Listeria monocytogenes as a Vaccine Vector: Virulence Attenuation or Existing Antivector Immunity Does Not Diminish Therapeutic Efficacy[1]

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The bacterium *L. monocytogenes* is a proposed vaccine carrier based upon the observation that this pathogen replicates within the intracytoplasmic environment facilitating delivery of Ag to the endogenous Ag processing and presentation pathway with subsequent stimulation of peptide specific MHC class I-restricted CD8$^+$ effector cells. In this report, we evaluate virulence-attenuated strains of *Listeria monocytogenes* as vaccine vectors and examine whether existing antivector (antilisterial) immunity limits or alters its efficacy as a therapeutic cancer vaccine. Following immunization with virulence-attenuated mutants, we found that the effectiveness of *L. monocytogenes* as a recombinant cancer vaccine remains intact. In addition, we found that antibiotic treatment initiated 24 or 36 h following therapeutic immunization with recombinant *L. monocytogenes* allows full development of the antitumor response. We also demonstrate that the vaccine vector potential of *L. monocytogenes* is not limited in animals with existing antilisterial immunity. For these latter studies, mice previously immunized with wild-type *L. monocytogenes* were infused with melanoma cells and then 5 days later challenged with recombinant tumor Ag expressing *L. monocytogenes*. Collectively, these results add additional support for the use of *L. monocytogenes* as a vaccine vector and underscore its potential to be used repeatedly for stimulation of recall responses concomitant with primary cell-mediated responses to newly delivered heterologous tumor-associated epitopes. ***The Journal of Immunology*****, 2004, 173: 420–427.**

The bacterium *Listeria monocytogenes* (Lm)[3] has been extensively studied for understanding mechanisms of intracellular pathogenesis as well as development of acquired cell-mediated immunity to pathogens that can reside and replicate within the cytoplasm of infected host cells (1, 2). Many of the bacterial determinants necessary for pathogenesis, including intracellular growth and spread of Lm, have been identified and are located within a 10-kb region of the chromosome termed the PrfA regulon which includes nine open reading frames (3). Control of Lm infection appears to have two distinct components: innate or constitutive immunity (nonspecific) and adaptive or protective immunity (*Listeria* specific). Following the initial wave of the constitutive response, *Listeria*-specific CD4$^+$ and CD8$^+$ T cell subsets are stimulated. CD4$^+$ T cells mediate delayed-type hypersensitivity reactivity (4) and a role for CD4$^+$ T cells in protective antilisterial immunity has been reported (5, 6). The CD8$^+$ T cell population is considered to be the subset responsible for protection, as shown using adoptive transfer studies and in vivo T cell subset depletion studies (4, 7–9).

Lm is proposed as a vaccine carrier based on the observation that this pathogen replicates within the intracytoplasmic environment, thus facilitating delivery of Ag to the endogenous Ag processing and presentation pathway, and a number of studies have been published that are in support of its vaccine vector potential. For example, immunization of mice with a Lm strain that secretes a lymphocytic choriomeningitis virus (LCMV) nucleoprotein (NP)-derived epitope stimulates NP-specific CD8$^+$ effector cells that protect against lethal challenge with LCMV (10), and rabbits immunized with a Lm strain that secretes a papilloma virus derived Ag are protected against papilloma virus infection (11). In murine tumor models, immunization with a Lm strain that secretes an influenza NP (flu NP) determinant stimulates CD8$^+$ CTL that are protective against renal or colon carcinomas engineered to also express flu NP (12). Regression of B16F10 solid tumors expressing flu NP has been shown following immunization with a Lm strain expressing flu NP (13) and immunization with Lm constructs that express the E7 protein of human papilloma virus (a protein associated with cervical cancer) stimulates a cell-mediated immune response that mediates regression of an E7-transduced tumor cell line (14).

Collectively, these data argue strongly for the continued assessment of the efficacy of recombinant Lm as a vaccine strain. Several issues that remain to be resolved prior to the general use of Lm as a vaccine vector include whether effectiveness is retained when using virulence-attenuated strains as the delivery vector for general safety concerns. Also, attenuated Lm may be desired for treating

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[3] Abbreviations used in this paper: Lm, *Listeria monocytogenes*; LCMV, lymphocytic choriomeningitis virus; NP, nucleoprotein; LLO, listeriolysin O; TRP-2, tyrosinase-related protein 2; Wt, wild type.

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cancer patients that are potentially immunocompromised due to prolonged chemo/radiotherapy. Also, given the unknown status of immunity to any vector within the general population, it is important to assess whether effectiveness is diminished in hosts with existing antivector immunity. The latter is a critical consideration as experiments with recombinant vaccinia or adenovirus virus show that existing immunity to the vector dramatically diminished primary responses and desired outcomes when their use is repeated (15–18). In this study using a murine pulmonary melanoma metastases model, we have found that virulence attenuation does not diminish the therapeutic effectiveness of Lm as a vaccine vector and also that existing antilisterial (antivector) immunity does not inhibit the development of therapeutic antimelanoma responses following subsequent immunization with recombinant Lm vaccine strains.

Materials and Methods

Bacteria

The following bacterial strains were used as immunogens for the described studies: Lm 10403 serotype 1 (originally obtained from Dr. E. Hall, Washington State University, Pullman, WA), a tyrosinase- related protein 2 (TRP-2)-expressing strain of Lm (the derivation of which is described in detail elsewhere[4]), an OVA-expressing wild-type (Wt) strain of Lm (derivation described previously (19)), an OVA-expressing *actA*$^-$strain of Lm (20), and an OVA expressing L461T listeriolysin O (LLO) strain of Lm (21). The pPL2 integration vector (22) containing an in-frame OVA-coding insert was used to derive the OVA-expressing recombinant *actA*$^-$ and L461T LLO strains containing a single copy of the OVA sequence integrated into an innocuous site of the *Listeria* genome. Virulence is maintained by repeated passage in BALB/c or C57BL/6 mice.

Mice and immunization

Six-week-old female C57BL/6 mice were purchased from The Jackson Laboratory (Bar Harbor, ME) and provided unrestricted access to food and water. Eight-week-old C57BL/6 mice were immunized by injection via the tail vein with ~0.1 LD_{50} of viable Lm in 0.2 ml of PBS. For secondary immunizations, mice were injected with ~8000 CFU in 0.2 ml of PBS by the same route. The pUbgp100 plasmid DNA vaccine construct was prepared by subcloning gp100 cDNA derived from a melanoma cell line into the pCMVi(-H3)Ubs plasmid construct by methodology previously described (23, 24). The pUbgp100 plasmid construct encodes an ubiquition/gp100 fusion protein, and the expression of this chimeric protein is regulated by the CMV promoter and CMV intron A enhancer sequences. Plasmid Giga kits (Qiagen, Chatsworth, CA) were used to generate large-scale plasmid DNA preparations (3–10 mg/ml). For immunization with the pUbgp100 plasmid construct, 6- to 8-wk-old C57BL/6 mice received the first of a series of three i.m. immunizations (via the tibialis anterior muscles) at 3-wk intervals with 100–125 μg of plasmid DNA in 50 μl of normal saline. All animal experiments were conducted with approval from the Institutional Animal Care and Use Committee.

Cell lines and reagents

The RMAS-K^d cell line (provided by Dr. M. Bevan, University of Washington, Seattle, WA) was maintained in RPMI 1640 (Life Technologies, Grand Island, NY) supplemented with 10% FCS and 200 μg/ml G-418 (Sigma-Aldrich, St. Louis, MO). The SIINFEKL-expressing melanoma tumor cell line (B16-MO5, provided by Dr. K. Rock, University of Massachusetts Medical Center, Worcester, MA (25)) was maintained in DMEM plus 10% FCS and 100 μg/ml G-418. The OVA-derived SIINFEKL peptide was purchased from SynPep (Dublin, CA), and the mouse TRP-2 aa180–188 peptide (K^b restricted, single letter code SVYDFFVWL), LCMV NP aa 396–404 peptide (D^b-restricted, single letter code FQPQNGQFI), and gp100 aa 25–33 peptide (D^b-restricted single letter code KVPRNQDWL) were synthesized at the Portland Veterans Affairs Medical Center (Portland, OR).

Enumeration of lung metastases

Experimental or control mice were infused with 7–10 $\times$ 10^5 B16-MO5 cells (SIINFEKL expressing) in a volume of 0.2 ml of DMEM via the tail vein. Nineteen to 21 days following tumor infusion, animals were killed by CO_2 inhalation and the lungs were removed. The lungs were placed in PBS and the number of individual visible tumor foci was counted using a dissecting microscope. In some cases, the lungs were immersed in a bleaching solution consisting of 67% ethanol, 9% formaldehyde, and 4% glacial acetic acid, and the number of visible tumor foci was enumerated 24–48 h later.

Antibiotic treatment

A stock solution of ampicillin (Sigma-Aldrich) was prepared at 10 mg/ml. Following immunization, animals were given 2 mg of drug in a volume of 0.2 ml by the i.p. route every 12 h for a total of six injections over a 72-h period.

ELISPOT analysis for enumeration of IFN-γ-secreting cells

Sixteen to 18 h before use, 96-well nitrocellulose plates (Millipore, Bedford, MA) were coated with 100–500 ng/well anti-mouse IFN-γ capture Ab (BD PharMingen, San Diego, CA) diluted in PBS and added in a volume of 100 μl. One-hour before use the plates were washed with sterile medium or sterile PBS and then blocked with cell culture medium (RPMI 1640 or DMEM) containing 5–10% FCS. For enumeration of peptide-specific cells, RMAS-K^d cells were held at room temperature for 16–18 h, then resuspended at 1 $\times$ 10^6 cells/ml and pulsed with 1 $\times$ 10^{-7} M of either the SIINFEKL, TRP-2, or LCMV NP peptides. The peptide pulsed cells were incubated at room temperature for 2 h, irradiated (2500 rad), washed twice with RPMI 1640, and then added to the ELISPOT plates at 100,000 cells/well in a volume of 100 μl. For enumeration of melanoma-specific effector cells, B16-MO5 melanoma cells were trypsinized from confluent T-25 flasks, irradiated with 6000 rad, and added to ELISPOT plates at 5–10,000 cells/well in a volume of 100 μl in RPMI 1640 supplemented with 10% FCS and 100 U/ml penicillin and 100 μg/ml streptomycin.

For enumeration of effector cell frequencies, single-cell suspensions of immune spleen cells from mice previously immunized with Lm were prepared and added to the ELISPOT plates at 25–100,000 cells/well to the peptide-pulsed RMAS-K^d cells. After a 24-h incubation at 37ºC, the plates were washed four times with 0.05% Tween 20-PBS and a biotinylated anti-mouse IFN-γ detection Ab (BD PharMingen) added at 500 ng/well in a volume of 100 μl. The plates were then incubated overnight at 4ºC, washed four times with 0.05% Tween 20-PBS, and 100 μl of a 1/1000 dilution of streptavidin AKP (BD PharMingen) was then added. After 1 h at room temperature, the plates are washed four times with 0.05% Tween 20-PBS and then the detection substrate BCIP/nitroblue tetrazolium (Kirkegaard & Perry Laboratories, Gaithersburg, MD) was added to each well. After 5–20 min, the plates are washed with distilled H_2O and allowed to dry. Spots were enumerated with a Zeiss microscopy unit equipped with KElispot software (Zeiss, Oberkochen, Germany).

Peptide-specific effector cells

Mice were immunized with either the OVA- or TRP-2-expressing strains of Lm or the gp100-containing plasmid and 10 days later spleens were removed and single-cell suspensions were prepared. Immune spleen cells were cocultured with irradiated (2500 rad) normal spleen cells (RBC free) that had been pulsed for 2 h with a 10^{-8} M concentration of either SIINFEKL, TRP-2, LCMV NP, or gp100 peptide. Cells were cocultured at a 1:1 ratio in T-75 cm^2 flasks at 1 $\times$ 10^8 total cells/flask. Cells were cultured in RPMI 1640 supplemented with nonessential amino acids (Life Technologies), 10% heat-inactivated FCS, 100 U/ml penicillin, 100 μg/ml streptomycin, 5 $\times$ 10^{-5} M 2-ME, and 30 U/ml hrIL-2 (Tecin; Biological Response Modifiers Program, National Cancer Institute, Frederick, MD). After 6 days of culture, the viable cells were collected and restimulated for an additional 6 days with peptide-pulsed normal spleen cells and identical coculture conditions. The viable cells were collected after the second culture phase and either used as indicator cells in ELISPOT assays or adoptively transferred at 8 $\times$ 10^6 cells/recipient via the tail vein in a volume of 0.2 ml.

Statistical analysis

The mean and the median between control and treatment groups was assessed (ANOVA *t* test) using JMP statistical software (SAS Institute, Cary, NC).

[4] K. W. Bruhn, B. D. Nguyen, N. Craft, J. Yip, A. De, S. S. Gambhir, and J. F. Miller. Stimulation of anti-self CD8 T cell responses and tumor protection following immunization with recombinant *Listeria monocytogenes* expressing the murine melanoma antigen TRP-2. *Submitted for publication.*

Results

Recombinant virulence-attenuated mutants of Lm stimulate therapeutic antitumor responses

The use of a Lm-based vector as a cancer vaccine is attractive due to the intrinsic ability of this intracellular pathogen to stimulate a strong $CD4^+$ and $CD8^+$ T cell response. For clinical application of Lm as a recombinant vaccine, various safety concerns need to be addressed. Since many of the critical virulence factors are known for Lm, defined mutants with attenuated phenotypes can be developed and assessed for vaccine vector potential. For these studies, we used recombinant strains of Lm expressing the model Ag OVA (19) and a variant of the B16 melanoma cell line that also expresses OVA (B16-MO5 cells) in the murine lung melanoma model (25). We chose to initially employ responses to the H2-K^b-presented SIINFEKL peptide of OVA as the antigenic target in this system since the $CD8^+$ T cell response to this determinant is well characterized and recombinant strains of Lm expressing OVA have previously been described (19).

We evaluated two virulence-attenuated strains of Lm for their ability to stimulate $CD8^+$ T cell responses that could inhibit tumor growth. For our initial studies, we used an *actA* deletion mutant strain that no longer spreads from cell-cell and has an LD_{50} in C57BL/6 mice of $\sim 2 \times 10^8$ CFU (20), and a strain with a leucine to threonine amino acid substitution in the LLO molecule at position 461, resulting in an LD_{50} in C57BL/6 mice of $\sim 1 \times 10^8$ CFU (21). C57BL/6 were first injected with 7×10^5 OVA-expressing melanoma tumor cells. This dose of melanoma cells is such that the pulmonary form of the disease is readily apparent, but is titrated to the lowest possible dose that gives reproducible lung disease and thus is likely to be similar to what is observed clinically. Five days later, the tumor-bearing animals were immunized i.v. with approximately a 0.1 LD_{50} dose of the various attenuated vaccine strains of Lm. Although the immunization dose for the attenuated strains is increased compared with that of Wt, the relative virulence of the attenuated strains, expressed as the 0.1 LD_{50} dose, is identical when compared with the Wt dose. The results in Table I show that similar levels of therapeutic antitumor immunity are evident following therapeutic immunization with the virulence-attenuated OVA-expressing mutants when compared with the Wt OVA-expressing positive control strain of Lm in tumor-bearing animals. The numbers of lung metastases in animals immunized in the therapeutic setting with *actA*$^-$ recombinant Lm (mean, 3 ± 3) or L461T LLO recombinant Lm (mean, 4 ± 2) are similar to the numbers of lung metastases in animals immunized therapeutically with Wt OVA-expressing Lm (mean, 8 ± 5). Immunization with the parental controls (non-OVA-expressing Lm) do not offer antitumor benefit (all groups with >250 tumor nodules/lung), suggesting that nonspecific inflammation and cell activation do not contribute to tumor eradication in this model. These results show that stimulation of therapeutic antitumor responses with virulence-attenuated Lm are indistinguishable from those following immunization with Wt Lm expressing the target Ag.

Table I. *Immunization with attenuated recombinant Lm strains stimulates therapeutic antitumor responses*

Therapeutic Immunization[a]	No. of Lung Metastases[b]	Mean[c]
Wild type	All >250	
Wild type-OVA	2,8,9,14	8 ± 5
ActA$^-$ parent	All >250	
ActA$^-$-OVA	0,1,1,2,3,4,5,9	3 ± 3
L461T LLO$^-$ parent	All >250	
L461T LLO$^-$-OVA	1,2,2,4,5,7	4 ± 2

[a] C57BL/6 mice were injected i.v. with 7×10^5 OVA expressing B16 melanoma cells and 5 days later were immunized with approximately 0.1 LD_{50} of the indicated Lm strains.

[b] Nineteen days after tumor challenge, the lungs were removed and the numbers of lung metastases were enumerated. Data shown reflect the tumor burden of each individual mouse.

[c] Mean ± 1 SD.

Antibiotic treatment and stimulation of antitumor responses

These strains of Lm retain sensitivity to a variety of antibiotics and can be easily controlled (if required) with antibiotic therapy (such treatment modalities are not available for recombinant viral vectors). Therefore, immunization regimens incorporating antibiotics can significantly reduce the risk to the patient. It has previously been determined that antibiotic treatment 24–48 h following immunization with Wt Lm does not diminish the stimulation of antilisterial immune effector cells (26). In pilot studies, we determined that antibiotic treatment 24 or 36 h after immunization with the Wt OVA-expressing strain of Lm does not alter stimulation of SIINFEKL-specific effector cells and also does not alter the antitumor effectiveness of this strain in a prophylactic tumor model, (data not shown). Because cancer vaccines are given in a therapeutic setting, we sought to determine in tumor-bearing animals whether immunization with a Lm vaccine strain followed by antibiotic treatment would alter antitumor efficacy. To conduct these experiments, animals previously injected with B16 melanoma cells expressing OVA were immunized with the Wt OVA-expressing strain of *L. monocytogenes* and then treated with ampicillin 24 or 36 h postimmunization. Enumeration of lung metastases shows very clearly that antibiotic treatment 24 or 36 h postimmunization does not alter the therapeutic efficacy of the vaccine as compared with tumor-bearing mice receiving the vaccine alone (Table II). There are no significant differences in the numbers of pulmonary metastases seen in the antibiotic-treated groups (mean, 18 ± 4.5 at 24 h or 26 ± 19 at 36 h) compared with that seen in the immunized control group (mean, 19 ± 16). As also shown, similar findings have been observed with OVA-expressing virulence-attenuated Lm strains following antibiotic treatment. These findings establish that the therapeutic efficacy of Lm as a recombinant vaccine is not compromised following antibiotic-mediated control of the infection.

Existing antilisterial immunity is not a barrier to vaccine efficacy

We previously determined that effector cell expansion to "memory" epitopes as well as priming of naive $CD8^+$ T cells to newly

Table II. *Antibiotic treatment following therapeutic immunization does not reduce efficacy of Lm as a recombinant vaccine*

Therapeutic Immunization[a]	Time of Ampicillin Administration[b] (h)	No. of Lung Metastases[c]	Mean[d]
—	—	All >250	
Wild type-OVA	—	6,9,18,41	19 ± 16
	24	11,18,20,21	18 ± 4
	36	4,17,35,47	26 ± 19
ActA$^-$-OVA	—	3,5,6,11,13	8 ± 4
	24	5,8,8,9,10	8 ± 2
L461T LLO$^-$-OVA	—	1,1,2,2,4	2 ± 1
	24	4,5,5,6,7	5 ± 1

[a] C57BL/6 mice were injected i.v. with 7×10^5 OVA-expressing B16 melanoma cells and 5 days later were immunized with approximately 0.1 LD_{50} of the indicated strains.

[b] At the indicated times postimmunization, ampicillin at 2 mg/animal was injected i.p. every 12 h, with six total injections given.

[c] Nineteen days after tumor challenge, the lungs were removed and the levels of tumor burden were determined. The data shown are from one of two experiments performed, with both experiments yielding similar data.

[d] Mean ± 1 SD.

delivered antigenic determinants can occur simultaneously following immunization with Lm (27). In humans, the majority of exposure to Lm is oral. However, because oral infection in mice is not efficient, systemic exposure and subsequent immunity is modeled following i.v. administration. Therefore, following i.v. primary infection with Wt Lm, we assessed antitumor responses following a second i.v. injection of recombinant Lm in the murine B16 melanoma model system. Injection of mice with Wt Lm results in uncontrolled replication of the bacteria within the first 72 h, after which time, splenic CFU begin to decline (Fig. 1, *left panel*). Six days following injection, splenic CFU are essentially absent. This finding is in marked contrast to the clearance of Lm from the spleen of immune animals where a rapid decline in splenic CFU is observed. By 72 h following challenge of immune mice, splenic CFU are below detection limits. To determine whether stimulation of a primary response to the recombinant heterologous Ag OVA occurs in Lm-immune mice, mice were immunized with Wt Lm (~800 CFU) and then 28 days later immunized with a Wt recombinant OVA-expressing strain of Lm (8000 CFU). The presence of SIINFEKL-specific effector cells in the spleen was assessed by ELISPOT assays 7 days later. We found that SIINFEKL-specific effector T cells were stimulated in Lm-immune animals subsequently immunized with a recombinant OVA-expressing strain of Lm, and the frequency of peptide-specific cells as detected by ELISPOT is consistent with the numbers of cells detected following a primary response in naive animals (Fig. 1, *right panel*). Also, when both immunizations are performed with the OVA-expressing Lm strain, there is a dramatic numerical expansion in the numbers of SIINFEKL-specific effector cells consistent with a memory response. The results from these experiments are in support of our previous findings (27) and extend the observation that existing antilisterial immunity does not hinder stimulation of effector cells to newly presented Lm-derived Ags in a distinct mouse strain.

To determine whether the antitumor effectiveness of the SIINFEKL-specific effector cells stimulated as a primary response in mice were altered by existing antilisterial immunity, mice were immunized with Lm and 23 days later these Lm-immune mice were injected with OVA-expressing melanoma tumor cells (B16-MO5). Five days later, the tumor-bearing mice were immunized with a Wt OVA-expressing vaccine strain of Lm. Positive controls include Lm-naive tumor-bearing mice injected with the OVA-expressing strain of Lm and negative controls included mice immunized with the parental Lm vector strain, infused with tumor cells, and then injected again with the parental Lm vector strain. Lungs from experimental and control mice were harvested 14 days later (19 days after tumor cell infusion), and the numbers of lung metastases were enumerated. The data presented in Table III show that the tumor burden in Lm-immune tumor-bearing animals injected with the vaccine strain is significantly reduced and the numbers of pulmonary metastases are equivalent to the control tumor-bearing vaccine-immunized group (mean, 12 ± 10 and 10 ± 10 tumor nodules). There is no reduction of tumor burden in the group of mice that received a primary and secondary injection with Wt Lm, providing additional evidence that tumor reduction is a function of specific rather than nonspecific inflammation. For the studies shown, the secondary dose of the OVA-expressing Lm (8000 CFU) was 10-fold greater than the primary immunization dose with Wt (800 CFU). We have also determined that following secondary immunization with the OVA-expressing Lm strain with a dose that is 10-fold less (800 CFU), antitumor responses to newly expressed tumor targets are indistinguishable from positive controls (data not shown). However, antitumor responses are less reproducibly evident following secondary

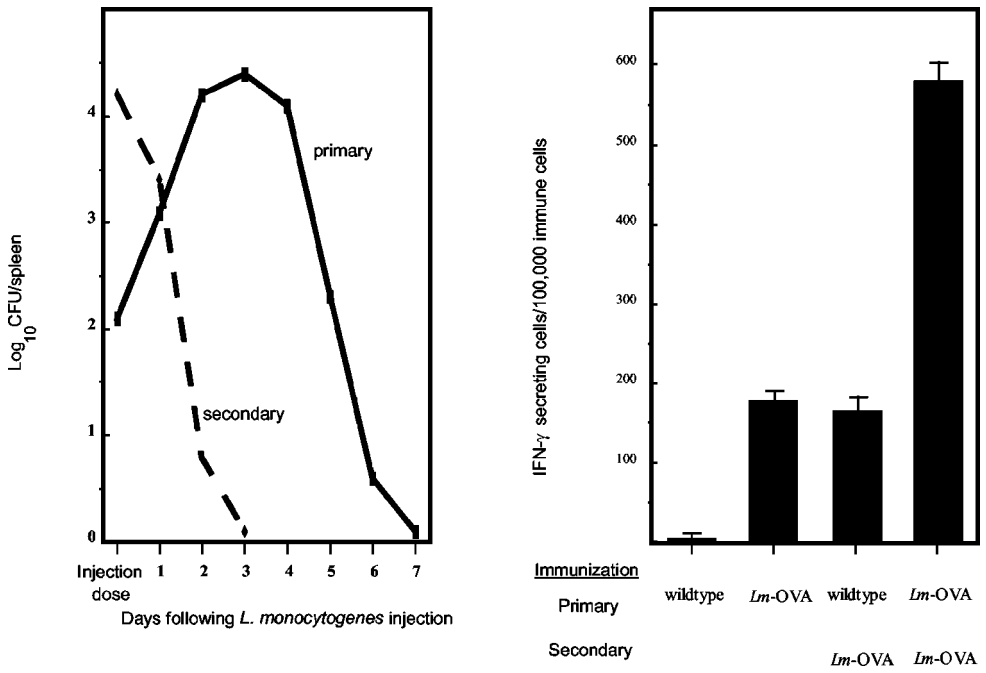

FIGURE 1. Existing antilisterial immunity does not alter priming to a newly expressed Lm-derived Ag following secondary infection. *Left panel*, C57BL/6 mice were immunized with ~800 CFU of Lm. Twenty-eight days later, these mice were injected with ~8000 CFU of the SIINFEKL-expressing Lm strain as a secondary dose, and the $\log_{10}$ CFU per spleen was determined on the days indicated (dashed line). A separate group of animals received ~800 CFU of the SIINFEKL-expressing Lm strain as a primary infection (solid line), and the $\log_{10}$ CFU per spleen determined simultaneously. *Right panel*, C57BL/6 mice were immunized with ~800 CFU of Lm. Twenty-eight days later, these mice were injected with ~8000 CFU of the SIINFEKL-expressing Lm strain (Lm-OVA) as a secondary dose and 7 days later the number of SIINFEKL-specific IFN-γ-secreting cells present in the spleen cell population was determined by ELISPOT. Controls include mice injected once with Wt, once with the SIINFEKL-expressing Lm strain, or animals injected twice with the SIINFEKL-expressing Lm strain. Data are representative of two independent experiments.

Table III. *Existing antilisterial immunity is not a barrier for utilization of Lm as a therapeutic recombinant vaccine*

Treatment[a]	No. of Lung Metastases[b]	Mean[c]
Melanoma cell control	All >250 ($n = 12$)	
Melanoma cells, Lm-OVA	1,1,2,3,10,16,16,18,18,22,30	12 ± 10*
Wt Lm, melanoma cells, Lm-OVA	0,1,2,2,5,6,7,13,14,22,25,27	10 ± 10*
Wt Lm, melanoma cells, Wt Lm	All >250 ($n = 12$)	

[a] C57BL/6 mice were immunized with approximately 800 CFU of the parental vector strain of Lm (Wt Lm) as indicated. Twenty-three days later, animals were infused with SIINFEKL-expressing melanoma cells and then immunized 5 days later with ~8000 CFU of either the SIINFEKL-expressing Lm strain (Lm-OVA) or Wt Lm as indicated.

[b] Nineteen days after tumor cell infusion, the numbers of lung metastases for each animal in the group were enumerated.

[c] Mean ± 1 SD.

*, $p < 0.001$ compared to controls.

immunization with lower doses such as 80 CFU (data not shown). The conclusion from these experiments is that existing antilisterial (antivector) immunity does not alter the development of therapeutic peptide-specific $CD8^+$ T cells following secondary immunization with a recombinant vaccine strain of Lm expressing a newly delivered antigenic target.

Lm vaccine strain expressing the TRP-2 melanoma tumor Ag

The results described above demonstrate 1) the therapeutic effectiveness of virulence-attenuated recombinant Lm as a tumor vaccine, 2) antibiotic treatment does not diminish the therapeutic antitumor response following immunization with recombinant Lm, and 3) existing antilisterial immunity does not alter the vaccine vector effectiveness of Lm in a model tumor Ag system. To determine whether these observations could be extended to an endogenous tumor target, we used a recombinant strain of Lm expressing a region of mouse TRP-2 that includes the MHC class I-presented TRP-2 target peptide (aa 180–188) as a vaccine to develop antimelanoma immunity. (The development and characterization of this recombinant Lm strain is detailed elsewhere.[4])

Immunization with the TRP-2-expressing strain of Lm (Lm-TRP-2) leads to the stimulation of TRP-2-specific responses, and in three independent experiments the TRP-2 response ranges from 46 to 77 IFN-γ-secreting cells per 100,000 immune cells. The Lm-TRP-2 strain also expresses a LCMV NP determinant and following immunization, the NP response ranges from 80 to 115 IFN-γ-secreting cells per 100,000 immune cells. Consistent with previous finding,[4] these results show that the Lm expressing TRP-2 stimulates the development of TRP-2-specific $CD8^+$ T cells.

To determine whether the TRP-2-specific cells recognize melanoma tumor cells, ELISPOT assays were performed with TRP-2-specific effector cells and B16-MO5 melanoma cells as the target. To derive TRP-2, LCMV NP, SIINFEKL, or gp100 (another endogenous melanoma tumor Ag (28, 29))-specific effector cells for these experiments, mice were immunized with either the TRP-2-expressing or OVA-expressing strains of Lm or a gp100-expressing plasmid DNA vaccination construct and 10 days later immune spleen cells were independently stimulated by coculture with TRP-2-, LCMV NP-, SIINFEKL-, or gp100 peptide-pulsed normal spleen cells as APC. The cells were cultured for 6 days and the stimulation-culture cycle was repeated. The cells were collected 6 days after the initiation of the second culture cycle and used as indicator cells in the ELISPOT assays. Because the melanoma cells also express OVA, responses to the MHC class I-presented SIINFEKL peptide target should also be evident. We found that the TRP-2-specific effector cells secrete IFN-γ following interaction with melanoma tumor cells and the efficiency of recognition is similar to that seen for SIINFEKL- or gp100-specific effector cells, with ~90 spots detected per 125 input effector cells. LCMV NP-specific effector cells do not secrete IFN-γ following interaction with melanoma cells. These results demonstrate that the TRP-2-specific cells as initially stimulated in Lm-TRP-2-immunized animals possess effector function (secrete IFN-γ) following coculture with melanoma tumor cells.

To determine whether TRP-2-specific cells possess antitumor activity in vivo, C57BL/6 mice were immunized with the Lm-TRP-2 strain, spleen cells obtained and stimulated by coculture with TRP-2 peptide-pulsed irradiated APC for 6 days as described previously. After the second stimulation cycle, the recovered cells were adoptively transferred at 8×10^6/recipients into animals that had been injected with melanoma cells 5 days earlier (Table IV). Fourteen days after T cell transfer, the animals were killed and numbers of pulmonary metastases were enumerated. The lungs of the untreated control animals contained >250 pulmonary metastases, while recipients of TRP-2-specific effector cells contained from 0 to 11 (mean, 3 ± 3) pulmonary metastases. (De novo responses do not occur as a result of in vitro stimulation as recipients of naive cells that have been stimulated for 2 cycles in culture with the TRP-2 target peptide show no reduction in the level of tumor burden (data not shown).) Recipients of "positive" control SIINFEKL-specific cells also contained markedly reduced numbers of pulmonary metastases (mean, 29 ± 26). This difference in therapeutic efficacy could be due to heterogenous expression of OVA on the B16-MO5 cells, while the endogenous TRP-2 Ag has a more homogenous expression. Recipients of negative control LCMV NP-specific effector cells show no reduction in the level of tumor burden. These results show that TRP-2-specific effector cells can mediate regression of established pulmonary metastases and that the regression response is peptide specific.

To determine whether immunization with Lm-TRP-2 results in the stimulation of long-lived or memory antitumor protection, C57BL/6 mice were initially immunized with the Lm-TRP-2 strain, then 3 wk later challenged with B16 melanoma tumor cells. Twenty days later, the lungs were removed and the numbers of metastases were enumerated. Consistent with earlier findings,[4] we found that the numbers of pulmonary metastases were markedly reduced in the Lm-TRP-2-immunized group compared with the naive tumor-bearing group (data not shown). To determine whether the Lm-TRP-2 vaccine strain could be used successfully in a therapeutic setting, mice were injected with B16-MO5 melanoma cells and then 5 days later immunized with the Lm-TRP-2 strain. As a positive control, a group of tumor-bearing mice was immunized with an OVA-expressing strain of Lm. The data presented in Fig. 2 show that the group immunized with the Lm-TRP-2 strain

Table IV. *TRP-2-specific effector cells mediate regression of established pulmonary metastases*

Specificity of the Adoptively Transferred Effector Cells[a]	No. of Lung Metastases[b]	Mean[c]
No effector cells	All >250	
SIINFEKL	4,7,7,9,33,54,55,65	29 ± 26
TRP-2	0,0,0,1,1,2,2,2,3,4,4,11	3 ± 3
LCMV NP	All >250	

[a] After two cycles of peptide-specific stimulation in vitro, SIINFEKL-, TRP-2-, or LCMV NP peptide-specific cells were adoptively transferred at 8×10^6 viable cells/recipient into 5-day tumor-bearing animals.

[b] Fourteen days after T cell transfer, the lungs were removed and the levels of tumor burden were determined for each individual animal in the group.

[c] Mean ± 1 SD.

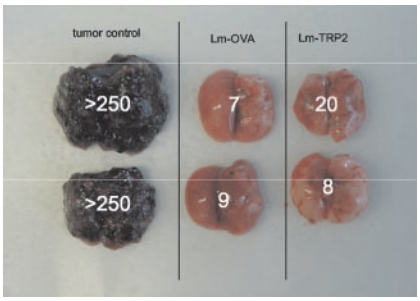

FIGURE 2. Recombinant Lm-expressing TRP-2 stimulates therapeutic antitumor responses. C57BL/6 mice were injected iv with 7×10^5 OVA-expressing B16 melanoma cells and 5 days later were immunized with either ~0.1 LD_{50} of the Lm-TRP-2 strain or the Lm-OVA strain (positive control). Nineteen days after the tumor injection, the lungs were removed and the levels of tumor burden were determined. The numbers of pulmonary metastases are superimposed on each lung sample.

have limited numbers of lung metastases, with the reduction in tumor burden similar in magnitude to the positive control group (animals immunized with the OVA-expressing strain of Lm). These lung data shown are representative of results obtained in identical experiments, with numbers of metastases enumerated to be 3, 5, 20, and 53 for the Lm-TRP-2-immunized animals. These results clearly support the therapeutic potential of the TRP-2-expressing strain of Lm and that the TRP-2-specific effector cells stimulated following immunization are clinically functional.

Existing antilisterial immunity and stimulation of antitumor responses following immunization with the TRP-2-expressing Lm strain

To determine whether immunization with the Lm-TRP-2 vaccine strain could stimulate therapeutic antitumor responses in Lm (antivector)-immune animals, C57BL/6 mice were immunized with Wt Lm and then 28 days later injected with melanoma cells. Five days later, these animals were immunized with the Lm-TRP-2 strain. Nineteen days after tumor challenge (14 days after secondary immunization with Lm-TRP-2), the animals were euthanized, lungs were removed, and the numbers of pulmonary metastases were enumerated. The positive control group included tumor-bearing mice given a therapeutic primary immunization with the Lm-TRP-2 strain, and the negative control group included Lm (vector-immune) tumor-bearing animals given a secondary injection with Lm. The therapeutic effectiveness of the Lm-TRP-2 vaccine strain was fully retained in the presence of existing antilisterial immunity (Fig. 3 and Table V). The level of the tumor burden in the antilisterial immune tumor-bearing animals given a secondary immunization with the Lm TRP-2 strain (mean, 7 ± 12 tumor nodules) is similar in magnitude to the level of tumor burden in tumor-bearing animals given a therapeutic primary injection with Lm-TRP-2 (mean, 5 ± 6 tumor nodules). Thus, even though Lm is rapidly cleared following the secondary injection because of the existing antilisterial response, the immunologic signals required to prime/stimulate "self"-TRP-2-reactive cells are not altered and these TRP-2-reactive cells are able to traffic to the tumor site and mediate tumor regression. It can be again noted that nonspecific events resulting from the Lm injection do not contribute to tumor eradication, as the level of the tumor burden in Lm-immune tumor-bearing animals given a secondary injection with Lm is similar in magnitude to the normal tumor-bearing control group.

Discussion

Vaccine strategies against cancer are based upon the requirement that a sufficient stimulus is delivered by the vaccine such that a

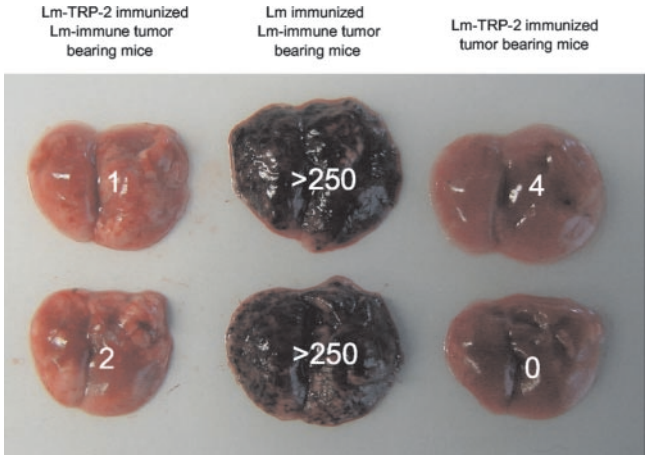

FIGURE 3. Therapeutic TRP-2-specific antitumor responses are stimulated in animals with existing antilisterial immunity. C57BL/6 mice were immunized with ~800 CFU of the parental Lm vector strain. Twenty-three days later, animals were infused with B16 melanoma cells and then immunized 5 days later with Lm-TRP-2 strain (Lm-TRP-2-immunized Lm-immune tumor-bearing mice). Controls include the Lm-immunized Lm-immune tumor-bearing mice and Lm-TRP-2 immunized tumor-bearing animals. For all groups, 19 days after tumor cell infusion, the lungs were removed and the levels of tumor burden were enumerated. The numbers of pulmonary metastases are superimposed on each lung sample.

tumor-specific response can be initiated and the development of tumor-specific effector cells that will interact with the tumor cell, leading to its destruction. In contrast to prophylactic bacterial vaccines where the immunogen is an exogenous bacterial product, the stimulation of an immune response to a tumor is problematic in that in most cases the response is specific for a tumor-associated self-Ag; most of the proteins or Ags expressed by tumors can be found elsewhere in the host. Thus, an additional requirement of a cancer vaccine is the need to provide a sufficient stimulus to overcome regulatory networks that may be in place to control or limit anti-self-directed responses. We have made the observation in a mouse melanoma model that when Lm-expressing TRP-2 is used as the vaccine vector to deliver the "self-tumor Ag" TRP-2, $CD8^+$ T cells develop that are TRP-2 specific and are therapeutic against established lung metastases. The finding that a response to self-tumor Ag can be initiated using Lm as the Ag delivery vector coupled with the observation that the stimulated cells are therapeutic is an important and necessary first step for the continued development of Lm as a vaccine vector.

For successful clinical application of Lm as a recombinant vaccine, various safety concerns need to be addressed. Virulence-attenuated mutant strains will most likely be used to minimize the potential infectious nature of the pathogen. Although reduced in virulence, attenuated strains of Lm maintain the capacity to stimulate protective antilisterial immunity, even at doses orders of magnitude below the LD_{50} (30). Furthermore, attenuated strains have shown efficacy in stimulating protective antilisterial immunity in immunocompromised hosts such as in IFN-γ knockout mice (31). In the current study, although virulence is reduced for the attenuated strains, peptide-specific $CD8^+$ effector cells develop and are functional in a therapeutic melanoma model setting, providing further support for the vaccine potential of attenuated strains of Lm. Recently, normal volunteers given an oral dose of an attenuated strain *L. monocytogenes* (defective in cell-cell spread) in a Phase I clinical trial showed that Lm was well tolerated with no untoward side effects observed (32).

The systemic use of a live attenuated Lm vaccine can be easily controlled (if required) with antibiotics. Previous reports show that

Table V. *Existing antilisterial immunity is not a barrier for utilization of Lm as a therapeutic recombinant vaccine*

Treatment[a]	No. of Lung Metastases[b]	Mean[c]
Tumor cell control	All >250 ($n = 9$)	
Melanoma cells, Lm-TRP-2	0,0,2,2,3,3,8,9,19	5 ± 6*
Wt Lm, melanoma cells, Lm-TRP-2	0,0,1,2,4,5,6,8,39	7 ± 12*
Wt Lm, melanoma cells, Wt Lm	All >250 ($n = 9$)	

[a] C57BL/6 mice were immunized with ~800 CFU of the parental vector strain of Lm (Wt Lm) as indicated. Twenty-three days later, animals were infused with B16 melanoma cells and then immunized 5 days later with ~8000 CFU of either the Lm-TRP-2 strain or Wt Lm as indicated.

[b] Nineteen days after tumor cell infusion, the numbers of lung metastases for each animal in the group were enumerated.

[c] Mean ± 1 SD.

*, $p < 0.001$ compared to controls.

antibiotic treatment 24–48 h following immunization with Lm does not diminish the stimulation of antilisterial effector cells (26). We have confirmed this finding and in this report show that the therapeutic anticancer vaccine potential of Lm is not altered or diminished following antibiotic treatment. The capacity to limit the infectious nature of Lm as a vaccine vector, either by virulence attenuation or antibiotic therapy, without the loss of therapeutic efficacy, strongly supports the continued development of this cancer vaccine platform.

An additional parameter for the development of recombinant Lm-based vaccines is related to observed benefits as a function of nonspecific vs specific effects, as *L. monocytogenes* is clearly a potent stimulator of inflammatory processes. Antitumor responses that are solely nonspecific in nature have been reported, as exemplified by findings showing regression of solid tumor as well as metastatic lung disease following immunization with control strains of Lm (13). The nonspecific nature of antitumor responses is supported by data demonstrating similar patterns of inflammatory cytokine release following recombinant Lm infection vs infection with Lm controls. In our studies, tumor reduction only occurred when a tumor-specific immune response developed following immunization with recombinant Lm vaccines expressing the defined Ag (Fig. 3 and Tables I, III, and V). The lack of nonspecific tumor reduction in our studies may be due in part the nature of the recombinant Lm strains used as the immunogen, the doses that were used for immunization (low vs high), or the B16 line used for the tumor model in this study.

Injection of naive mice with Lm results in uncontrolled replication of the bacteria within the first 72 h, after which time, splenic CFU begin to decline. Six days following injection, splenic CFU are essentially absent, mediated by proinflammatory $CD4^+$ T cells and protective $CD8^+$ CTL responses (4, 9). This finding is in marked contrast to the clearance of Lm from the spleen of immune animals. By 72 h following challenge, splenic CFU are typically below detection limits (33). In general, rapid immune-mediated clearance of a vector prevents its repeated use in the clinical setting. Thus, when considering Lm as a vaccine carrier, a key question is whether there is sufficient time for stimulation of a primary response to the recombinant Ag of interest, given the narrow window that the vector would be expected to persist in an environment of existing antivector immunity.

Since clearance of Lm is primarily a cell-mediated event, with neutralizing Ab playing little to no role in its clearance, repeated immunization should be possible. Our previous studies have shown this rapid clearance (short exposure time) of Lm as a consequence of existing antilisterial immunity does not preclude stimulation of recall responses to Lm-derived peptides and existing antilisterial immunity does not hinder development of $CD8^+$ T cell responses to newly expressed Ags contained as a component of the secondary immunization (27). In this report, we extend these findings to additional determinants, the SIINFEKL peptide of OVA and the self-peptide TRP-2, and confirm that priming responses and recall responses can occur independently and simultaneously. The capacity to prime a cellular immune response against a self-peptide in an environment of existing antivector immunity shows that the rapid clearance of recombinant Lm following secondary exposure does not diminish the nature or strength of the inflammatory signals that allow for stimulation of a $CD8^+$ response against the self-derived Ag. Our findings are supported by studies showing that $CD8^+$ T cells are activated and proliferate following brief (as little as 2 h) exposure to peptide plus MHC (34, 35). In further support of our findings, it has been shown that mice with existing influenza NP peptide-specific CTL (H2-K^d restricted) develop H2-K^d-restricted responses and H2-L^d-restricted responses following immunization with a recombinant vaccinia virus that codes for the influenza peptide as well as additional target CTL peptides (36). In these experiments, existing immunity is present as an anti-influenza response. The presence of existing anti-influenza CTL does not alter the initial vaccinia infection or priming to additional peptides presented by the same MHC class I-restricting element, as well priming to CTL peptide targets presented by distinct MHC class I-restricting elements. The influenza NP-specific response was enhanced following the booster injection, further demonstrating that primary $CD8^+$ effector responses and recall memory responses can occur independently and simultaneously. The antibiotic studies would further support the fact that a relatively short exposure of the host to Lm is sufficient to prime a functional cellular immune response.

Although our data as presented in Tables III and V and Fig. 3 support the premise that existing antivector immunity is not a barrier for therapeutic efficacy of Lm as a recombinant vaccine, data have been reported that are not in keeping with our original finding (37). In this study, pre-existing antilisterial immunity appeared to inhibit priming to newly expressed Lm-derived determinants as delivered following secondary infection. A possible explanation for these different results may be found in the dose of Lm used for the secondary infections. The number of Lm CFU used for the secondary injection in our studies is ~8000 CFU. In contrast, for the study that failed to detect priming within an environment of existing antilisterial immunity, an ~100,000 CFU dose of Lm was used for the secondary injection inoculum. Thus, it appears that the success of the priming event to the new determinant may be associated with the magnitude of the secondary infection. Using tetramer analysis of peptide-specific $CD8^+$ T cells following secondary Lm, it has been shown that the magnitude of the subsequent recall response is inversely related to the level of the secondary infection dose (19), thus adding some support to the notion that unknown events occurring at the higher infection doses may not be the most favorable for stimulation of immune responses, either at

the priming level or at the memory cell/recall response level. We are continuing with experiments in the melanoma tumor model to assess this phenomenon in greater detail.

A recent study has shown regression of B16F10 solid tumors expressing flu NP following immunization with a Lm strain expressing the same flu NP Ag (13). An interesting feature of this study was that repeated immunizations with the influenza-expressing strain of Lm did not appear to be of additional benefit in this model. To account for this finding, the rapid clearance of Lm in immune animals was considered as a likely mechanism that may prevent the further stimulation or expansion of flu NP-specific effector cells. This interpretation would suggest that this rapid clearance event may limit the efficacy or repeated usage of Lm as a recombinant vaccine. However, this interpretation seems to be in disconnect with the many studies that have demonstrated numerical increases in effector cell populations following secondary Lm infection (38). It is possible that these results can be explained on the basis of the use of a recombinant Lm vaccine strain in which the determinant of interest is plasmid vs genomically encoded. For the studies assessing stimulation and therapeutic benefit of flu NP-specific responses, flu NP was plasmid encoded, allowing for potential plasmid loss in vivo. For the studies presented in this report, the heterologous determinant was genomic, thus reducing the concern for in vivo loss of expression.

In summary, the data presented in this report show two important findings for the use of Lm as a cancer vaccine vector in a clinical setting. First, in a therapeutic melanoma model, the use of virulence-attenuated strains as the vaccine vector, or antibiotic therapy to limit the infection following immunization, does not diminish the capacity of recombinant Lm to stimulate antitumor responses. Second, unlike viral vectors such as vaccinia or adenovirus, repeated injections with Lm are immunoenhancing and do not appear to limit the continued development of primary CTL effector cells. Of significance and in the therapeutic model setting, the priming response evident following immunization can be developed against a self-derived determinant. These findings are in support of the continued evaluation of Lm as a vaccine delivery vector.

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