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Antiviral Cytotoxic T-Cell Memory by Vaccination with Recombinant *Listeria monocytogenes*

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Listeria monocytogenes **is a facultative intracellular bacterium that is able to escape phagocytic vesicles and replicate in the cytoplasm of infected cells. As with viral vectors, this intracytoplasmic life cycle provides a means for introducing foreign proteins into the major histocompatibility complex class I pathway of antigen presentation. Using recombinant *L. monocytogenes* (rLM) strains expressing the full-length nucleoprotein (NP) or a single cytotoxic T-lymphocyte (CTL) epitope from lymphocytic choriomeningitis virus (LCMV), we analyzed antiviral CTL responses induced by rLM vaccination. After vaccination, rLM was cleared from the host within 7 days while inducing an LCMV-specific ex vivo $CD8^+$ effector CTL response. Virus-specific CTL memory was maintained for 6 months postvaccination, as demonstrated by vigorous secondary CTL responses after in vitro stimulation. A single immunization with rLM that expressed either the full-length NP gene or the CTL epitope alone resulted in LCMV NP-specific CTL precursor frequencies of approximately $1/10^4$ $CD8^+$ T cells. A second rLM vaccination resulted in enhanced virus-specific CTL activity and in vitro proliferation. rLM-vaccinated mice were protected against chronic viral infection by an accelerated virus-specific memory CTL response. These mice cleared infectious virus as well as viral antigen, suggesting that sterilizing immunity was achieved. In contrast to mice that received wild-type LM, rLM-vaccinated mice were protected from virally induced immunosuppression and splenic atrophy associated with chronic LCMV infection. The ability to elicit long-term cell-mediated immunity is fundamental in designing vaccines against intracellular pathogens, and these results demonstrate the efficacy of recombinant LM vaccination for inducing protective antiviral CTL memory.**

Experimental *Listeria monocytogenes* infection has been used as a model system for the study of intracellular parasitism and cell-mediated immunity (14, 18, 28, 36, 42). This gram-positive intracellular bacterium is able to infect mammalian cells and escape into the cytoplasm, where it multiplies and spreads directly from cell to cell without encountering the extracellular environment (41, 50). This ability to grow and spread intracellularly allows the organism to escape humoral defenses, and efficient clearance of the bacterial infection requires a cell-mediated immune response, including the induction of *L. monocytogenes*-specific cytotoxic $CD8^+$ T lymphocytes (CTL) (19, 20, 26, 28, 31, 44). Recent studies have shown that recombinant *L. monocytogenes* (rLM) expressing foreign proteins can induce $CD8^+$ T-cell responses to heterologous polypeptides (17, 23, 46).

To investigate the potential of rLM vaccination in preventing viral infection and disease, we have used the lymphocytic choriomeningitis virus (LCMV) model system. LCMV infection of mice is one of the best-characterized models for studying the interaction of a virus with the immune system of its natural host (34, 43, 57). LCMV infection is well characterized with respect to both antiviral immunity and T-cell memory, and it is well documented that clearance of LCMV is mediated by $CD8^+$ CTL (2, 5, 8, 9, 24, 27, 32, 33, 39, 53, 56).

Our initial studies describing rLM vaccination focused on the establishment of a genetic system for expressing LCMV proteins by rLM vaccine strains as well as demonstrating protection against chronic LCMV infection (47). The current study is the first to use limiting-dilution analysis to quantitatively compare virus-specific CTL memory induced by rLM vaccination with CTL memory induced by the natural viral infection. We have (i) compared the in vivo growth and clearance of rLM vaccine strains with that of wild-type *L. monocytogenes* (wt-LM), (ii) identified direct ex vivo virus-specific CTL responses induced by rLM vaccination, (iii) quantitated long-term antiviral CTL memory, (iv) demonstrated that rLM immunization can be successfully repeated and results in enhanced antiviral immunity, and (v) examined the mechanisms underlying the antiviral protection provided by rLM vaccination after viral challenge with immunosuppressive LCMV variants.

MATERIALS AND METHODS

Mice. BALB/cByj (*H-2*^{d}) mice were purchased from Jackson Laboratory, Bar Harbor, Maine, or bred in our colony at the University of California at Los Angeles.

Virus. The Armstrong CA 1371 strain of LCMV and variants clone 28b and clone 13 (derived from this strain) were used in this study. The isolation and phenotypic characterization of these variants have been previously described (3, 5, 38, 55). LCMV-immune mice were obtained by injecting 5- to 8-week-old mice intraperitoneally with 2×10^5 PFU of LCMV-Armstrong and used in experiments at >90 days postinfection. For protection studies, mice were challenged intravenously (i.v.) with 2×10^6 PFU of clone 28b or clone 13.

Determination of viral titers. Infectious LCMV was quantitated by plaque assay on Vero cell monolayers as previously described (5).

L. monocytogenes. rLM strains MSL223 (rLM-NP_{actA}), EJL243 (rLM-NP_{LLO}), and HSL236 (rLM-$NP_{118\text{-}126}$) were derived from parental strain 10403S (wt-LM) (10) as previously described (47). rLM-NP_{actA} and rLM-NP_{LLO} contain the

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full-length LCMV nucleoprotein (NP) gene regulated by the ActA and hemolysin (LLO) promoters, respectively. rLM-$NP_{118-126}$ expresses the immunodominant CTL epitope consisting of NP amino acids 118 to 126 [NP (118-126); flanked on each side by three amino acids] embedded within an LLO′-PhoA fusion protein. rLM-vaccinated mice were obtained by injecting 7- to 8-week-old mice i.v. with 10^3 CFU of rLM; when noted, mice were boosted i.v. with 10^4 CFU of the same rLM strain 4 weeks later.

Determination of *L. monocytogenes* CFU. *L. monocytogenes* CFU were determined by homogenization of infected tissues in sterile H_2O containing 1% Triton X-100 followed by plating of dilutions of homogenate onto brain heart infusion plates. Colonies were scored after 24 h of growth at 37°C.

CTL assay. Major histocompatibility complex class I-restricted LCMV-specific CTL activity was determined by using BALB clone 7 cells as targets in a 6-h ^{51}Cr release assay as previously described (5).

Limiting-dilution analysis. Splenocytes were cultured in RPMI supplemented with 10% fetal calf serum (HyClone) and 5×10^{-5} M β-mercaptoethanol (Sigma). Spleen cells were cultured in graded doses (12 to 24 wells per dose) with 8×10^5 syngeneic spleen cell feeders (1,200 rad) from uninfected mice and 2×10^5 syngeneic spleen stimulator splenocytes (1,200 rad) from LCMV carrier mice in 96-well flat-bottom plates. Recombinant human interleukin-2 (Cetus Corp.) was added at 50 U/ml (final concentration). After 8 days, the contents from each well were split to test CTL activity against LCMV-infected and uninfected targets in a 6-h ^{51}Cr release assay (32, 49).

Proliferation assay. Splenocytes (5×10^5 per well) in a 200-μl volume were grown in medium alone (RPMI, 10% fetal calf serum, 5×10^{-5} M β-mercaptoethanol), medium and peptide NP(118-126), or medium and 2×10^5 irradiated (1,200 rads) syngeneic stimulator splenocytes from LCMV carrier mice in 96-well flat-bottom plates. Peptide was used at a final concentration of 1 μg/ml. Cells were pulsed for 24 h with [^{3}H]thymidine (NEN) at 5 μCi/ml (final concentration). Cells were harvested with a Titertek Cell Harvester 530 and counted on a Beckman LS1800 scintillation counter.

Antisera and T-cell depletion. Monoclonal antibody (MAb) 2-43 was used for depleting $CD8^+$ T cells in vivo. Mice were given four injections of partially purified MAb 2-43 (0.3 ml i.p.) on days −2, 0, 2, and 4. This protocol resulted in approximately 95% depletion of circulating $CD8^+$ T cells as shown by fluorescence-activated cell sorting analysis. In vitro depletion of $CD8^+$ T cells was performed with MAb AD4-15 and LOW-TOX-M rabbit complement (Cedarlane, Hornby, Ontario, Canada).

Immunoperoxidase staining and histology. To stain for viral antigen, frozen tissue was cut to 6-μm sections, fixed, and stained as previously described (25). Tissues for histology were fixed in neutral-buffered formalin, and sections were stained with hematoxylin and eosin.

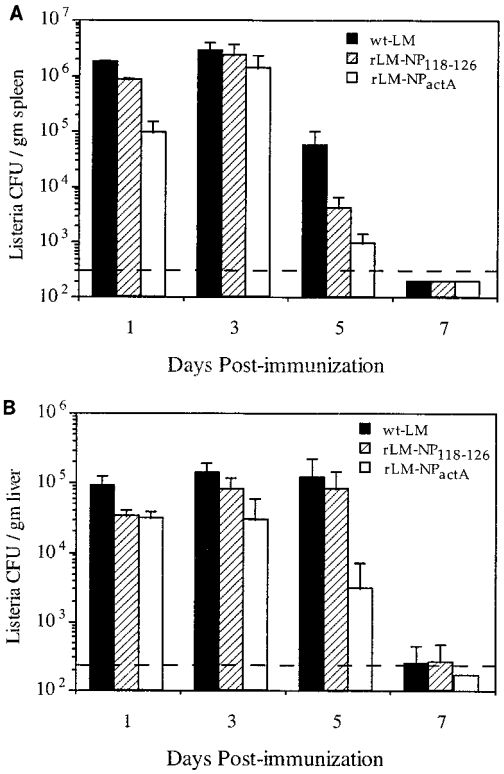

FIG. 1. Kinetics of bacterial persistence in spleens and livers of immunized mice. Adult BALB/c mice (three per group) were immunized i.v. with 10^3 CFU of wt-LM, rLM-$NP_{118-126}$, or rLM-NP_{actA}. The number of CFU and standard deviations were determined per gram of spleen (A) and liver (B) over a 7-day period.

RESULTS

In vivo persistence of rLM inoculum. We constructed rLM vaccine strains that express either the full-length LCMV NP gene or a single immunodominant CTL epitope [NP(118-126)] under the regulation of two different *L. monocytogenes* virulence gene promoters and their respective secretion signal sequences (see Materials and Methods). Expression of recombinant proteins may attenuate the growth of *L. monocytogenes*, and so rLM strains were characterized by comparing the in vivo persistence of rLM-$NP_{118-126}$ and rLM-NP_{actA} with that of wt-LM in adult BALB/c mice. As shown in Fig. 1A, wt-LM and rLM-$NP_{118-126}$ grew to approximately 10^6 CFU/g of spleen within 24 h whereas rLM-NP_{actA} inoculation initially resulted in about 10-fold-lower values at that time point. LM CFU titers in the spleen were similar among all three strains by day 3. At day 5, CFU titers began to decline, and titers of both rLM vaccine strains were 10- to 50-fold lower than wt-LM titers. By day 7, LM CFU titers had dropped below the level of detection in the spleen. Figure 1B shows that LM immunization resulted in substantial bacterial colonization of the liver on days 1 and 3. By day 5, however, rLM-NP_{actA} showed nearly 40-fold-lower CFU per gram of liver compared with the other strains. rLM-NP_{actA} CFU dropped below the level of detection by day 7, while wt-LM and rLM-$NP_{118-126}$ were almost cleared. These results show that expression of full-length LCMV NP decreases rLM persistence at later time points during infection and that both recombinant and wild-type strains are cleared by approximately 7 days postinoculation.

Primary ex vivo virus-specific CTL response after rLM vaccination. In vitro restimulation is often required to detect specific CTL responses. We determined if virus-specific effector CTL responses could be induced directly by rLM vaccination without further in vitro restimulation. After immunization of adult BALB/c mice with rLM-NP_{actA}, direct ex vivo virus-specific CTL activity was observed at both 5 and 8 days after rLM immunization and then dropped to baseline levels by 15 days (Fig. 2). In contrast, naive mice (Table 1) and mice vaccinated with wt-LM (Table 1; Fig. 2) did not show appreciable virus-specific CTL activity. We determined that the ex vivo cytolytic response was $CD8^+$ T-cell mediated by depleting $CD8^+$ cells in vitro prior to performing the CTL assay (Table 1). After depletion of $CD8^+$ T cells, the LCMV-specific CTL response was reduced to background levels. This result shows that $CD8^+$ virus-specific effector CTL were directly elicited by rLM vaccination.

Analysis of antiviral CTL memory. To determine if the antiviral CTL response generated by rLM vaccination was long-lived, we tested splenocytes of rLM-vaccinated mice for LCMV-specific CTL activity at late time points after immunization. After in vitro stimulation with virus, splenocytes from mice vaccinated with rLM-NP_{actA} demonstrated substantial antiviral CTL activity that showed no decline from 59 to 129 days postvaccination (Table 2). Likewise, rLM-$NP_{118-126}$ vaccination induced LCMV-specific CTL responses that were detected 170 days later. When relative levels of antiviral CTL memory were compared in terms of lytic units (LU), rLM-vaccinated mice had five- to sixfold-lower virus-specific CTL activity than LCMV-immune mice. wt-LM-vaccinated mice, on

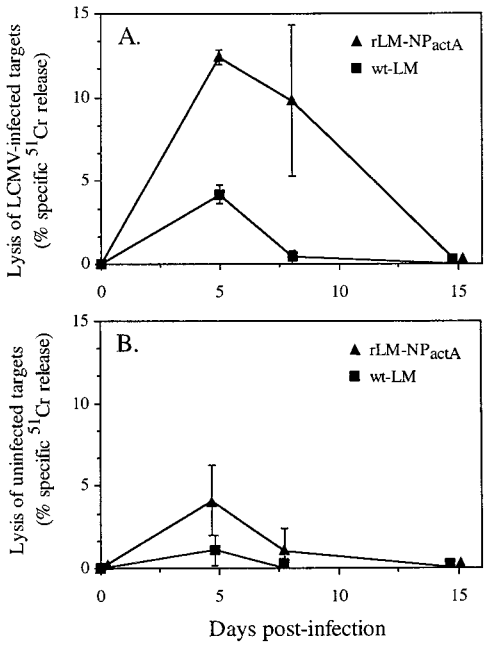

FIG. 2. Kinetics of the primary ex vivo virus-specific CTL response in rLM-vaccinated mice. Adult BALB/c mice (four to six per group) were vaccinated i.v. with 10^3 CFU of either rLM-NP_{actA} or wt-LM. At the indicated time points, splenocytes from immunized mice were assayed for LCMV-specific CTL activity. The figure represents CTL activity at an effector-to-target ratio of 100:1. (A) Lysis of LCMV-infected targets; (B) lysis of uninfected targets.

the other hand, showed essentially no LCMV-specific CTL activity (<1 LU/10^6 cells).

As a more precise and quantitative assessment of T-cell memory, we used limiting-dilution analysis to determine the number of LCMV-specific CTL precursors (CTLp) induced by rLM immunization. Table 3 shows that comparable CTLp frequencies in the spleen were obtained with both rLM-NP_{actA} and rLM-$NP_{118-126}$ vaccination (1/4.0 × 10^4 and 1/2.1 × 10^4 $CD8^+$ T cells, respectively) and that antiviral CTL memory persisted at least 170 days postvaccination. LCMV-specific CTLp frequencies in axillary and inguinal lymph nodes of rLM-vaccinated mice were approximately twofold lower than the CTLp frequency observed in the spleen (data not shown). wt-LM-immunized mice and naive mice had CTLp frequencies of less than 1/1.6 × 10^5 $CD8^+$ T cells. Virus-specific CTLp frequencies of LCMV-immune mice, on the other hand, were 10- to 20-fold higher than that observed in rLM-vaccinated mice. This result indicates that rLM vaccination induced considerable virus-specific CTL memory, but the relative magnitude of the response was lower than that achieved by acute LCMV infection.

TABLE 1. Induction of LCMV-specific $CD8^+$ effector CTL following vaccination with rLM

Group[a]	In vitro treatment[b]	% Specific ^{51}Cr release[c]					
		Infected targets			Uninfected targets		
		100:1	30:1	10:1	100:1	30:1	10:1
rLM-NP_{actA}							
1	Complement	14.5	6.4	0.7	3.7	2.0	0.8
2	Complement	17.0	8.2	0.8	2.2	1.6	0.2
1	Anti-CD8 + complement	2.3	1.7	0.1	2.6	1.6	0.2
2	Anti-CD8 + complement	2.5	0.9	0	2.8	1.6	0.2
wt-LM							
1	None	0.8	1.1	0	0	0	0
2	None	0.8	0.7	0	0	0	0
Naive							
1	None	0	0	0	0	0	0
2	None	0	0	0	0	0	0

[a] Adult BALB/c mice were vaccinated 8 days previously with 10^3 CFU of either rLM-NP_{actA} or wt-LM.
[b] Before determination of CTL activity, $CD8^+$ T cells were depleted in vitro by treatment with antibody and complement.
[c] CTL activity in the spleen was determined by measuring specific ^{51}Cr release from either uninfected or LCMV-infected targets at the indicated effector/target ratios.

TABLE 2. Effects of rLM immunization on virus-specific CTL memory

Group[a]	Days post-immunization	% Specific ^{51}Cr release[b]							LU[c]/10^6 cells
		Infected targets				Uninfected targets			
		30:1	10:1	3:1	1:1	30:1	10:1	3:1	
rLM-NP_{actA}									
2 pooled	59	73	60	28	8	0	2	2	16
1	129	68	45	20	8	8	4	3	12
2	129	84	66	32	12	9	4	2	17
rLM-$NP_{118-126}$									
1	170	68	63	40	16	1	0	0	30
2	170	57	48	24	7	0	0	0	14
3	170	71	54	29	11	1	0	0	16
wt-LM									
1	101	25	11	4	2	18	8	3	<1
2	170	3	0	0	0	1	0	0	<1
LCMV immune									
1	80	80	79	73	58	29	16	5	114
2	92	82	83	82	51	37	16	4	72

[a] Adult BALB/c mice were immunized i.v. with 10^3 CFU of the indicated strain. The LCMV-immune mice were injected intraperitoneally with 2 × 10^5 PFU of LCMV-Armstrong. At the indicated time points, mice were euthanized and spleen cells were cultured in vitro with LCMV-infected carrier splenocytes for 5 days.
[b] CTL activity was determined by measuring specific ^{51}Cr release from either uninfected or LCMV-infected targets at the indicated E:T ratios.
[c] Number of effector cells required to exhibit 30% lysis of infected targets.

Boosting with rLM increases antiviral $CD8^+$ T-cell responses. It is well documented that secondary (booster) vaccinations increase humoral responses to specific antigens, but in general, little is known about the efficacy of booster vaccinations on improving $CD8^+$ CTL responses. With live vaccines in particular, one of the inherent difficulties involved with multiple vaccinations is that the immunity induced against the vector itself often limits its usefulness for revaccination. Further vaccination attempts are often hindered by vector-specific preexisting antibody that inhibits the infectivity and initial effectiveness (take) of the secondary vaccination (13). Since preexisting antibody plays a relatively minor role in immunity against *L. monocytogenes* (36), we determined if secondary rLM vaccination could boost the magnitude of the antiviral CTL response initiated by primary rLM vaccination. Secondary vaccination resulted in a substantial increase in virus-specific $CD8^+$ T-cell responses as measured by both cytotoxicity and proliferation assays (Fig. 3). After 5 days of in vitro stimulation, a >3-fold increase in LCMV-specific LU/10^6 cells in rLM-NP_{actA}-boosted mice was detected (Fig. 3A), and limiting-dilution analysis further confirmed that LCMV-specific CTLp frequencies increased from an average of 1/1.7 × 10^5 to

TABLE 3. Quantitation of virus-specific CTLp frequency after rLM vaccination

Group[a] (no. of mice used)	Days post-immunization	LCMV-specific CTLp[b]		
		Frequency/CD8$^+$ T cells (range)	Frequency/spleen cells	Total CTLp/spleen (range)
rLM-NP$_{actA}$ (4)	129–170	$1/4.0 \times 10^4$ (2.3–7.8)	$1/2.6 \times 10^5$	441 (197–783)
rLM-NP$_{118\text{-}126}$ (3)	170	$1/2.1 \times 10^4$ (1.4–3.2)	$1/1.3 \times 10^5$	916 (486–1,205)
wt-LM (2)	101–170	$<1/1.6 \times 10^5$	$<1/1.0 \times 10^6$	<100
LCMV immune (6)	100–180	$1/1.7 \times 10^3$ (1.3–2.0)	$1/1.0 \times 10^4$	9,860 (9,160–10,200)

[a] Adult BALB/c mice were immunized i.v. with 10^3 CFU of the indicated strain.
[b] Determined as previously described (32).

$1/4.6 \times 10^4$ spleen cells. Figure 3B shows that spleen cells from rLM-boosted mice have higher proliferative responses upon in vitro stimulation with either LCMV-infected cells or LCMV CTL epitope NP(118-126). The proliferative response during in vitro stimulation with LCMV could be due to a combination of CD8$^+$ and CD4$^+$ T cells, whereas the response to the CTL epitope peptide alone demonstrates CD8$^+$ T-cell-specific proliferation. These results therefore indicate that secondary rLM vaccination can boost the magnitude of virus-specific CD8$^+$ T-cell responses.

Protective antiviral immunity in rLM-vaccinated mice is due to accelerated CD8$^+$ CTL responses. CTL memory does not prevent viral reinfection per se, because virus-specific CTL responses must first be stimulated by presentation of CTL peptides on virally infected cells. Once reinfection has occurred, however, antiviral CTL responses often play a major role in viral clearance and prevention or reduction of disease. We have previously shown that rLM-vaccinated mice are protected against viral challenge with immunosuppressive LCMV variants and that protection requires CD8$^+$ T cells (47). However, the nature of this protective immunity is not well understood. Whether the level of initial viral infection and replication (i.e., viral take) was the same in naive and rLM-vaccinated mice, or whether vaccinated mice were protected by accelerated antiviral CTL responses, was an unresolved question. To address this, we challenged mice with LCMV clone 28b, a variant strain that causes chronic, immunosuppressive infections in naive mice (3, 5, 38). We compared the kinetics of the ex vivo effector CTL responses of rLM- and wt-LM-vaccinated mice with that of LCMV-immune mice. In parallel, we monitored viral growth kinetics by quantitating the level of infectious virus in several tissues.

On days 3, 5, and 8 after viral challenge, splenocytes were tested for direct killing of LCMV-infected targets. rLM-vaccinated mice mounted early effector CTL responses with virus-specific ex vivo CTL activity as early as day 3 postchallenge (Fig. 4). These CTL responses continued to increase on days 5 and 8 postchallenge. In contrast, wt-LM-vaccinated mice did not initiate an LCMV-specific CTL response until day 5, and this relatively low CTL response was lost by day 8. Comparing these CTL responses with the respective levels of infectious virus in each group, it is apparent that the rLM-vaccinated mice which mounted higher and more rapid CTL responses were better able to control viral infection (Fig. 5). It is noteworthy that all groups of mice had comparable levels of virus in the spleen ($\sim 10^7$ PFU/g) at day 1. This represents substantial viral growth during the first day of infection, since the input level of virus in the spleen is $\sim 10^4$ PFU/g at 4 h postinfection (data not shown). The day 1 time point therefore shows that the initial rate of viral replication from 4 to 24 h postinfection was the same in both rLM- and wt-LM-vaccinated mice. By day 8, however, rLM-vaccinated mice showed between 100- and 1,000-fold-lower viral titers than wt-LM-vaccinated mice and were able to eventually clear the viral infection. wt-LM-vaccinated mice were unable to mount a protective CTL response (Fig. 4) and became chronically infected with high titers of infectious virus in the serum, spleen, and liver (Fig. 5). LCMV-immune mice mounted the most rapid ex vivo CTL response in our study; this was expected since these mice had a 10- to 20-fold-higher virus-specific CTLp frequency than did the

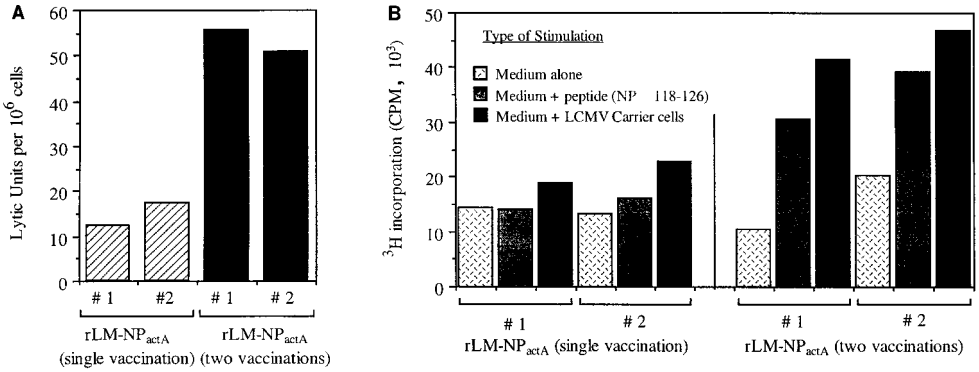

FIG. 3. Secondary vaccination with rLM enhances antiviral immunity. BALB/c mice were immunized with 10^3 CFU of rLM-NP$_{actA}$ and boosted with 10^4 CFU 4 weeks later. CTL and proliferation assays were performed with spleen cells from immunized mice between 101 and 129 days after primary or secondary vaccination. (A) LCMV-specific CTL activity was calculated as LU per 10^6 cells after 5 days of in vitro stimulation with LCMV. (B) LCMV-specific proliferative responses were determined by stimulating spleen cells with medium alone, medium plus CTL epitope peptide NP(118-126), or irradiated (1,200 rads) LCMV-infected syngeneic carrier cells. Cells were pulsed with [^{3}H]thymidine from 24 to 48 h after in vitro stimulation.

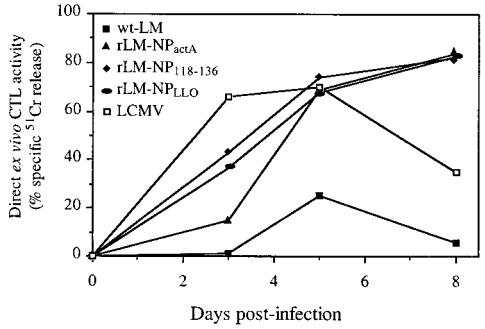

FIG. 4. rLM-vaccinated mice exhibit a rapid and enhanced LCMV-specific CTL response after viral challenge. At 50 days postvaccination, mice immunized with wt-LM, rLM-NP$_{actA}$, rLM-NP$_{LLO}$, or rLM-NP$_{118-126}$ or LCMV-immune mice were challenged with 2×10^6 PFU of LCMV clone 28b. Virus-specific CTL responses in the spleen were measured by direct ex vivo CTL assays, and each datum point represents the average of two to three mice. The percent CTL activity is shown at an effector/target ratio of 50:1, and cytotoxicity on uninfected targets at the same ratio has been subtracted.

rLM-vaccinated mice. This effector CTL response peaked between 3 and 5 days postchallenge and then declined substantially by 8 days postchallenge. LCMV-immune mice were susceptible to initial viral infection and at day 1 showed the same level of infectious virus in the spleen as both rLM- and wt-LM-vaccinated mice. However, LCMV-immune mice mounted a more effective CTL response (Fig. 4) and cleared the systemic viral infection within 3 days postchallenge (Fig. 5). This finding suggests that after viral challenge, the accelerated antiviral CTL response of LCMV-immune and rLM-vaccinated mice was the determining factor in protecting against chronic viral infection.

A successful vaccination strategy not only should protect the host against infection but ideally should provide sterilizing immunity as well. Since viral antigen may remain for some time after infectious virus has fallen below limits of detection by plaque assay, we determined the presence of viral antigen by staining for LCMV in liver sections of wt-LM- and rLM-NP$_{actA}$-vaccinated mice (4, 25, 29, 38). As shown in Fig. 6, LCMV antigen was efficiently cleared from rLM-NP$_{actA}$-vaccinated mice by 28 days postinfection, whereas wt-LM-vaccinated mice harbored high levels of viral antigen including infectious virus.

rLM-vaccinated mice are protected against virally induced splenic atrophy. We have shown that rLM vaccination induced antiviral CD8$^+$ CTL memory and protection against chronic viral infection, but can immunization with rLM protect against virally induced disease and immunopathology? We addressed this question by challenging wt-LM- and rLM-NP$_{actA}$-vaccinated mice with a chronic LCMV variant, clone 13. Like LCMV clone 28b, clone 13 is an immunosuppressive strain that causes splenic degeneration in naive mice, including the destruction of normal splenic architecture and a sharp decrease in lymphoid cellularity (5, 7, 38, 45, 55). rLM-vaccinated mice ($n = 7$) clear infectious virus from the serum (<50 PFU/ml of serum) by 8 days postinfection. In contrast, wt-LM-vaccinated mice ($n = 4$) become chronically infected, averaging 2.5×10^5 PFU/ml of serum. At 28 days after clone 13 infection, spleen sections from wt-LM- and rLM-NP$_{actA}$-immunized mice were stained with hematoxylin and eosin for morphological comparison. As illustrated in Fig. 7A, wt-LM-immunized mice exhibited deterioration of splenic architecture and notable acellularity resulting from chronic clone 13 infection. In contrast, spleens of rLM-NP$_{actA}$-vaccinated mice retained their splenic architecture, as noted by intact follicles that have normal follicular organization and dense lymphoid cellularity (Fig. 7B). Splenic degeneration was most striking when total cells per spleen were calculated. Viable cells recovered from the spleens of rLM-vaccinated mice at 8 days after clone 13 infection averaged 1×10^8 to 2×10^8 total cells per spleen, whereas wt-LM-vaccinated mice averaged less than 5×10^6 total cells per spleen, indicating >95% loss in viable cells due to the immunopathology associated with chronic LCMV clone 13 infection. These results show that the antiviral CTL memory induced by rLM vaccination not only provides sterilizing immunity (Fig. 6) but protects against virally induced immunopathology and disease.

DISCUSSION

Our results show that rLM vaccination induces long-term, virus-specific CTL memory. We previously showed that rLM vaccination provides protection against chronic LCMV infection (47); in the current study, we quantitated the level of

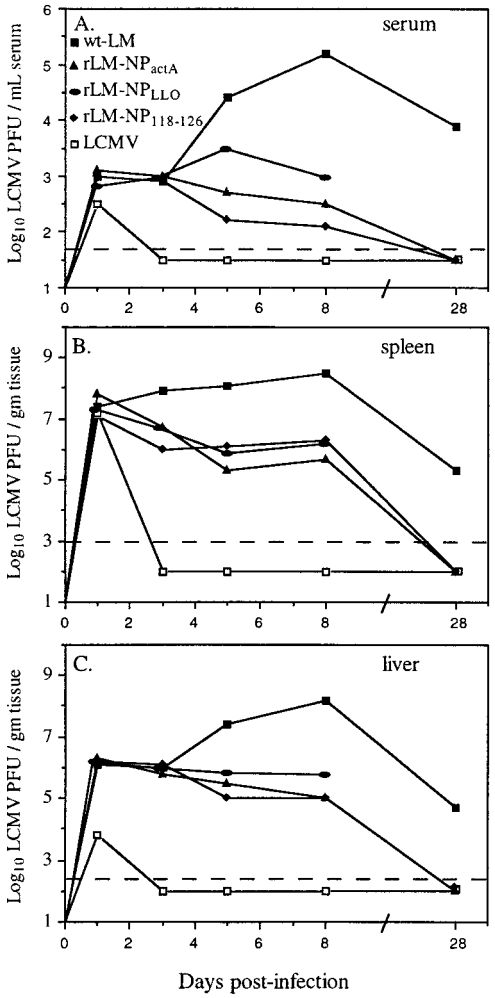

FIG. 5. Level of infectious virus after challenge with LCMV clone 28b. At 50 days postvaccination, mice immunized with wt-LM, rLM-NP$_{actA}$, rLM-NP$_{LLO}$ or rLM-NP$_{118-126}$ or LCMV-immune mice were challenged with 2×10^6 PFU of LCMV clone 28b. Organ titers represent the averages of two to four mice per group, and although error bars have been omitted to improve clarity of the graph, the range for each time point was <0.5 log$_{10}$ PFU/g of tissue or ml of serum. rLM-NP$_{LLO}$ virus titers were not determined at 28 days postinfection.

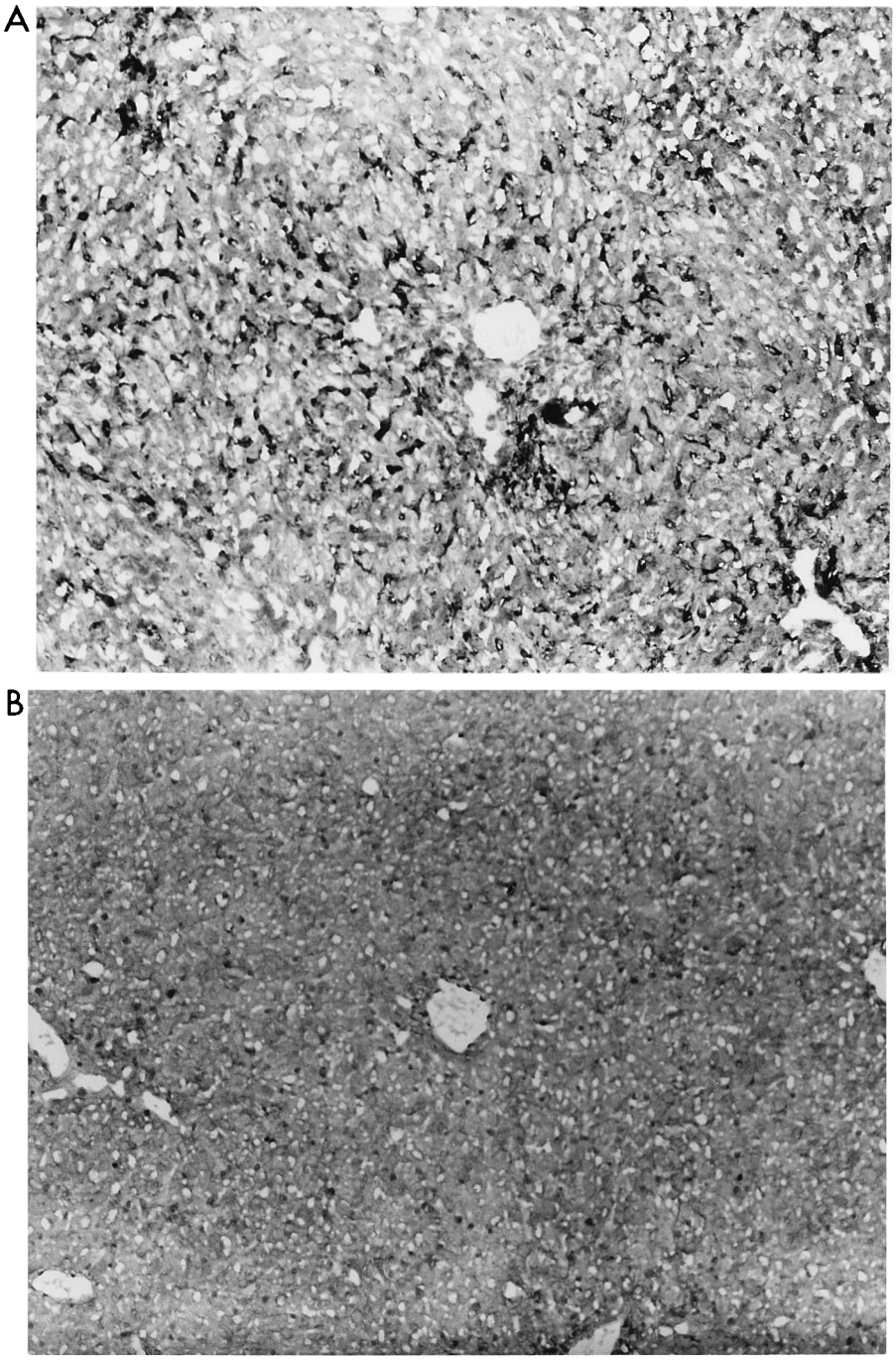

FIG. 6. Clearance of viral antigen from rLM-vaccinated mice. (A) Distribution of viral antigen in the liver of wt-LM-vaccinated mice at 28 days after viral challenge; (B) clearance of infectious virus in rLM-NP_{actA} vaccinated mice, with no detectable viral antigen remaining at 28 days after clone 28b challenge. The levels of infectious virus were 4.8 $\log_{10}$ PFU/g (A) and below detection (<2.4 $\log_{10}$ PFU/g) (B). Magnification, ×100.

virus-specific CTL memory induced by rLM vaccination and showed that it was maintained for at least 170 days postvaccination. We compared the in vivo growth and persistence of rLM strains with that of wt-LM and found that expression of full-length LCMV NP decreased bacterial persistence. However, both rLM and wt-LM were cleared within about 1 week postvaccination. This short growth period elicited virus-specific effector CTL from rLM-vaccinated mice without requiring further in vitro restimulation. The magnitude of antiviral CTL memory generated by rLM vaccination was measured by limiting-dilution analysis, and the duration of CTL memory lasted at least 6 months. The level of CTL memory acquired after a single rLM vaccination could be boosted further by a secondary rLM vaccination. After rLM-vaccinated mice were challenged with chronic LCMV variants, protection was mediated by rapid and enhanced CTL responses that eventually led to resolution of viral infection and sterilizing immunity. rLM-vaccinated mice were spared from immunopathological dis-

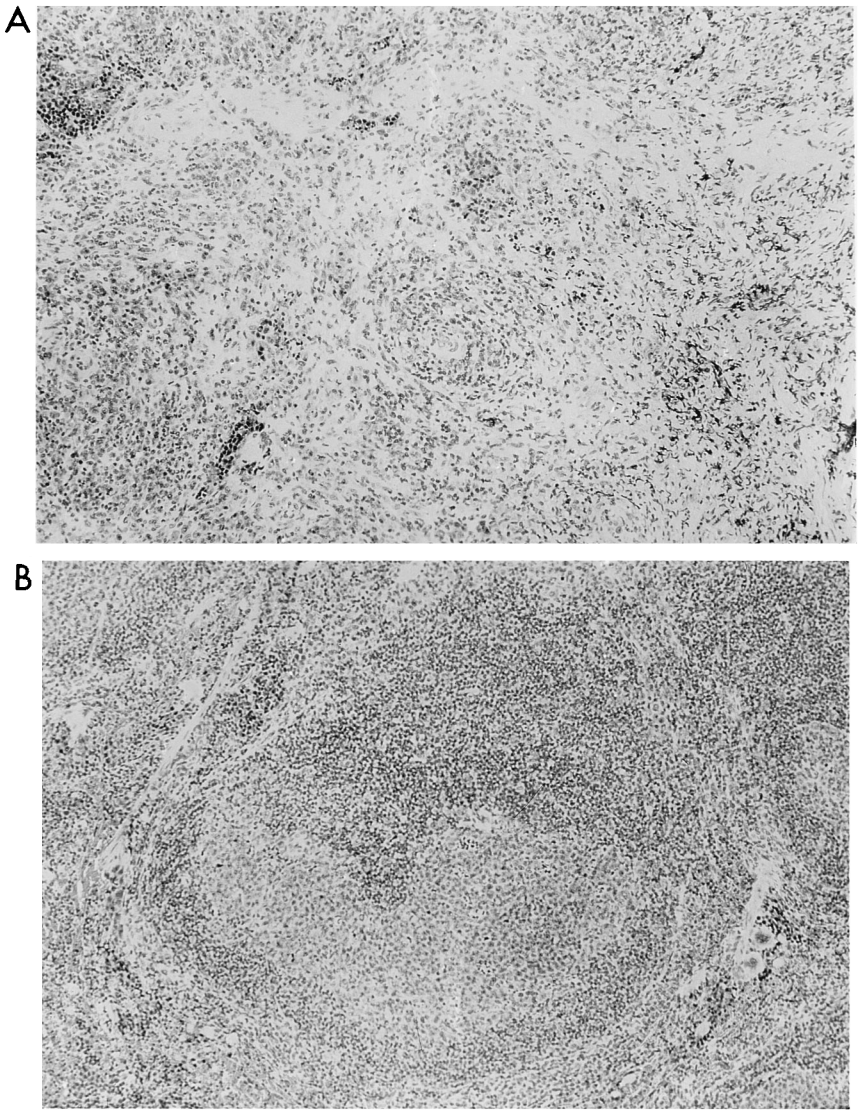

FIG. 7. Protection of rLM-vaccinated mice against virally induced splenic atrophy. BALB/c mice vaccinated and boosted 4 weeks previously with wt-LM or rLM-NP_{actA} were challenged with 2×10^6 PFU of LCMV clone 13. Hematoxylin-and-eosin staining was performed on spleen samples collected at 28 days after viral challenge. (A) Spleens of wt-LM-immunized mice display typical acellularity, disorganization, and loss of follicular structure. (B) Spleens of rLM-NP_{actA}-vaccinated mice have highly active follicles that maintain normal follicular structure and cellularity. Magnification, ×400.

ease associated with chronic LCMV infection, whereas wt-LM-vaccinated mice were susceptible to chronic infection and showed greatly degenerated splenic morphology.

A concern with rLM vaccination is the degree of bacterial persistence in the immunized host. Studies have shown that within 10 min after an i.v. injection of LM into a mouse, approximately 90% of the inoculum can be recovered from the liver and 10 to 20% of the inoculum can be recovered from the spleen (11, 12, 14). We therefore determined the rate of rLM clearance in these two organs after i.v. vaccination. In our studies, both wt-LM and rLM strains rLM-$NP_{118\text{-}126}$ and rLM-NP_{actA} readily colonized the spleen and liver for several days but were then efficiently cleared from the host within about 1 week (Fig. 1). This growth period allowed priming of naive mice with an LCMV-specific effector CTL response and induced long-term antiviral CTL memory (Table 1 to 3).

We quantitated the relative strength and duration of antiviral CTL memory induced by rLM vaccination by using limiting-dilution analysis. Our results showed that rLM-vaccinated mice maintained high CTLp frequencies ranging from $1/2.1 \times 10^4$ to $1/4.0 \times 10^4$ $CD8^+$ T cells for more than 5 to 6 months following a single vaccination (Table 3). Expression of a single virus-specific CTL epitope (rLM-$NP_{118\text{-}126}$) was as efficient as expression of the full-length viral nucleoprotein (rLM-NP_{actA} and rLM-NP_{LLO}) in eliciting antiviral CTL memory and protecting against chronic viral infection (Table 3; Fig. 5).

An important parameter of antiviral CTL immunity provided by rLM vaccination is the relative level of CTL memory generated. For example, how does the frequency of LCMV-specific CTLp induced by these recombinant bacterial vaccines compare with that induced by a viral vaccine such as recombinant vaccinia virus (rVV)? rVV ranks among the best viral vectors used to elicit CTL responses and has been well characterized (6, 21, 37, 40). Mice vaccinated with rVV expressing a nearly full-length LCMV NP gene (VVΔSTY) which contains the same CTL epitope as rLM-NP_{actA} had CTLp fre-

quencies of approximately 1/7.5 $\times$ 10^4 $CD8^+$ T cells (31a). Since rLM-NP_{actA} vaccination elicited a CTLp frequency of 1/4.0 $\times$ 10^4 $CD8^+$ T cells (Table 3), this finding indicates that rLM vaccination is able to induce CTL memory comparable to that obtained from a recombinant viral vector. In contrast, natural LCMV infection induced about 10- to 20-fold higher CTLp frequencies than either rLM or rVV vaccination. The majority (>95%) of the CTL response to LCMV in BALB/c mice is specific for LCMV-$NP_{118-126}$ (54), and so the acute LCMV infection did not provide an increased number of CTL epitopes. Other possible reasons accounting for the higher CTLp frequency observed in LCMV-immune mice may include differences in antigenic load as well as the possibility of antigenic competition. LCMV typically causes a systemic infection with copious amounts of viral antigen being expressed and presented by infected cells (30). In this regard, LCMV infection may stimulate a stronger immune response because more cells are potentially infected. Antigenic competition probably plays a very minor role in the immune response to LCMV infection, since LCMV is a small RNA virus encoding only four proteins. However, antigenic competition may play a more important role in the immune response to foreign antigens expressed in rVV, which has >100 proteins, and rLM, which encodes several thousand proteins.

The antiviral cell-mediated immunity of rLM-vaccinated mice was substantially increased by secondary vaccination. This result implies that rLM vaccination may be used more than once to either augment a recall response (Fig. 3) or possibly initiate new immune responses to novel antigens. This may be an advantage over other recombinant vectors such as rVV. Neutralizing antibody to vaccinia virus can persist for more than 15 years, and long-term immunity against vaccinia virus limits the effectiveness of rVV used after a primary vaccination (13). Cell-mediated immunity to the *L. monocytogenes* vector itself would sharply limit its in vivo growth during secondary vaccinations, but if an initial infection can take, then a cell-mediated immune response to new antigens is possible. This observation suggests that rLM may be a useful vector for studying the efficacy of multiple vaccinations on increasing specific $CD8^+$ CTL responses. The preliminary results of our study are promising, but a more quantitative assessment of revaccination strategies should be performed to determine the overall effectiveness of rLM vaccination in *L. monocytogenes*-immune hosts.

After viral challenge, LCMV-immune and rLM-vaccinated mice mounted rapid antiviral CTL responses that conferred protection against chronic infection. In contrast, wt-LM-vaccinated mice mounted a CTL response and were susceptible to persistent viral infection. LCMV-immune mice cleared virus from the serum and tissues by 3 days postinfection as a result of a potent and rapid CTL response that declined sharply by day 8 postchallenge. This result shows that the effector phase of the CTL response is transient and decreases once the viral infection has been resolved. rLM-vaccinated mice had intermediate levels of CTL activity at day 3 (Fig. 4) which restricted viral growth (Fig. 5), but since viral replication occurred for a longer period than in the clone 28b-challenged LCMV-immune group, rLM-vaccinated mice took longer to clear this proportionally larger viral load. This may explain why rLM-vaccinated mice sustained a higher level of effector CTL activity for an extended period of time and were still able to clear the viral infection and achieve sterilizing immunity (Fig. 6).

The rLM strains used in this study were specifically designed to secrete recombinant antigens into the host cell cytoplasm for the induction of an antiviral CTL response. The LCMV model system is best suited for the study of vaccines that induce $CD8^+$ CTL responses, since $CD8^+$ immunity is required for resolving this viral infection (2, 5, 8, 9, 24, 27, 32, 33, 39, 53, 56). We were, however, unable to detect a specific antibody response against LCMV NP by enzyme-linked immunosorbent assay (data not shown). Future studies should be designed to optimize a rLM vaccination strategy for the induction of specific antibody responses in a model system in which humoral immunity plays a prominent role in protection.

A number of bacterial vaccine vectors, including recombinant *Salmonella* strains, *Mycobacterium bovis* BCG, and *L. monocytogenes*, have been shown to induce cell-mediated immunity (15, 17, 23, 46–48). The effective priming of CTL responses by *L. monocytogenes* is most likely due to its cytoplasmic life cycle since, unlike most intracellular bacteria that reside in endocytic vesicles, LM escapes into the host cell cytoplasm, which allows more efficient loading of the major histocompatibility complex class I pathway. Another key attribute of *L. monocytogenes* is its ability to induce macrophages to produce interleukin-12, a cytokine linked to enhanced cell-mediated immunity (1, 22, 51). Interleukin-12 enhances cytolytic lymphocyte activity and stimulates gamma interferon production in vivo (16, 52). Therefore, a distinct advantage may be gained when *L. monocytogenes* is used as a vaccine vehicle, since it may simultaneously present foreign antigens in the proper (cytoplasmic) environment for induction of cell-mediated immunity as well as stimulate an advantageous TH1-type cytokine profile as a result of the adjuvancy of the bacterial vector itself. To address the safety concerns involved with a live vaccine vector, avirulent vaccine strains of LM have recently been engineered, and we are currently studying the immunogenicity of these attenuated rLM strains with respect to antiviral protection.

LM combines the advantages of both viral and bacterial vaccines. Like a viral vaccine, LM presents proteins cytoplasmically, allowing efficient loading of the major histocompatibility complex class I pathway, and induces a TH1-type immune response for strong cell-mediated immunity. Similar to other vaccine strains of bacteria, LM is amenable to genetic manipulation (with the potential to incorporate large or multiple DNA constructs), can be safely attenuated genetically, and is susceptible to antibiotics, and vaccination may be performed by several routes, including oral administration for possible induction of mucosal immunity. These advantages, as well as our results demonstrating that rLM vaccination induces long-lived antiviral CTL memory, indicate that LM has potential as a live vaccine vector for inducing protective cell-mediated immunity.

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