type *L. monocytogenes* [41]. Those cats immunized with LM-*gag*/pND14-Lc-*env* maintained normal percentages of CD4+ and CD8+ T-cells in lymph nodes and gut associated lymphoid tissues. The ability of orally delivered LM-*gag*/pND14-Lc-*env* to elicit FIV-specific humoral and cellular responses at mucosal and inductive sites justifies further study to determine whether attenuated recombinant *L. monocytogenes* can induce protective immunity as a single dose, in multiple doses, or in a prime-boost vaccination strategy.

**Acknowledgements**
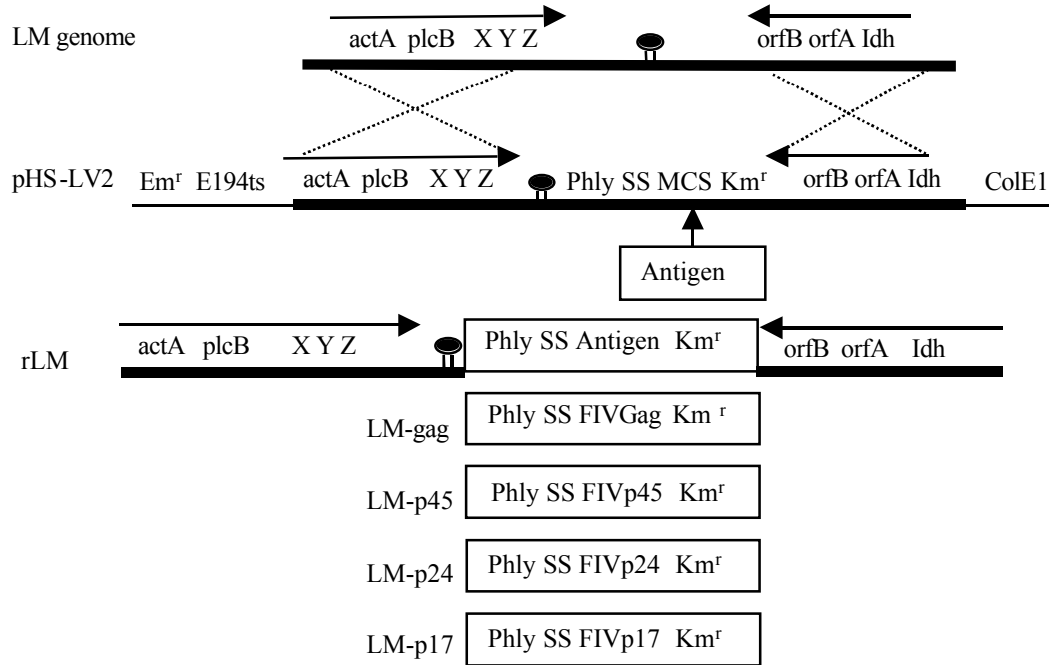
Figure 1.

Figure 1. Strategy for introducing FIV sequences into the *Listeria* genome. The FIV *gag* sequence of interest was cloned directionally into the multiple cloning site (MCS) of pHS-LV2. Through selective culturing involving temperature sensitive plasmid replication and erythromycin resistance, a double cross-over event occured, resulting in the stable integration of the antigen cassette into the *Listeria* genome. Four recombinant *L. monocytogenes* containing FIV *gag* genes were constructed (LM-*gag*, LM-p45, LM-p24, and LM-p17).
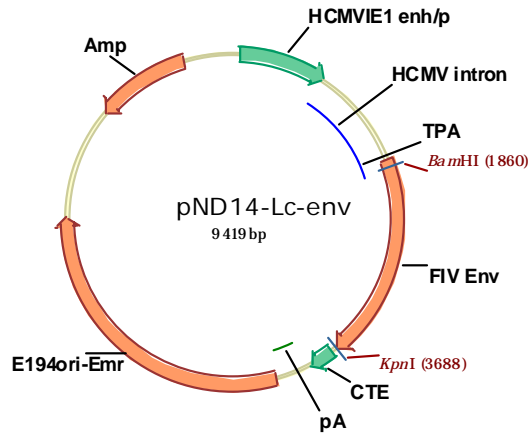
Figure 2.

A.
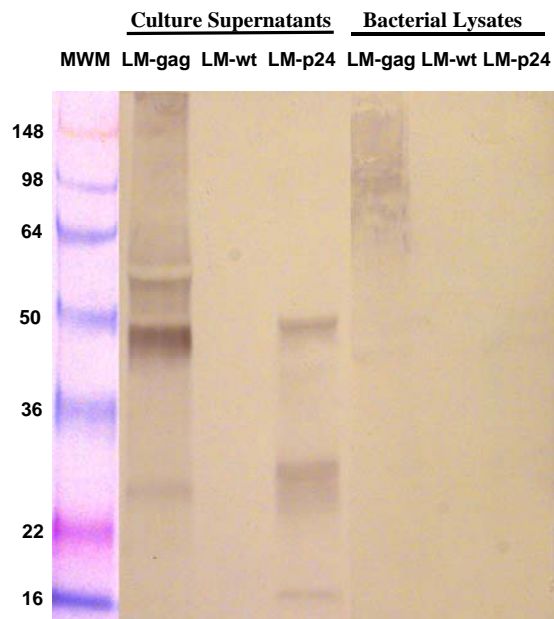
B.
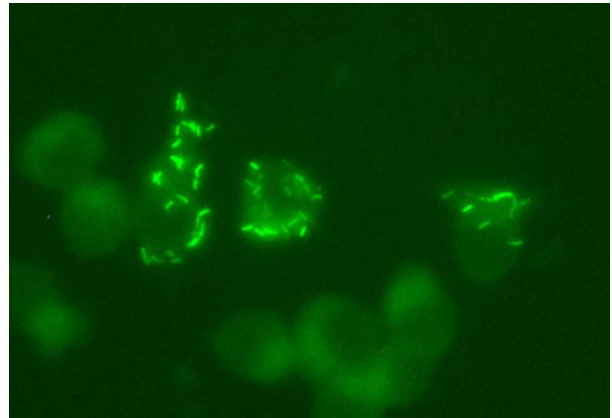
Figure 2. Expression of soluble recombinant FIV Gag by LM-*gag*. LM-*gag* and LM-p24 were grown in BHI broth then the bacteria were pelleted and lysed. The culture supernatants and lysed bacterial pellets were analyzed by western blot using anti-p24 monoclonal antibody 51G11.1. Gag protein and p24 were present in the supernatants (bands at about 48kD and 26kD) (A). P388D1 cells were infected with LM-*gag* and after 4 hours in culture were stained with an anti-Gag monoclonal antibody followed by anti-mouse IgG-FITC. Bacteria expressing the FIV Gag appear as green fluorescent rods in the cytoplasm of the macrophages (400x mag) (B).
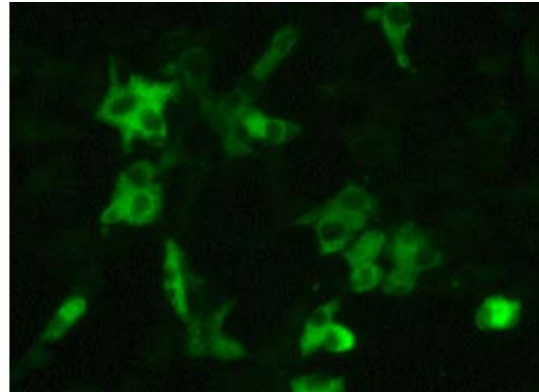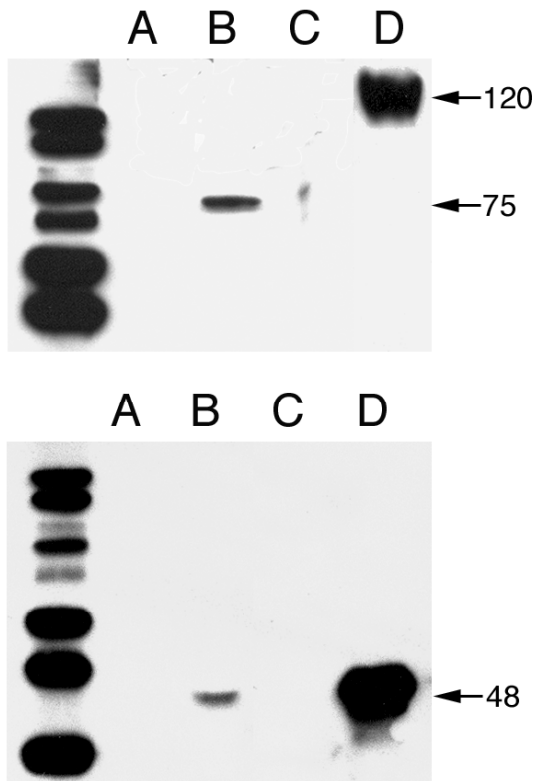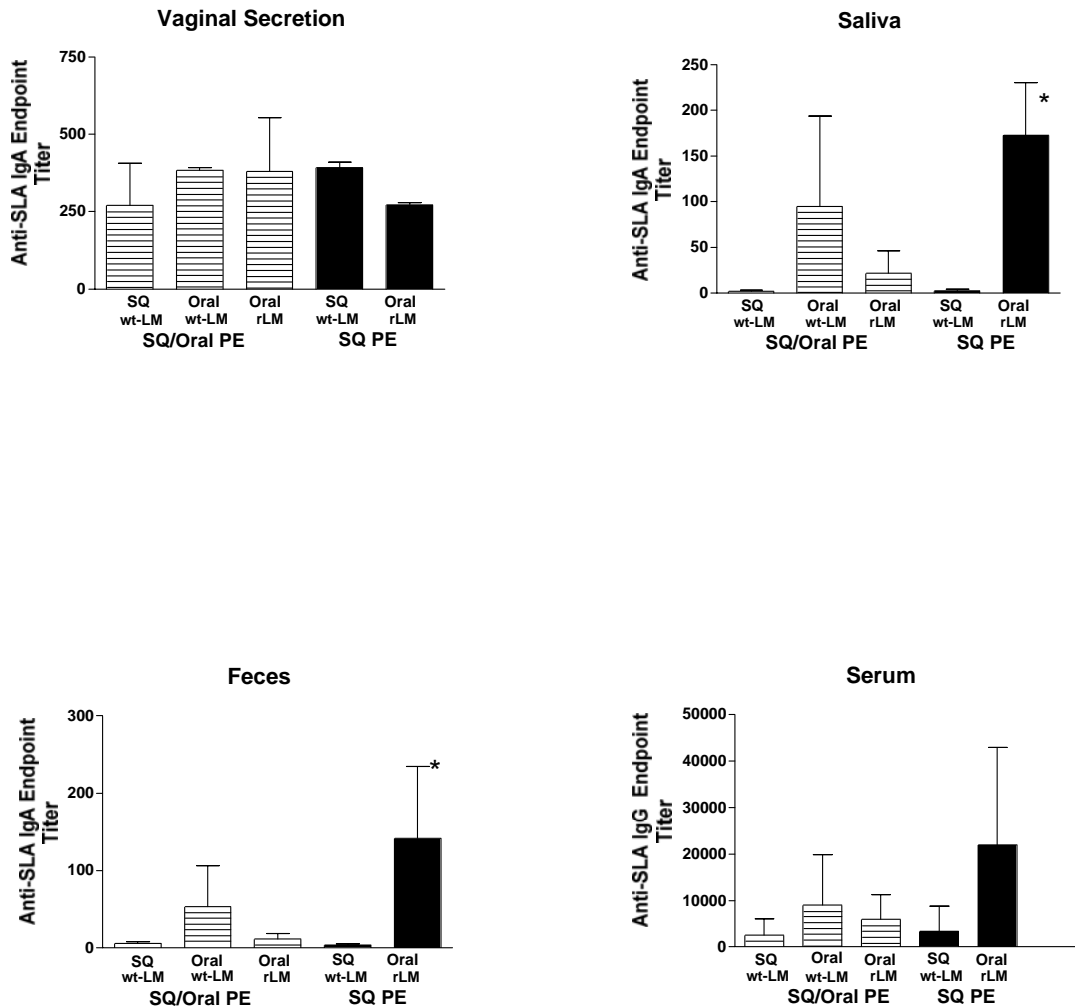
Figure 3.

A.

B.

Figure 3. Plasmid map of pND14-Lc-*env* (A). Fluorescence photomicrograph of pND14-Lc-*env* transfected COS-7 cells stained with antibodies against FIV Env (200x magnification) (B).
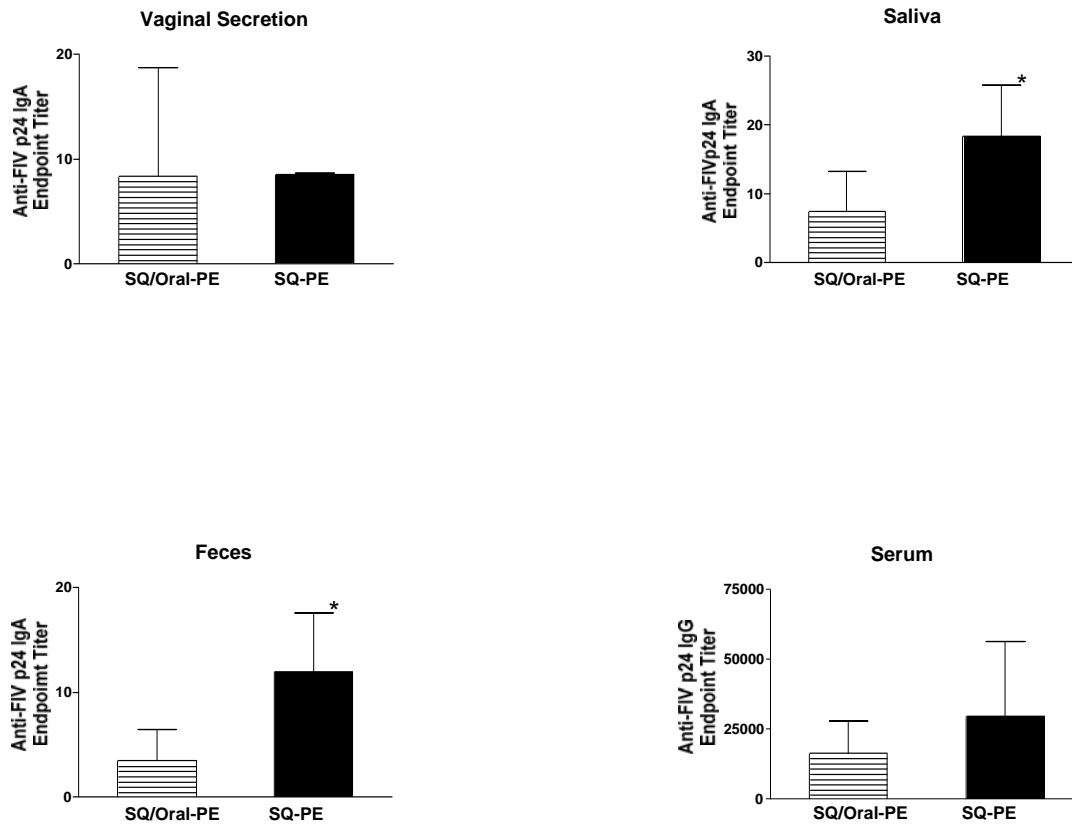
Figure 4.

Figure 4. *In vitro* validation of LM-*gag*/pND14-Lc-*env*. The human myeloid cell line THP-1 was infected with LM-*gag*/pND14-Lc-*env*, cultured as described in methods, then the cells were lysed. The soluble fraction of the cell lysate was evaluated by western blot for expression of FIV Env (top) and FIV Gag (bottom). Lane A: Lysate from uninfected THP-1 cells. Lane B: Lysate from LM-*gag*/pND14-Lc-*env* infected THP-1 cells. Lane C: Lysate from LM-wt infected THP-1 cells. Lane D: Positive controls showing FIV SU from purified FIV (top) and recombinant FIV p24-GST (bottom). The truncated FIV Env product representing the ectodomain of gp100 was evident at the predicted 75kD size, and the FIV Gag was evident at approximately 48kD.
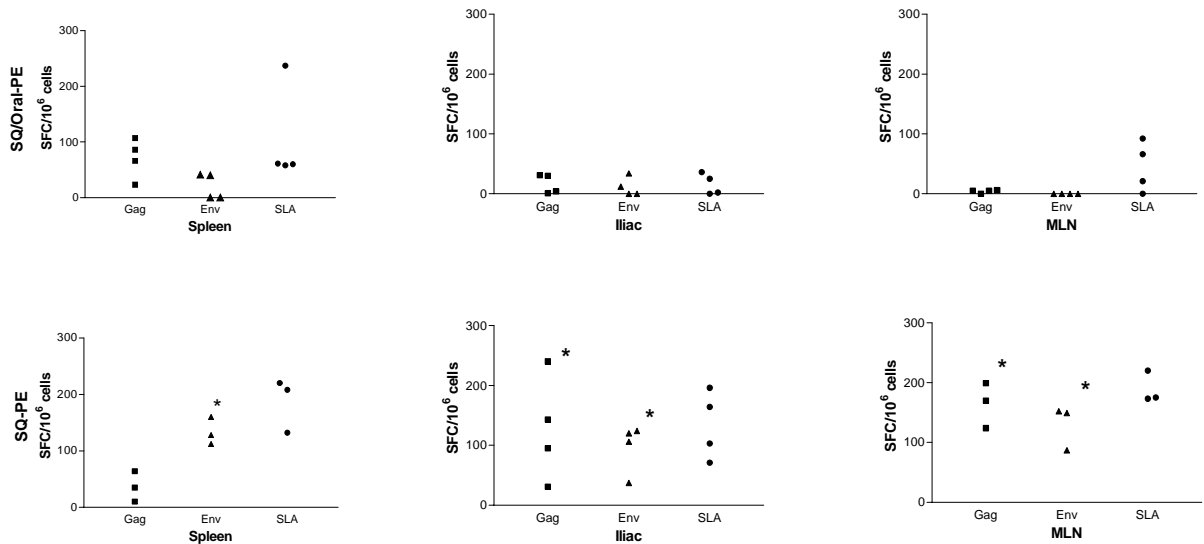
Figure 5.

Figure 5. Anti-SLA antibody responses. Cats were exposed to 4×10^6 cfu wild-type *L. monocytogenes* by subcutaneous route (SQ). Four months later cats in group SQ/oral were orally exposed to 5×10^6 cfu wild-type *L. monocytogenes* (wt-LM). Two months later, cats from both groups were orally immunized with 1×10^8 LM-*gag*/pND14-Lc-*env* (rLM). SLA-specific IgA was measured in vaginal secretions, feces and saliva by ELISA, 4 months post SQ administration of wild-type *L. monocytogenes* (SQ LMwt), 30 days after oral exposure to wild-type *L. monocytogenes* (for SQ/oral group), and after oral immunization with LM-*gag*/pND14-Lc-*env*. Serum SLA-specific IgG was similarly measured. IgA data is shown as endpoint titer per microgram of total IgA ± SD. IgG data is expressed geometric mean endpoint titers ± SD. *Denotes statistically significant difference between SQ/Oral-PE and SQ-PE values for saliva and feces after immunization with LM-*gag*/pND14-Lc-*env* (p=0.003 and p=0.03, respectively).

Figure 6.

Figure 6. Anti-p24 antibody responses. Cats were treated as described in Figure 5. Vaginal secretions, feces and saliva were evaluated for p24-specific IgA and serum for p24-specific IgG by ELISA 30 days post-oral immunization with $1x10^8$ LM-*gag*/pND14-Lc-*env*. IgA data is expressed as endpoint titers per microgram of total IgA ± SD. * Denotes statistically significant difference in anti-FIV p24 IgA antibody between SQ/Oral-PE and SQ-PE for saliva and feces (p=0.04 and p=0.03 respectively). IgG data is expressed as geometric mean endpoint titers ± SD.

Figure 7

Figure 7. Oral immunization with LM-*gag*/pND14-Lc-*env* induces T-cell responses. Two months post oral immunization with $1x10^8$ LM-*gag*/pND14-Lc-*env* cats were euthanatized and spleens, medial iliac (iliac) and mesenteric lymph nodes (MLN) were collected and processed. Spleen, iliac and MLN mononuclear cells were stimulated with FIV Gag and envelope (Env) peptides (1µM) or SLA antigen (1µg/ml) and analyzed by IFN-γ ELISPOT. Results shown are the mean of triplicate wells with unstimulated control wells subtracted. All values were normalized to $1x10^6$ cells. * Denotes statistically significant difference between SQ/Oral-PE and SQ-PE in splenic Env IFN-γ response (p=0.02), medial iliac Gag, Env and SLA (p=0.04, p=0.01, and p=0.001 respectively) as well as MLN Gag, Env and SLA (p=0.01, p=0.02, p=0.01 respectively).

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Evaluation of antigen-specific T cell responses following oral immunization with a recombinant *Listeria monocytogenes* vaccine by IFNγ ELISPOT

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Abstract

Previous work from our laboratory has shown the effects of single and multiple previous exposure(s) to *Listeria monocytogenes* (LM) on the generation of LM*gag*/pND14-Lc-*env*-specific immune responses. Previous systemic exposure to *Listeria monocytogenes* does not prevent generation of LM*gag*/pND14-Lc-*env*-specific immune responses. However, systemic and oral pre-exposure to LM reduces vaccine-specific CD8 T cell responses of splenic and lamina propria lymphocytes. As spleen and mucosal tissues can only be evaluated in terminal studies, we evaluate, by IFNγ ELISPOT, peripheral blood mononuclear cells (PBMC) as an indicator of vaccine induced FIV Gag, Env and *Listeria*-specific T cell responses in feline splenic and lamina propria lymphocytes. Our results show that extended culture is required to quantify low-frequency antigen-specific T cell responses in peripheral blood. While measurement of vaccine-specific T cell responses in peripheral blood is convenient, linear regression analysis revealed that there was no correlation between PBMC response and responses observed in splenic and lamina propria lymphocytes.

Introduction

For almost a decade, recombinant *Listeria monocytogenes* has been evaluated as a vaccine vector against human immunodeficiency virus (HIV) (4,7,9,19,30). Using the feline model of HIV, we have continued to explore LM as a vaccine vector with emphasis on 1) sites to assess for vaccine efficacy and 2) the effect of route of previous LM exposure on the generation of mucosal vaccine-specific CD8 T cell responses. The goal of a vaccine is to generate an immune response that will protect individuals from disease

upon exposure to the pathogen. To validate the efficacy of an oral vaccine, both systemic and mucosal samples must be assayed. In terminal animal studies, important immunological sites such as the lamina propria and spleen are relatively straightforward to assess. For human and non-terminal vaccine trials these sites are generally not accessible. However, PBMC are readily available during the course of a vaccine trial and are frequently the only site sampled in human and non-terminal animal studies. Importantly, the appropriateness of this site as an indicator of mucosal immune response induced by oral immunization has yet to be determined. In this study, we evaluated vaccine induced T cell responses by feline INFγ ELISPOT. With this assay, we were able to quantify antigen-specific CD8 immune responses to our orally delivered, recombinant LM (rLM) vaccine at the single-cell level. LM*gag*/pND14-Lc-*env* is the recombinant strain of LM that carries a full-length copy of the FIV molecular clone NCSU$_1$ *gag* gene stably integrated into the *L. monocytogenes* genome. The LM*gag*/pND14-Lc-*env* also carries a DNA vaccine plasmid with the NCSU$_1$ FIV envelope (SU and ectodomain of TM) cloned into pND14-Lc for eukaryotic expression of the FIV Env under the control of a CMV promoter. The construction and validation of LM*gag*/pND14-Lc-*env* have been previously described (30).

L. monocytogenes is a Gram positive, enteric intracellular pathogen found in soil and water. The natural route of infection is through consumption of unwashed fruits and vegetables, unpasteurized dairy products and contaminated processed meats. *L. monocytogenes* is different from other intracellular bacteria in that upon phagocytosis it can escape the phagolysosome, enter the cytosol and replicate (11,14,26). *L. monocytogenes* secreted proteins are then presented by the MHC class I pathway leading

to the strong CD8 T cell response that is critical for clearance of infection. Bacteria that remain in the phagolysosome are presented through the MHC class II pathway allowing for interaction with and activation of B cells and CD4 T cells (14,15,21,26).

Until recently, most *L. monocytogenes* studies focused on the pathogenesis of systemic infection rather than oral infection, which is the natural route. However, studies conducted by K. Rosenthal and L. Lefrancois have shown that while systemic immunization can induce mucosal CTL memory, that protection is short-lived (5,17,28). The generation of long-term antigen-specific memory within mucosal tissues is dependent on the homing of effector and memory lymphocytes and the route of immunization (5,18,28) It has been shown that systemically administered *L. monocytogenes* actively replicates in the liver and spleen, with these sites later containing the majority of *Listeria*-specific tetramer positive memory CD8 T cells. It has also been shown by tetramer and cytolytic analysis that while a small percentage of *Listeria*-specific CD8 T cells home to the lamina propria after systemic infection and retain effector capabilities, oral inoculation results in a larger antigen-specific population with enhanced lytic capacity (8,13). The generation and maintenance of memory CD8 T cells at mucosal effector sites might be critical for protection upon mucosal challenge.

To further characterize LM*gag*/pND14-Lc-*env* as a vaccine vector, we evaluated several critical parameters by IFNγ ELISPOT: 1) length of *ex vivo* stimulation to detect low-frequency antigen-specific cells, 2) the localization of antigen-specific T cell effector responses in the spleen, mesenteric lymph node and small intestinal lamina propria and 3) relevance of PBMC as an indicator of vaccine-induced splenic and mucosal antigen-specific immune response. Here we describe the necessity for extended culture of PBMC

to quantify low-frequency antigen-specific T cell responses. We also show that systemic followed by oral exposure to LM-wt reduces the FIV-specific CD8 T cell response of lamina propria lymphocytes (LPL).

2. Materials and Methods

2.1. Cats

Eight specific pathogen free, 16-20 week old cats (4 male, 4 female) were randomly divided into two groups consisting of 2 male and 2 females each. The inoculation schedule is shown in Figure 1. Briefly, all cats were exposed to 4×10^6 colony forming units (cfu) wild-type *L. monocytogenes* (LM-wt, strain 10403S) delivered subcutaneously (SQ). Four months post SQ infection, group 1 (designated SQ/Oral-pre-exposure (PE)) was given 5×10^6 cfu LM-wt delivered orally, followed two months later by a single oral dose of 1×10^8 cfu LM*gag*/pND14-Lc-*env*. The second group (designated SQ-PE) received 1×10^8 cfu LM*gag*/pND14-Lc-*env* orally. All cats were euthanized two months post LM*gag*/pND14-Lc-*env* immunization. Blood was collected monthly during the study and PBMC were isolated by Histopaque-1077 (Sigma, St. Louis, MO) and processed as previously described (30). Spleen, MLN and small intestine (SI) were collected at the time of necropsy and processed as previously described (30). All animals were housed and cared for in accordance with the standards of the Association for Assessment and Accreditation of Laboratory Animal Care and the guidelines of the Institutional Animal Care and Use Committee.

2.2 Peptides

Peptide pools for FIV strain NCSU$_1$Gag and extracellular portion of Env (subunit (SU) and transmembrane (TM)) consisting of nine 15-mers overlapping by 10 amino acids were

used at a concentration of 1μM. The Gag and Env peptides used in this study have previously been shown to be predominantly CD8 T cell restricted (2).

2.3 Soluble Listerial Antigen (SLA)

An overnight culture of *L. monocytogenes* (strain 10403S) was centrifuged at 1700x *g* for fifteen minutes to pellet the bacteria. The pellet was washed three times with sterile PBS then resuspended in sterile PBS. The bacterial suspension was sonicated five times for one minute each with 20 second cooling on ice between each round of sonication. The sonicate was clarified by centrifugation for 50 minutes at 39,000 x *g*. The supernatant was then passed through a 0.45μm filter and protein concentration was determined by Bradford assay. Soluble listerial antigen contains a multitude of listerial proteins including p60, Listeriolysin-O (LLO) and phospholipase C.

2.4 IFNγ ELISPOT

For FIV-specific stimulation, peptide pools representing FIV NCSU$_1$ Gag and Env were used at 1μM as previously described (30). SLA was used at 1μg/ml to stimulate cells. The Feline IFNγ Detection Module (R&D Systems, Minneapolis, MN) was used according to manufacturer's instructions. Briefly, the ELISPOT assay was performed as previously described (22) with several modifications to the protocol. For four-day stimulation, cells were cultured in 96 well culture plates at a concentration of 5×10^5 cells/well. After culture, triplicate wells were pooled, counted and added to ELISPOT plate at 3.5×10^5 cells/well. Overnight culture cells were added at a concentration of 5×10^5 cells/well. Control cells were stimulated with PMA (50ng/ml) and ionomycin (300ng/ml) or left unstimulated and incubated for 24 hours at 37°C, 5% CO_2. Spot forming cells (SFC) were counted and analyzed on Immunospot Series 1 Analyzer (Cellular

Technologies Ltd., Cleveland, OH). To determine the number of spot-forming cells (SFC), triplicate wells were averaged, background counts from unstimulated wells were subtracted and the total was normalized to 1×10^6 cells.

2.5 Statistics

Two-tailed student *t*-test and analysis of variance (ANOVA) with Bonferroni post-test was performed using GraphPad Prism version 3.0 for Windows to determine the significance of differences between the two study groups (GraphPad Software, San Diego, CA). Significance was defined as p≤0.05.

3. Results

3.1. *Prolonged stimulation is required to detect low-frequency LM-specific T cell response*

To evaluate the utility of PBMC as a measure of vaccine induced antigen-specific T cell responses, we isolated PBMC from SQ/Oral-PE cats two months post oral immunization with 5×10^6 cfu LM-wt and from SQ-PE cats one month post 1×10^8 cfu LM*gag*/pND14-Lc-*env* immunization (Fig.2). Cells were stimulated with 1μg/ml SLA for one four days before addition to IFNγ ELISPOT plate. SQ/Oral-PE cats did not respond to one day stimulation, but after four day stimulation there was a significant, albeit low frequency, increase in SLA-specific spots (p=0.01). One month post oral immunization with LM*gag*/pND14-Lc-*env,* SQ-PE cats exhibited a significantly increased number of spot-forming cells after four day stimulation (p=0.03). SQ-PE cats also had a significantly greater number of SLA-specific spots after their higher oral

immunization with LM*gag*/pND14-Lc-*env* than SQ/Oral-PE cats had after receiving LM-wt (p=0.02). Based on these results, we determined that all PBMC would be stimulated for four days to better measure low-frequency antigen-specific spots.

3.2. *Four day stimulation of PBMC detects Gag and SLA-specific T cell responses*

We evaluated the presence of FIV antigen-specific responses induced by LM*gag*/pND14-Lc-*env* immunization in PBMC (Fig. 3). SQ/Oral-PE and SQ-PE cats were orally immunized with $1x10^8$ cfu LM*gag*/pND14-Lc-*env*. Two months later, PBMC were isolated and stimulated with FIV strain $NCSU_1$ Gag and Env-specific peptide pools (1μM) or SLA (1μg/ml) for four days and then analyzed by IFNγ ELISPOT. All cats responded to Gag and SLA stimulation but had minimal to no response to the Env peptide pool. Due to individual cat differences, there were no statistical differences between the two groups.

3.3. *Evaluation of anti-vector response at time of LMgag/pND14-Lc-env immunization*

We next determined the anti-Listerial immune response present at the time of LM*gag*/pND14-Lc-*env* oral immunization (Figure 4). Cryopreserved PBMC were thawed, allowed to rest overnight and were then stimulated for four days with SLA (1μg/ml) before analysis by ELISPOT assay. Two months post oral immunization with $1x10^6$ cfu LM-wt, SQ/Oral-PE cats had a greater response to SLA stimulation (173±29.6) than SQ-PE cats who were five months post SQ LM-wt inoculation (92.2±2.7). The anti-vector response at time of recombinant immunization was significantly greater for SQ/Oral-PE cats than SQ-PE cats (p=0.034).

3.4. *LMgag/pND14-Lc-env-specific responses localize in spleen and lamina propria.*

To determine antigen-specific T cell localization upon oral immunization, we evaluated splenic (Figure 5a), MLN (5b) and small intestine lamina propria lymphocytes (LPL, Figure 5c) for FIV and listerial-specific T cells responses by IFNγ ELISPOT Two months post $1x10^8$ cfu orally delivered LM*gag/*pND14-Lc-*env* cats were sacrificed and spleen, MLN and small intestine sections from SQ/Oral-PE and SQ-PE cats were collected and processed. Mononuclear cells were isolated and stimulated overnight with SLA, FIV Gag and Env peptide pools.

In the spleen, SQ-PE cats had a significantly greater response to Env and SLA stimulation than SQ/Oral-PE cats ($p<0.04$, $p<0.0014$ respectively). However, there was no statistical difference in splenic response between SQ/Oral-PE cats and SQ-PE cats upon Gag peptide stimulation (167.5 ± 11.73, 204.7 ± 18.37, Figure 5a). In MLN, there was no statistical difference between SQ/Oral and SQ-PE cats upon Gag and SLA stimulation. Gag-specific spots detected were low for both SQ/Oral-PE (36.33 ± 9.244) and SQ-PE (57.50 ± 7.77) cats. Although, SLA-specific response was greater for SQ-PE immunized cats (87.75 ± 9.86) compared to SQ/Oral-PE cats (48.33 ± 16.41) it was not statistically significant. Interestingly, neither group had a detectable anti-Env response in the MLN (Figure 5b).

Among LPL's, SQ-PE cats had a significantly greater response to Gag, Env and SLA stimulation compared to SQ/Oral-PE cats ($p<0.0003$, $P<0.005$, $p<0.0001$ respectively). Average spot forming cells detected for SQ-PE cats upon Gag, Env and SLA stimulation was 347.0 ± 26.38, 69.6 ± 19.6 and 421.2 ± 29.6 respectively (Figure 5c).

For SQ-PE cats, there were significant differences between the tissues upon Gag-peptide stimulation (Figure 5 a-c) (p<0.001 ANOVA). The CD8 T cell response to Gag peptide stimulation was significantly greater in the spleen and LPL than in MLN (p<0.01, p<0.001respectively). Additionally, LPL response to Gag-stimulation was significantly greater than that measured in the spleen (p=0.001). There was no significant difference between the spleen and LP upon SLA stimulation. However, SLA-specific T cell responses were significantly greater in the spleen and LP compared to MLN (p< 0.001 for both). For SQ-PE cats, the optimal site to measuring vaccine–induced FIV Gag-specific response by IFNγ ELISPOT was the lamina propria. For FIV Env-specific response, there was no statistical difference between the spleen and LPL.

Next, we evaluated the effect of multiple exposures to LM (systemic LM-wt exposure followed by oral LM-wt) on the localization and magnitude of LM*gag*/pND14-Lc-*env*-specific responses. Response to Gag, Env and SLA stimulation was measured in the spleen, LPL and MLN. SQ/Oral-PE Gag-specific response was significantly greater in the spleen compared to LPL and MLN (p<0.001 for both). The splenic SLA T cell response was also significantly greater than that of LPL and MLN (p<0.001 for both). For this group of cats, the spleen was the optimal site to measure vaccine-induced Gag and SLA T cell responses.

Finally, to determine whether PBMC CD8 T cell response to FIV Gag-peptides is indicative of responses observed in the spleen, MLN and lamina propria, we compared sfc/10^6 cells of 4-day stimulated PBMC to overnight stimulation of tissue mononuclear cells and analyzed by ANOVA (Fig 3 and 5). For SQ/Oral-PE cats, Gag-specific sfc/10^6 PBMC significantly underestimated antigen-specific response in the spleen (p<0.03) and

over-estimated response in the MLN (p<0.02). SQ-PE cats Gag-specific PBMC response under-estimated the response seen in the LP (p<0.001) and over-estimated response in MLN. For both groups, linear regression analysis revealed no connection between observed PBMC response to Gag stimulation to that found in the spleen (r^2 0.0004, p=0.95) and LPL (r^2= 0.3, p= 0.19).

4. Discussion

The goal of vaccination is to induce an antigen-specific immune response in a location most likely to require protection from the target pathogen. FIV disease pathogenesis is similar to human immunodeficiency virus and is an excellent model to evaluate vaccination strategies. HIV's primary route of transmission is mucosal, so vaccines should target the mucosal immune system (23,28,29,31). Rosenthal and others have shown that while systemically delivered vaccines can induce mucosal immunity, mucosal immunity derived from systemic vaccination is short-lived (6,28). It is thought that to effectively control viral replication and disease progression, both cell-mediated and humoral immune responses against HIV antigens will need to be generated (3,10,12,25). We have previously shown that a single, oral, low-dose immunization with LM*gag*/pND14-Lc-*env* generates Gag-specific antibodies in serum and vaginal secretions (30). To evaluate LM*gag*/pND14-lc-*env*'s potential to induce anti-FIV CD8 T cell responses, we optimized the IFNγ ELISPOT assay (22) and identified appropriate peptide pools to measure *gag* and *env*-specific CD8 T cell responses (2). In this study, we use the IFNγ ELISPOT assay to determine the level of anti-vector and FIV-specific responses in peripheral blood, spleen, MLN and LPL. We also determined whether peripheral blood

could be used as an indicator of vaccine-induced splenic and mucosal antigen-specific immune response.

L. Lefrancois and others have shown that at the peak of LM infection, peripheral blood is an appropriate site to sample, but that antigen-specific CD8 T cells rapidly decline in the periphery and home to the spleen and tertiary tissues (8,13,18). We determined that extended culture was required to identify listeria-specific T cell responses in the peripheral blood. We modified an ELISPOT assay used to identify low-frequency antigen-specific cells and cultured our PBMC for four days before plating in the ELISPOT (20). For both groups of cats, there was significantly enhanced spot formation upon four-day culture (Fig.2). We then analyzed the peripheral response induced by immunization with LM*gag*/pND14Lc-*env* by stimulating PBMC with FIV Gag and Env-specific peptides for four days (Fig. 3). Cats who had two previous exposures to LM-wt, (SQ/Oral-PE) before oral immunization with LM*gag*/pND14Lc-*env* demonstrated no significant change in FIV-specific CD8 T responses as measured in the periphery versus cats who had only one previous exposure to LM-wt (SQ-PE). All cats had minimal to no response to Env peptide stimulation. We then analyzed the anti-vector response at the time of LM*gag*/pND14-Lc-*env* immunization to evaluate its effect on the generation of FIV-specific T cell responses (Figure 4). While both groups of cats generated an anti-SLA T cell response, the SQ/Oral-PE response was significantly greater. We believe this is due to a boosting effect due to the subsequent LM-wt oral exposure. This phenomenon has been noted in other LM studies (1,24,27).

We next evaluated antigen specific responses in the spleen, MLN and LPL to determine localization of vaccine-induced T cell responses (Fig.5). Extended culture of

lymphocytes derived from these tissues did not enhance SFC as found in the PBMC. One factor may be that LPL did not survive the four-day culture (data not shown). We determined that overnight culture with fresh tissue cells was sufficient to elicit an effector response in LPL, spleen and MLN. We ascertained the effect of previous LM exposures on the magnitude and localization of T cell responses in MLN, spleen and LPL. SQ-PE cats had significantly greater responses in LPL to SLA, Gag and Env stimulation than SQ/Oral-PE cats. We theorize that this is due to rapid clearance of LM*gag*/pND14Lc-*env* in the gut resulting from previous systemic followed by oral exposures to LM-wt. SQ-PE cats also had a greater response to SLA in the spleen and LPL. We believe the heightened response to SLA in the spleen of cats who were only systemically exposed to LM-wt (SQ-PE) can be explained in two ways: 1) low-dose SQ infection followed by single high-dose recombinant led to a greater priming of the spleen and 2) delayed clearance of LM*gag*/pND14Lc-*env* in the gut compared to SQ/Oral-PE cats (data not shown). Previous studies on the localization of response to LM-delivered antigens have relied on tetramer analysis. Others have found that functional assays such as the ELISPOT typically have lower percentages of responding CD8 T cells than might be expected based on tetramer analysis (8,13,16,18). We evaluated antigen-specific response in the spleen, LPL and MLN. Compared to SQ/Oral-PE cats, SQ-PE cats had significantly greater response to Gag and Env peptides in the LPL. As observed in other LM studies, our results show the lamina propria to be the optimal site to measure response generated by oral LM immunization (8,13,18).

Evaluation of spleen and LPL requires sacrificing the animals. To circumvent this problem, we determined that extended culture of PBMC could measure vaccine induced

T cell responses. As we did not discover a statistical correlation between PBMC response and that seen in the spleen or LPL, PBMC response can only be considered a poor indicator of responses measured in other sites. In this study, while peripheral blood analysis under-estimated Gag-specific CD8 T cell response of LPL for SQ-PE cats, there was no statistical difference for SQ/Oral-PE cats. We believe, for cats with no previous exposure to LM, PBMC response would probably underestimate that found in spleen and LPL. Further analysis of fresh mucosal tissue from single-vaccinated cats will need to be performed to fully elucidate the magnitude and localization of response to LM*gag*/pND14-Lc-*env* immunization.
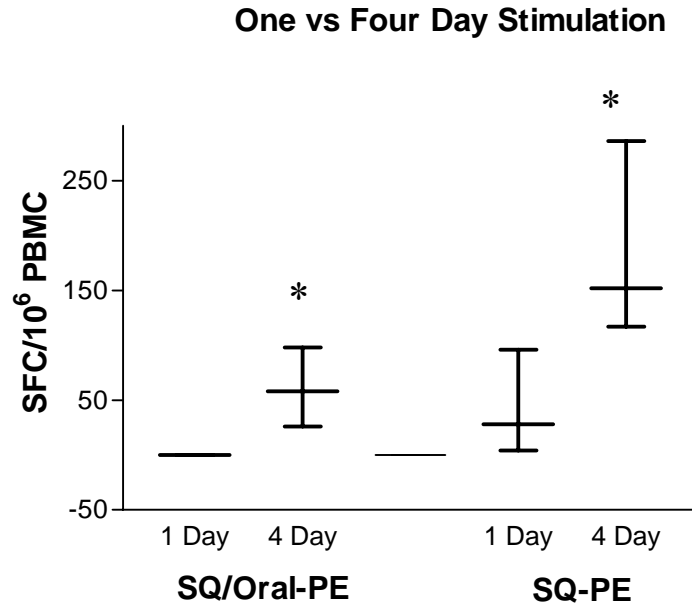
Figure 1

Month	0	4	5	6	7	8
SQ/Oral-PE	SQ 4x10^6 LM-wt	Oral 5x10^6 LM-wt	No TX	1x10^8 rLM	No TX	Necropsy
SQ-PE	SQ 4x10^6 LM-wt	No TX	1x10^8 rLM	No TX	Necropsy	

Figure 1. Immunization design. Eight cats were immunized subcutaneously (SQ) with 4x10^6 cfu wild-type *Listeria monocytogenes* (LM-wt) on month 0. Four months later cats were divided into two groups, subcutaneous LM-wt/oral LM-wt pre-exposure (SQ/Oral-PE) and subcutaneous pre-exposure (SQ-PE). SQ/Oral-PE cats were orally immunized with 5x10^6 cfu LM-wt followed 2 months later with 1x10^8 cfu orally delivered Lm*gag*/pND14-Lc-*env* (rLM). Group SQ-PE was orally immunized with 1x10^8 cfu Lm*gag*/pND14-Lc-*env* five months post SQ exposure. All cats were euthanatized two months post rLM immunization. No TX= No treatment.
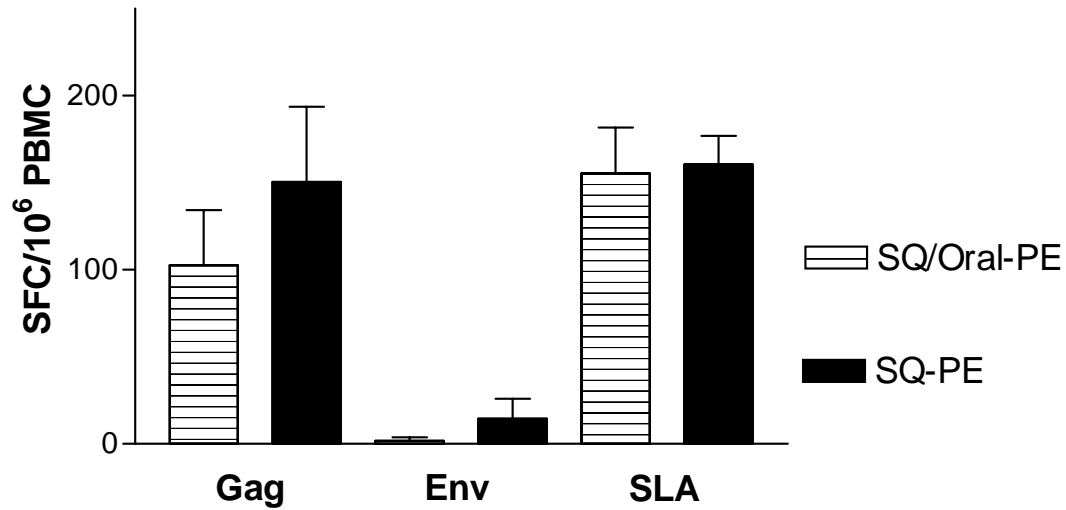
Figure 2

One vs Four Day Stimulation

Figure 2. Extended culture required to detect anti-vector response in PBMC.

PBMC were isolated two months post oral immunization with $5x10^6$ cfu LM-wt (SQ/Oral-PE) or one month post oral immunization with $1x10^8$ cfu LMgag/pND14-Lc-env (SQ-PE). $5x10^5$ PBMC/well were stimulated for both one and four days with SLA (µg/ml). Four day stimulated cells were added to ELISPOT plate at concentration of $3.5x10^5$ cell/well whereas one day stimulated cells were added at concentration of $5x10^5$ cells/well. All cells were incubated in ELISPOT plate for 24 hours. Results shown are mean of triplicate wells with unstimulated control wells subtracted. All spot forming cell (SFC) values were normalized to $1x10^6$ cells. * Denotes statistical difference between four and one day stimulation for SQ/Oral-PE (p=0.01) and SQ-PE (p=0.03).
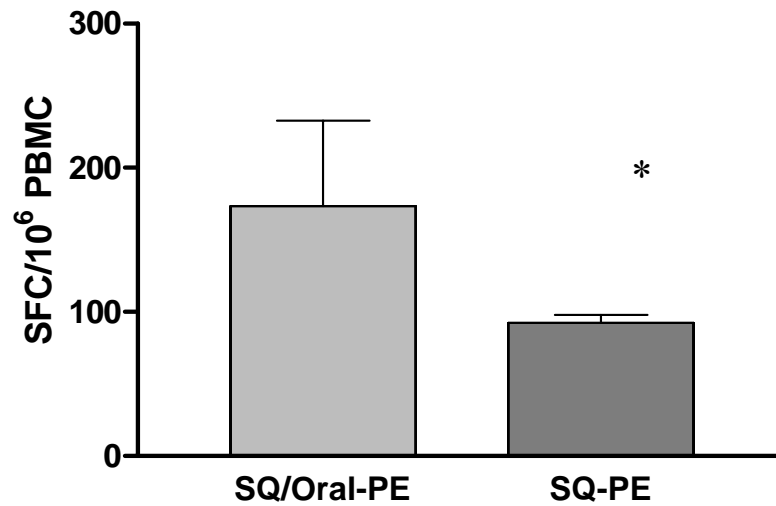
Figure 3

Figure 3. SLA and FIV Gag and Env-specific T cells responses in PBMC

PBMC from SQ/Oral-PE (striped) and SQ-PE (solid) were isolated two months post $1x10^8$

oral immunization with LMgag/pND14-Lc-env and stimulated for four days with FIV Gag and Env peptide

pools (1 μM) and SLA (1 μg/ml). Cells were added to ELISPOT plate at a concentration of $3.5x10^5$

cells/well and incubat for 24 hours. Results shown are mean of triplicate wells with unstimulated control

wells subtracted. All spot forming cell (SFC) values were normalized to $1x10^6$ cells.
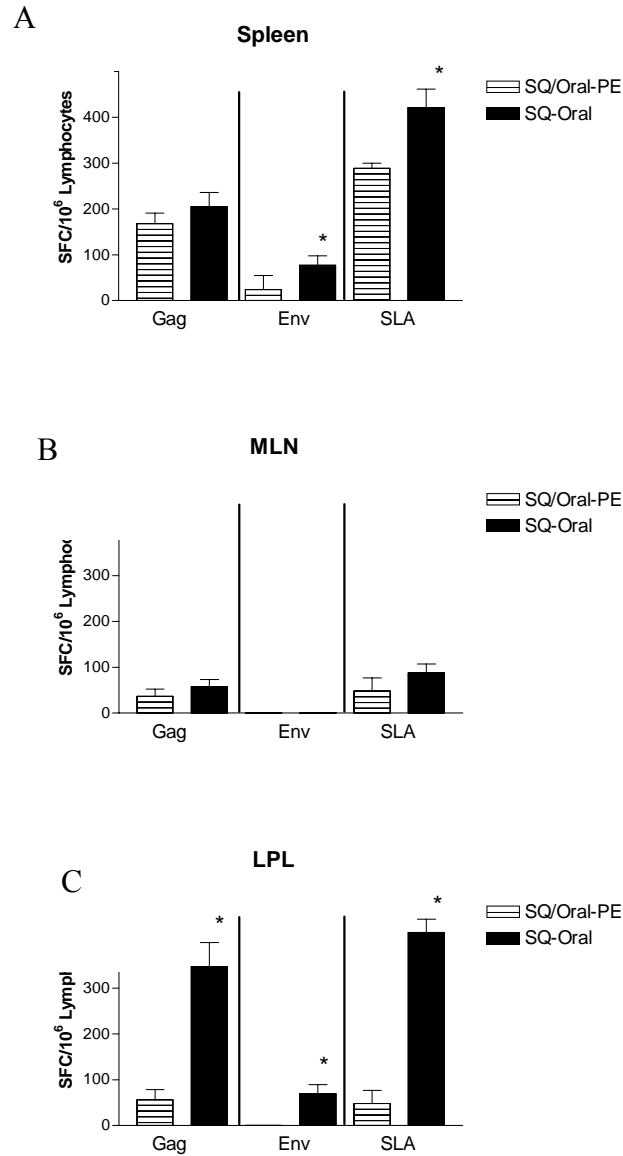
Figure 4

Figure 4. Anti-vector response at time of oral LM*gag*/pND14-Lc-*env* immunization

PBMC were isolated at time of LMgag/pND14-Lc-env immunization and stimulated with SLA as stated in Figure 3. Results shown are mean of triplicate wells with unstimulated control wells subtracted. All spot forming cell (SFC) values were normalized to $1x10^6$ cells. * Denotes statistical difference in anti-vector response between SQ/Oral-PE and SQ-PE cats (p=0.0013).

Figure 5

Figure 5. LM*gag*/pND14-Lc-*env*-specific responses in Spleen, MLN and LP
Lymphocytes were isolated from spleen (A), MLN (B), and LP (C) two months post oral
immunization with $1x10^8$ cfu LM*gag*/pND14-Lc-*env,* stimulated overnight then added to
ELISPOT plate. All spot forming cell (SFC) values were normalized to $1x10^6$ cells. * Denotes statistical
difference between SQ/Oral-PE and SQ-PE cats in (A) SLA and Env T cell responses (p<0.004, 0.04) and
(C) Gag, Env and SLA T cell responses (p<0.003, 0.005, 0.0001).

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Summary

The preceding experiments have demonstrated the following in LM*gag*/pND14-Lc-*env* immunized cats.

1. Single oral immunization with LM*gag*/pND14-Lc-*env* controls viral replication and disease progression by:

- Generating vaginal anti-Gag antibodies

- Limiting viral reservoirs

- Controlling viral replication in a CD8 T cell mediated manner

- Maintaining normal CD4 and CD8 T cell populations in MLN and intestinal mucosa

2. Single previous oral exposure to *L. monocytogenes* does not negate utility of LM*gag*/pND14-Lc-*env* as a vaccine vector against FIV.

- All cats generated serum, salivary, fecal and vaginal anti-Gag antibodies in spite of pre-existing anti-Listerial antibodies.

- All cats had measurable splenic FIV-specific CD8 T cell responses.

- SQ-PE cats had significantly greater FIV-specific CD8 T cell response in LPL than cats that had two previous exposures to LM-wt.

3. Evaluation of peripheral blood mononuclear cell response to LM*gag*/pND14-Lc-*env* immunization revealed:

- PBMC require extended culture to measure low-frequency antigen-specific T cell responses.

- FIV-specific CD8 T cell responses can be measured in peripheral blood.

- There is no correlation between antigen-specific peripheral blood response and responses observed in splenic and lamina propria lymphocytes.

Conclusion

Based on the experiments outlined here, a single oral LM*gag*/pND14-Lc-*env* immunization controls virus entry and replication. Cats that had a single SQ exposure to LM-wt generated significantly greater Env and SLA-specific T cell responses in the spleen than cats that had multiple previous exposures to LM-wt. SQ-PE cats also generated significantly greater vaccine-specific T cell response as measured in LPL. While cats that received both systemic and mucosal exposures to LM-wt had reduced FIV-specific CD8 T cell responses in the LPL, they generated FIV-specific antibodies and had similar responses to Gag peptide stimulation as SQ-PE cats.

PBMC can be used to evaluate vaccine-induced T cell responses by ELISPOT with the understanding that the response measured is just an indicator of vaccine-induced T cell responses and has no correlation to potential mucosal immune response.

These experiments show recombinant *Listeria monocytogenes* to be an excellent vaccine vector against FIV and worthy of further evaluation.

Appendix:

Objective: Develop a plasmid construct that expresses full-length FIV strain NCSU$_1$.

Source of virus: Virus was isolated from PBMC of a cat that was naturally infected with NCSU$_1$. Genomic DNA was isolated, partially digested with *Sau3*AI and then cloned into EMBL3 lambda vector (See Joo-Sung Yang dissertation and J. Virol 70:3011-3017).

Design outline:

1) LTR primers were designed to amplify the LTR fragment of NCSU$_1$ and add Not1 and Sal1 restriction sites to the end of the LTR fragments.

2) Clone fragment directionally into multiple cloning site of pWKS30 generating a 5.7kb plasmid, pWKS30-LTR.

3) Verify pWKS30-LTR plasmid by PCR and sequencing

4) Cut full-length NCSU$_1$ from Lambda phage with restriction enzyme Nhe1 generating a 9kb fragment.

5) Linearize pWKS30-LTR with Nhe1 and alkaline phosphatase treat to prevent self-ligation.

6) Evaluate different ligation ratios of plasmid to vector, 1:1, 1:3 and 3:1.

7) Ligate and transform into Stbl2 competent cells.

8) Incubate aliquots of transformants on LB/ampicillin plates at 30°C overnight.

9) Pick colonies and screen by PCR.

10) Colonies positive for insert will be screened for size by gel electrophoresis.

11) Colonies that contain insert and are the appropriate size (14.7 kb) with be sequenced to verify directionality and proper sequence of insert.

A) Grow phage

1) Set up overnight culture of *E.coli* host (LE392) in T-TYN media
supplemented with maltose at 37°C. * See Note 1.

Liquid lysate method: * See Note 2.

2a) Mix 500µL of phage stock with 500µL LE392 and incubate for 20-25
minutes in 37°C water bath. * See Note 3.

3a) Inoculate above solution into 50-100 ml LB T-TYN medium and incubate
overnight at 37°C in shaking incubator. Cover with aluminum foil cover to
seal off flask.

4a) After overnight incubation, you should see lysis of bacterial host (clearing
of culture). Add 500µto 1ml of chloroform to lyse any remaining bacteria.

5a) To pellet out cell debris, centrifuge phage culture at 3700 rpm twice in
table-top or super-speed floor centrifuge. (Keep phage- containing
supernatant).

6a) To pellet out any remaining cell debris, transfer phage culture to Oak
Ridge tubes and centrifuge 10,000 rpm (12,100 x *g*) for 10 minutes at 4°C.
To isolate phage DNA: QIAGEN Lambda phage kit (Cat# 12523) or
Qbiogene Lambda Quick Kit (Cat # 2055-400).

Lambda phage plate method:

When starting overnight culture, pour 10-12 bottom agar plates.

2b) For each plate, mix 100µL O/N LE392 culture with 100µL phage stock in
15ml tube, and incubate for 10-15 minutes in 37°C water bath.

3b) Add 15ml top agarose to each tube, vortex and pour onto bottom agar plates prepared the night before.

4b) Let top agarose set, invert plates, then put into 37 C incubator for 16-20 hours (or until plaque lawn is observed).

5b) Overlay plates with 5ml suspension medium (SM) and let shake at room temperature for several hours or overnight at 4 C (shake).

6b) Transfer liquid to clean polypropylene tubes, wash plates with additional ml of SM and transfer to tubes.

7b) Add chloroform (2%v/v) to tubes and vortex vigorously. Centrifuge tubes at > 10,000 x g for ten minutes. Retain the supernatant.

8b) To isolate phage DNA: QIAGEN Lambda phage kit (Cat# 12523) or Qbiogene Lambda Quick Kit (Cat # 2055-400).

B) Generate pWKS30-LTR.

1) Amplify LTR fragment with primers.

Primers used:

NCSU1-LTR/1 5' ATA AGA ATG CGG CCG CTG GGA TGA GTA TTG GGA CCC 3'

NCSU1-LTR/2 5' ACG CGT CGA CTG CGA AGT TCT CGG CCC GGA T 3'

2) Digest pWKS30 with Not1 and Sal1.

3) Directionally clone LTR-Not1 and LTR-Sal1 into plasmid (See Figure1).

4) Digest 9kb $NCSU_1$ fragment from phage with Nhe1 (See Figure 2).

5) Digest pWKS30-LTR with Nhe1 and alkaline phosphatase treat.

6) Ligate insert and vector in various ratios into Stabl2 cells, spread on LB/amp plates and incubate overnight at 30 C.

C) Results:

Propagated phage by both liquid and plate methods.(mention what worked better)

Digested 9kb fragment from phage.

Generated pWKS30-LTR plasmid.

Ligated insert into vector and transfected Stbl2 cells.

Screened colonies, but colonies were never PCR positive for $NCSU_1$.

Recipes:

T-TYN Media + Mg+2

10 g tryptone

5 g yeast extract

5 g NaCl

10 ml 1M Tris pH 7.2

900 ml ddH_2O, adjust pH to 7.2 with NaOH.

QS to 1 liter with ddH_2O

Autoclave, cool and add 10 ml sterile 1M $MgCl_2$.

NOTE: FOR GROWING UP CULTURES OF LE392- ADD 1 ml 20% MALTOSE PER 100 ml medium.

T-TYN Bottom Agar

Make 1 liter T-TYN media

Add 15 g agar

Autoclave, cool and add 10 ml 1M $MgCl_2$

Pour 10 large plates (100mm)

T-TYN Top Agarose

Make 1 liter T-TYN media

Add 7 g ultrapure agarose (agarose is required)

Autoclave, cool and add 10 ml 1M MgCl$_2$

Use 9-10 ml per large plate

<u>Suspension media</u>

NaCl - 2.9 grams

MgSO$_4$ - 1 gram

1 M Tris pH 7.5 - 25ml

Add enough ddH$_2$O water to bring vol to 500 ml

Autoclave

Can also purchase SM from QIAGEN.

Notes:

1) T-TYN medium is not as rich as LB medium leading to slower *E. Coli* growth. This is important as *E. coli* can easily overgrow the phage and prevent the formation of plaques. Maltose is added to the medium to induce expression of lambda receptor on the bacterial cell surface. The lambda receptor is part of the maltose-usage pathway.

2) It might be better to start with the Lambda plate method first for two reasons. 1) You will generate fresh plaques for propagation and 2) It is less likely that bacteria will overgrow the phage by the plate method.

3) If overgrowth occurs, simply decrease amount of bacteria that is incubated with the phage.

Figure 1. Validation of pWKS30-LTR
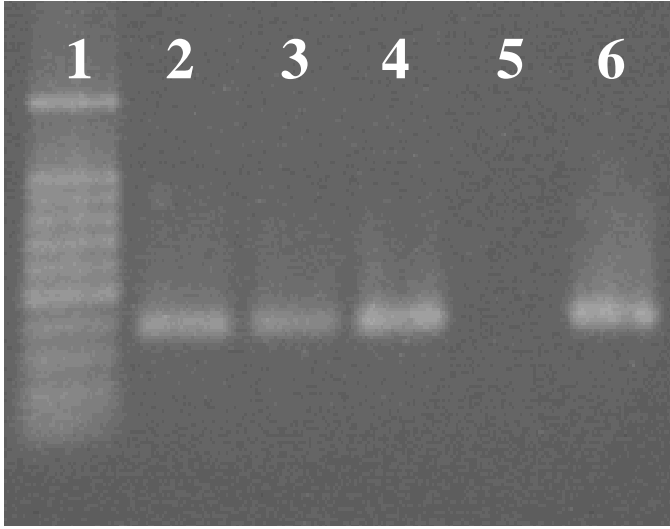
Figure 1. Lane 1: 100 bp molecular weight marker.
Lane2-4: pWKS30 clones positive for LTR's.
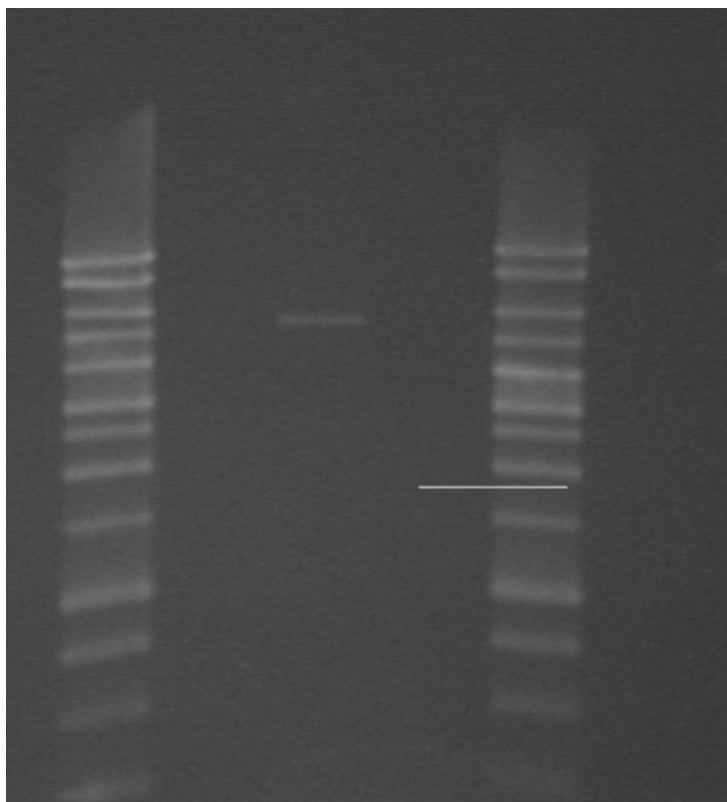Lane 6: positive control

Figure 2. Gel purified NCSU$_1$

Figure 2. Lane 1 and 5 MWM. Lane: 3 approx 9kb fragment
Gel-purified from Nhe1 digested Lambda phage DNA.