

Directed antigen delivery as a vaccine strategy for an intracellular bacterial pathogen

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We have developed a vaccine strategy for generating an attenuated strain of an intracellular bacterial pathogen that, after uptake by professional antigen-presenting cells, does not replicate intracellularly and is readily killed. However, after degradation of the vaccine strain within the phagolysosome, target antigens are released into the cytosol for endogenous processing and presentation for stimulation of CD8⁺ effector T cells. Applying this strategy to the model intracellular pathogen *Listeria monocytogenes*, we show that an intracellular replication-deficient vaccine strain is cleared rapidly in normal and immunocompromised animals, yet antigen-specific CD8⁺ effector T cells are stimulated after immunization. Furthermore, animals immunized with the intracellular replication-deficient vaccine strain are resistant to lethal challenge with a virulent WT strain of *L. monocytogenes*. These studies suggest a general strategy for developing safe and effective, attenuated intracellular replication-deficient vaccine strains for stimulation of protective immune responses against intracellular bacterial pathogens.

CD8⁺ T cell | replication-deficient | *Listeria monocytogenes*

Bacterial pathogens that replicate intracellularly, such as *Mycobacterium tuberculosis*, *Salmonella enterica* serovar Typhi, *Listeria monocytogenes* (*Lm*), and *Chlamydia trachomatis*, cause significant morbidity and mortality worldwide. One successful vaccine strategy against intracellular pathogens is immunization with live-attenuated strains that retain the ability to replicate within host cells, allowing for processing and presentation of endogenous pathogen-derived antigens. Nonetheless, this approach is hindered by difficulties in generating attenuated variants that effectively stimulate protective immune responses yet do not cause disease, especially in immunocompromised individuals. Additional vaccine strategies against intracellular pathogens include intracellular replication-deficient variants that do not produce infectious progeny after immunization yet stimulate protective immunity (1) or component vaccines where defined antigenic determinants are engineered for delivery to appropriate antigen-presenting cells (APCs) (2). An advantage of component vaccines is enhanced patient safety; a disadvantage is that stimulation of responses against multiple determinants may be required, and the identities of protective antigens for many pathogens are unknown.

For many intracellular pathogens, CD8⁺ T cells play a critical role in protective immunity. Priming of CD8⁺ effector T cells occurs most efficiently after antigen delivery into the cytosol of professional APCs [i.e., dendritic cells (DCs)] for processing and presentation by the endogenous MHC Class I pathway (3). We have reported a cytolysin-mediated antigen-delivery strategy whereby nonpathogenic *Escherichia coli* engineered to express a cytoplasmic-localized pore-forming cytolysin, and a model T cell antigen can stimulate CD8⁺ effector T cells *in vivo* (4–6). Although this antigen-delivery strategy has proven successful in priming CD8⁺ T cells against ovalbumin (OVA) as a model antigen, stimulation of protective immunity against an infectious agent has not been demonstrated.

Lm is a facultative intracellular bacterial pathogen of humans and animals (7) and has been extensively studied by using the

murine infection model. Many facets of pathogenesis and protective immunity to *Lm* are well characterized. After uptake by APCs, *Lm* escapes from the phagocytic vacuole into the cytosol, an event facilitated by a secreted pore-forming cytolysin, listeriolysin O (LLO) (8). After escape from the vacuole, *Lm* replicates freely within the cytosol, allowing bacterial products to access the endogenous MHC Class I presentation pathway. Consistent with this cytosolic replication niche, protective antilisterial immunity depends on *Lm*-specific CD8⁺ effector T cells, with antibody playing no significant role (9). Stimulation of protective antilisterial immunity readily occurs after immunization with viable virulent *Lm* (10) but not nonviable (heat-killed) *Lm* (11, 12). In addition, injection with heat-killed *Lm* also does not enhance existing antilisterial immunity (13). Combining the benefits of cytolysin-mediated delivery of target antigens with an attenuated variant of a pathogen that expresses an array of native protective antigens, we have constructed a *Lm* vaccine strain that, after uptake by APCs, remains localized within phagocytic vacuoles and does not replicate intracellularly, yet facilitates delivery of multiple listerial proteins into the cytosolic MHC Class I pathway. In this report, we show that immunization with the intracellular replication-deficient *Lm* strain stimulates *Lm*-specific CD8⁺ effector T cells that mediate protective immunity. This approach of directed antigen delivery suggests a general strategy for developing safe, intracellular replication-deficient vaccine strains for stimulating protective cellular immunity to intracellular bacterial pathogens.

Results

Construction of a cytoLLO *Lm* Strain. The *hly* gene encoding LLO but lacking its N-terminal secretion signal sequence (cytoLLO), was cloned into a *Lm* site-specific integration vector (14), maintaining the native *hly* transcriptional and translational control elements (Fig. 1A). This cytoLLO expression vector was integrated into the chromosome of *Lm* strain DH-L377. DH-L377 is an avirulent *Lm* strain containing a deletion of the *hly* gene (Δ LLO). DH-L377 also contains a mutation in the PrfA transcriptional activator, resulting in constitutive overexpression of the integrated cytoLLO construct and all other PrfA-controlled virulence genes (15, 16). The resulting cytoLLO *Lm* strain (DH-L1233) allows for elevated levels of LLO to be produced in the bacterial cytoplasm. The presence of cytoplasmic LLO did not alter the replication of cytoLLO *Lm* in broth culture compared with WT *Lm* (data not shown). Western blot analyses showed that cytoplasmic-localized LLO was produced by the cytoLLO *Lm* strain, with no LLO protein detected in the supernatant of cytoLLO *Lm* bacterial cultures (Fig. 1B). In contrast, LLO protein was found in the supernatant of WT cultures. Retention of cytoLLO pore-forming activity was confirmed by

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Abbreviations: APC, antigen-presenting cell; CFU, colony-forming units; BM-MAC, bone-marrow-derived macrophage; DC, dendritic cell; LLO, listeriolysin O; cytoLLO, cytoplasmic LLO; *Lm*, *Listeria monocytogenes*; OVA, ovalbumin; SCID, severe combined immunodeficient.

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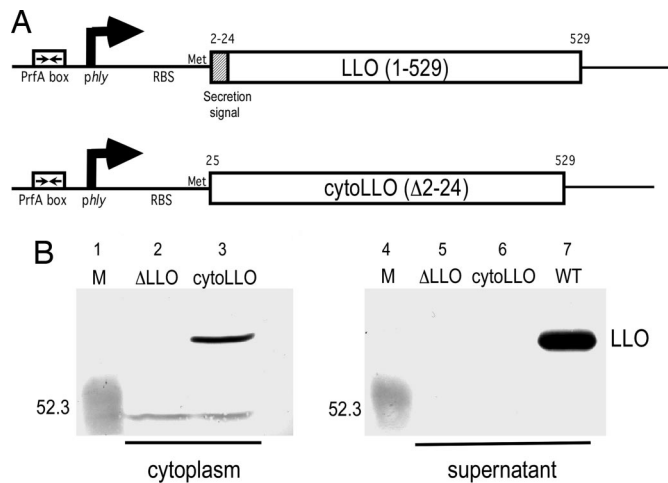


Fig. 1. Generation of cytoLLO *Lm*. (A) Schematic of LLO expression constructs. LLO is a 529-aa polypeptide containing a secretion signal sequence at the N terminus. Transcription of the *hly* gene encoding LLO initiates from the PrfA-regulated *phly* promoter. The cytoLLO expression construct contains a 23-aa deletion of the secretion signal sequence and maintains the native transcriptional and translational control elements. The locations of the PrfA-binding site (PrfA box), *hly* promoter (*phly*), ribosome-binding site (RBS), initiating methionine (Met), and secretion signal are indicated. (B) Cytoplasmic fractions or secreted proteins present in culture supernatants of WT SLCC-5764 (WT), LLO-negative DH-L377 (Δ LLO), and the cytoLLO DH-L1233 (cytoLLO) *Lm* strains were analyzed by Western blot with a monoclonal anti-LLO antibody. Lanes 1 and 4 (M) show the mobility of a mass marker with size given in kDa.

using hemolytic activity assays after mechanical lysis of cytoLLO-expressing bacteria (data not shown).

CytoLLO *Lm* Do Not Replicate Intracellularly *In Vitro* and Are Cleared Rapidly *In Vivo*. A critical aspect for vaccine safety is the absence of bacterial replication within the cytosol. We examined the ability of the cytoLLO *Lm* strain to grow within mammalian host cells. In murine bone-marrow-derived macrophages (BM-MAC) the cytoLLO *Lm* strain behaved identically to Δ LLO *Lm*; both strains failed to escape the phagosome and did not replicate within BM-MAC (Fig. 2A). Consistent with the ability of primary macrophages to kill phagocytosed bacteria retained in the phagosome, the numbers of intracellular Δ LLO and cytoLLO bacteria declined ≈ 10 -fold over the assay period. Microscopic examination confirmed no increase in intracellular Δ LLO or cytoLLO bacteria (Fig. 2C and D). In contrast, WT *Lm* escaped primary phagosomes and grew intracellularly ≈ 10 -fold (Fig. 2A and B). In addition, cytoLLO *Lm* did not replicate within murine hepatocytes or bone-marrow-derived DCs (see Fig. 5, which is published as supporting information on the PNAS web site).

To determine whether cytoLLO *Lm* replicates *in vivo*, groups of BALB/c or severe combined immunodeficient (SCID) mice were injected with WT [≈ 600 colony-forming units (CFU)] or cytoLLO ($\approx 2 \times 10^8$ CFU) *Lm*, and the numbers of CFU per spleen were determined at selected days postinfection. Consistent with infection by WT *Lm* (9), the numbers of CFU per spleen increased during the first 3 days postinjection in BALB/c and SCID mice, yet complete bacterial clearance was observed by day 8 for BALB/c mice (Fig. 2E). No additional time points after day 3 were assessed in SCID mice because of the chronic unresolved nature of *Lm* infection in these animals (17). In contrast, after injection with cytoLLO *Lm*, the numbers of CFU per spleen declined ≈ 4 logs by 24 h postinjection in both BALB/c and SCID animals, with complete clearance observed by 8 days postinjection.

CytoLLO *Lm* Delivers Native Antigens to the MHC Class I Pathway. We hypothesize that after degradation of cytoLLO *Lm* within the

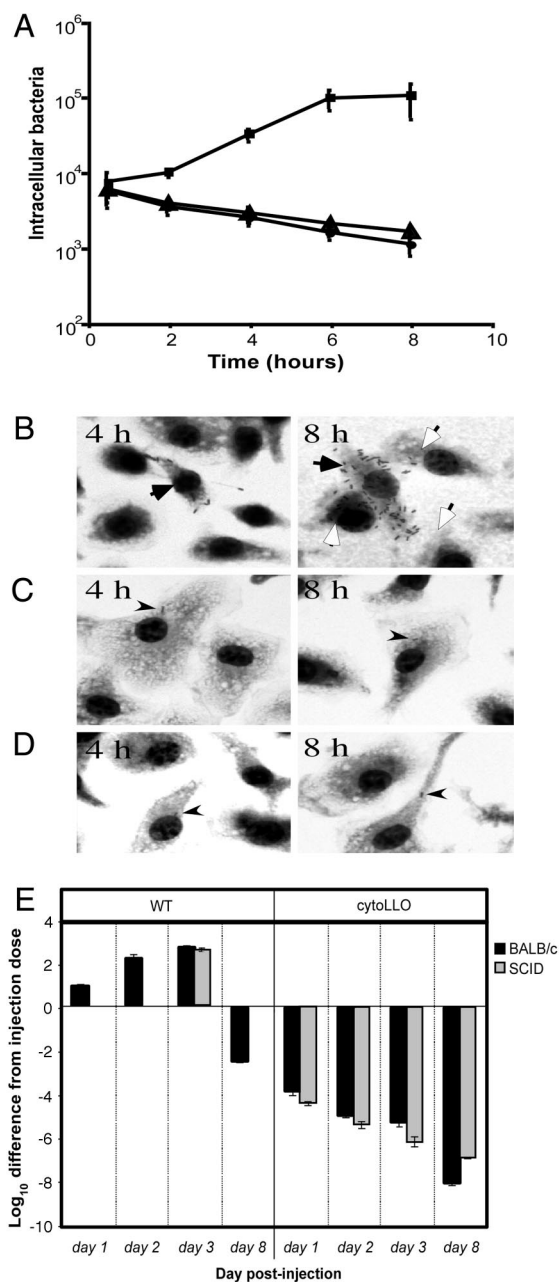


Fig. 2. Intracellular growth of *Lm* in BM-MAC. (A) BALB/c BM-MAC were infected with WT (squares) or pulsed with Δ LLO (circles) or cytoLLO (triangles) *Lm* and the number of intracellular bacteria determined. Results are the means \pm SD of one of two experiments performed in triplicate with similar results. (B–D) At the indicated times postinfection, BM-MAC infected with WT (B) or pulsed with Δ LLO (C) or cytoLLO (D) *Lm* were fixed, stained, and examined by light microscopy. (B) Filled arrows indicate primary infected BM-MAC with replicating cytosolic bacteria. Open arrows indicate neighboring BM-MAC infected by cell-to-cell spread of WT *Lm*. (C and D) Arrowheads indicate phagocytosed Δ LLO (C) or cytoLLO (D) *Lm*. (E) BALB/c or SCID mice were injected with WT (≈ 600 CFU) or cytoLLO ($\approx 2 \times 10^8$ CFU) *Lm*. At the times indicated postinjection, the numbers of splenic CFU were determined. Data represent the difference between the numbers of CFU per spleen and the injection dose at the indicated time point. Determination of CFU for SCID mice injected with WT *Lm* was performed only at day 3 postinjection.

phagosome, LLO-mediated perforation of the vacuole will allow for native *Lm* antigens to be released into the cytosol for processing and presentation via the conventional MHC Class I pathway. We constructed a cytoLLO *Lm* strain that expresses cytoplasmic OVA

(cytoLLO-cOVA) and determined whether C57BL/6 (H-2^b) APCs that have internalized cytoLLO-cOVA *Lm* are targets of OVA-(SIINFEKL)-specific effector T cells. We found that *lacZ*-inducible B3Z cells (18) produced β -galactosidase after coculture with APCs infected with an OVA-secreting WT *Lm* strain (WT-sOVA) or that had internalized cytoLLO-cOVA *Lm*, but not APCs that had internalized Δ LLO *Lm* expressing cytoplasmic OVA (Δ LLO-cOVA) (Fig. 3 *A* and *B*). MHC Class I presentation of OVA was via the conventional pathway, because *TAP1*^{-/-}-derived APCs (H-2^b) that had internalized cytoLLO-cOVA *Lm* were not recognized by B3Z T cells (Fig. 3*C*).

To determine whether native *Lm*-derived antigens were targeted to the MHC Class I pathway, we assessed whether BALB/c-derived BM-MAC that had internalized cytoLLO *Lm* were recognized by H-2K^d-restricted LLO₉₁₋₉₉-specific effector T cells. LLO₉₁₋₉₉-specific effector T cells secreted IFN- γ after coculture with BM-MAC that had internalized either WT or cytoLLO *Lm* but not Δ LLO *Lm* (Fig. 3*D*). Collectively these data demonstrate that APCs that have internalized cytoLLO *Lm* are immunologic targets of MHC Class I-restricted effector T cells.

Immunization with cytoLLO *Lm* Primes Effector T Cells and Stimulates Protective Antilisterial Immunity. To determine whether MHC Class I-restricted effector T cells are stimulated after immunization with cytoLLO *Lm*, BALB/c mice were injected (prime-boost) with WT, Δ LLO, or cytoLLO *Lm*. Twenty-eight days after the booster injection, spleen cells were collected and the frequencies of LLO₉₁₋₉₉ and p60₂₁₇₋₂₂₅ effector T cells determined (19). Animals immunized with cytoLLO *Lm* developed LLO₉₁₋₉₉- and p60₂₁₇₋₂₂₅-specific effector T cells, with responses increased in magnitude after the booster injection (Fig. 4*A*). Unlike WT immunization, the p60₂₁₇₋₂₂₅ response was immunodominant, with the LLO₉₁₋₉₉ response below the level seen after a single injection with WT *Lm*. Animals immunized with Δ LLO *Lm* did not develop LLO₉₁₋₉₉-specific effector T cells, with the response of p60₂₁₇₋₂₂₅-specific effector T cells minimally above background after prime-boost. This result is not surprising, because Δ LLO *Lm* does not escape from the phagosome to the cytosol and, therefore, would not be expected to efficiently stimulate CD8⁺ T cells.

We next determined whether immunization with cytoLLO *Lm* stimulates effector cells with *in vivo* CTL function. Immunized mice received fluorescently labeled LLO₉₁₋₉₉ or p60₂₁₇₋₂₂₅ peptide-pulsed target cells 6 or 10 days after booster injection, and, 18 h later, reduction of peptide-bearing cells was evaluated (Fig. 4*B-D*). Mice immunized with WT *Lm* showed a reduction of LLO₉₁₋₉₉- and p60₂₁₇₋₂₂₅-pulsed targets by up to 70% and 50%, respectively. For cytoLLO *Lm*-immunized mice, p60₂₁₇₋₂₂₅-pulsed targets were cleared to similar levels as WT, with LLO₉₁₋₉₉-pulsed targets cleared less efficiently. These findings are consistent with ELISPOT data (Fig. 4*A*) and confirm *in vivo* cytolytic function within these effector T cell populations.

We evaluated whether effector T cells stimulated after cytoLLO *Lm* immunization protected animals against lethal challenge with a highly virulent strain of *Lm* (10403) (20, 21). BALB/c mice were immunized with WT, Δ LLO, or cytoLLO *Lm*. Twenty-eight days after a booster injection, animals were challenged with a 10-LD₅₀ dose of *Lm* strain 10403 and the splenic CFU burden determined 48 h later. WT *Lm*-immunized mice showed a 4-log₁₀ reduction in the splenic bacterial burden when compared with nonimmunized controls (Fig. 4*E*). Animals immunized with Δ LLO (prime-boost) or a single injection of cytoLLO *Lm* showed no reduction in the splenic bacterial burden; however, animals given a prime-boost immunization with cytoLLO *Lm* showed an approximate 2-log₁₀ reduction in the splenic bacterial load, demonstrating stimulation of protective immunity. When cytoLLO *Lm*-immunized animals were challenged 8 weeks after the boost, the level of reduction in the splenic bacterial burden was 1.3-log₁₀ (data not shown), demonstrating long-lived immunity. Depletion studies in cytoLLO *Lm*-

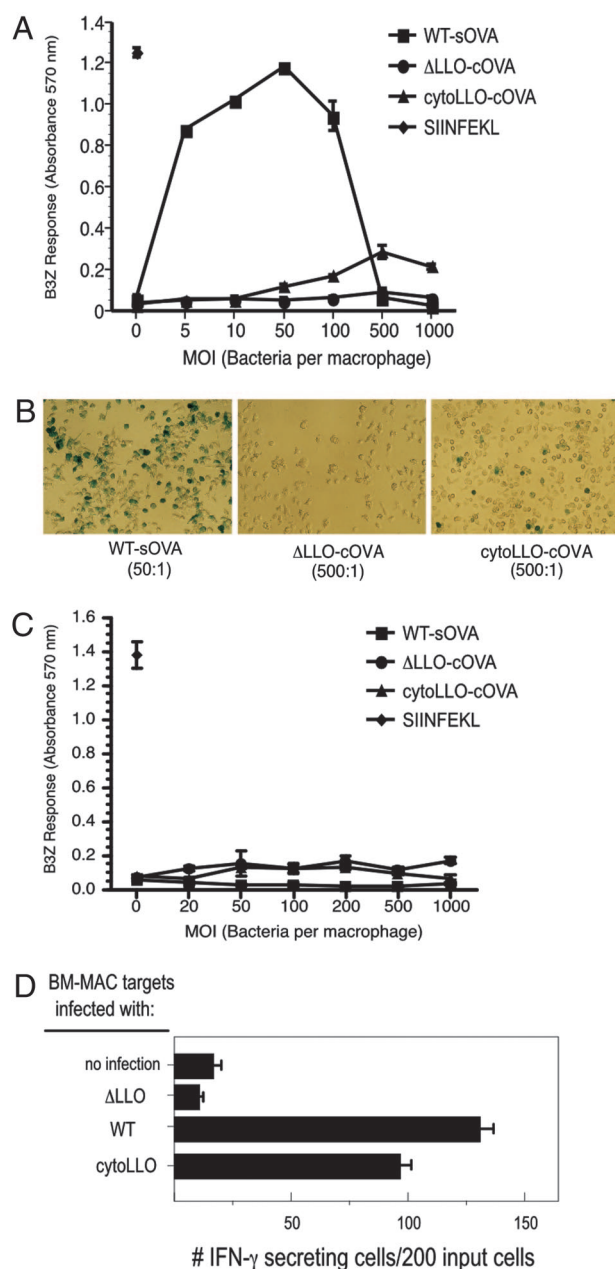


Fig. 3. Presentation of listerial antigen to CD8⁺ effector T cells. (A) C57BL/6 BM-MAC were pulsed with 100 nM SIINFEKL peptide, infected with WT *Lm* secreting OVA (sOVA), or pulsed with Δ LLO or cytoLLO *Lm* expressing cytoplasmic-localized OVA (cOVA) and used as APCs in B3Z T cell-activation assays. (B) Cocultures of APCs and B3Z cells in (A) were examined by light microscopy after staining with X-gal. The multiplicity of infection used is indicated below each panel. (C) *TAP1*^{-/-} BM-MAC were infected with WT *Lm* secreting OVA (sOVA) or pulsed with Δ LLO or cytoLLO *Lm* expressing cytoplasmic-localized OVA (cOVA) and used as APCs in B3Z assays. (D) BALB/c BM-MAC infected with WT or pulsed with Δ LLO or cytoLLO *Lm* were cocultured with LLO₉₁₋₉₉-specific effector T cells in ELISPOT assays. Results presented in A, C, and D are the means \pm SD of one of three independent experiments performed in triplicate with similar results. Results presented in B are representative of one of three independent experiments with similar results.

immunized mice showed that CD8⁺ effector T cells were a key component of protective immunity, because anti-CD8 treatment before challenge reduced the level of the antilisterial response \approx 98% compared with nontreated controls (see Fig. 6, which is published as supporting information on the PNAS web site).

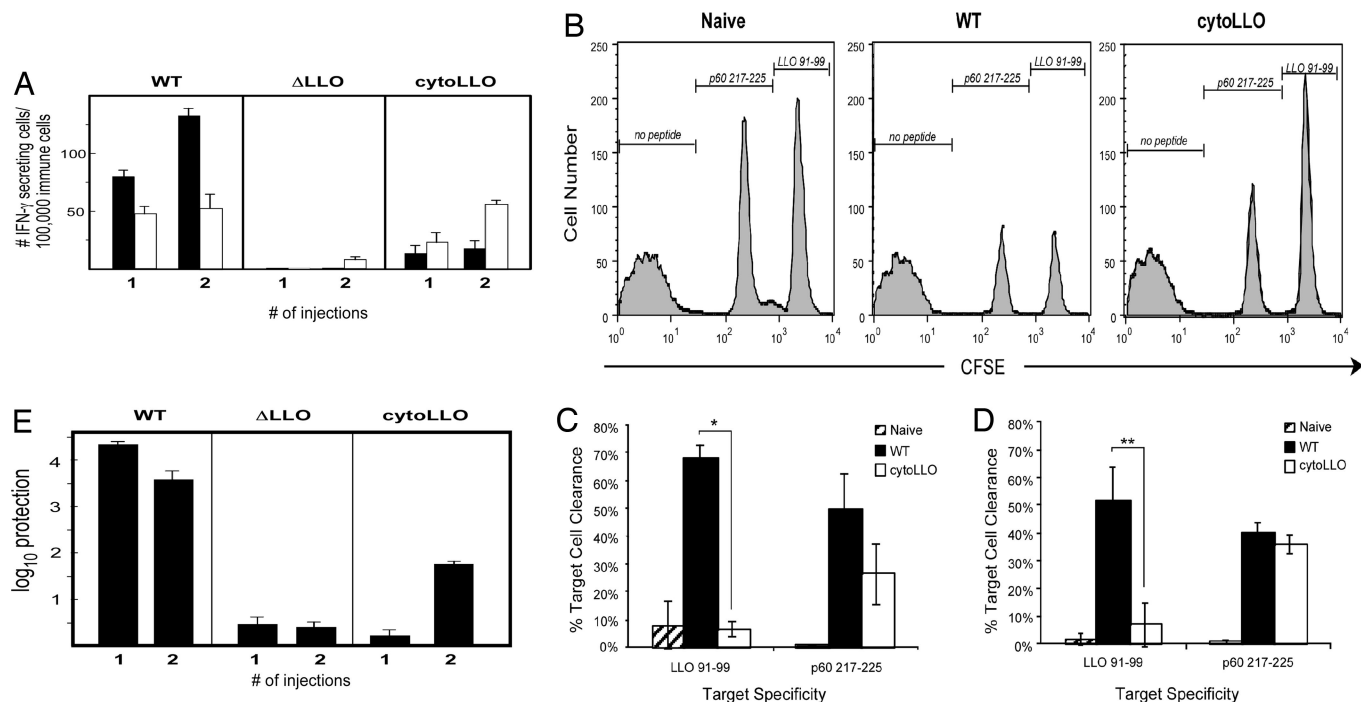


Fig. 4. Immunization with cytoLLO *Lm* primes *Lm*-specific effector T cells. (A) BALB/c mice were immunized with one or two doses of WT, Δ LLO, or cytoLLO *Lm*, and the frequencies of LLO₉₁₋₉₉ (dark bars) or p60₂₁₇₋₂₂₅ (open bars) specific effector T cells were determined by ELISPOT. Results are presented as the numbers of IFN- γ -secreting cells per 100,000 spleen cells and represents the means \pm SD of one of five independent experiments performed in triplicate with similar results ($n = 4$ mice per group). (B) The clearance of peptide-pulsed, fluorescently labeled target cells was determined 18 h after injection in immunized mice relative to naïve animals. The response of a representative mouse evaluated 10 days after booster injection is also presented. The mean responses of immunized mice evaluated 6 (C) or 10 (D) days after booster injection are also presented. Data represent the means \pm SD ($n = 2-4$ mice per group). *, $P = 0.000037$; **, $P = 0.0469$. (E) BALB/c mice were immunized with one or two doses of WT, Δ LLO, or cytoLLO *Lm*. Mice were challenged with a 10-LD₅₀ dose of *Lm* strain 10403 and the numbers of splenic CFU determined. Data are presented as log₁₀ protection compared with the naïve challenged group, and results are the means \pm SD of three independent experiments ($n = 4$ mice per group).

Animals immunized (prime-boost) with 10-fold less cytoLLO *Lm* ($\approx 2 \times 10^7$ CFU) did not show a significant reduction of the splenic bacterial burden after challenge with *Lm* strain 10403 (data not shown).

Additionally, we determined whether cytoLLO *Lm*-immunized

Table 1. Immunization with cytoLLO *Lm* stimulates protective antilisterial immunity

	Immunization*	Boost†	Challenge dose‡	
			10 LD ₅₀	40 LD ₅₀
BALB/c				
	WT <i>Lm</i>	–	10 of 10	4 of 4
	Δ LLO <i>Lm</i>	–	0 of 6	ND
	Δ LLO <i>Lm</i>	+	0 of 6	ND
	cytoLLO <i>Lm</i>	–	0 of 6	ND
	cytoLLO <i>Lm</i>	+	9 of 10	4 of 4
C57BL/6				
	WT <i>Lm</i>	–	4 of 4	ND
	Δ LLO <i>Lm</i>	+	0 of 4	ND
	cytoLLO <i>Lm</i>	–	0 of 4	ND
	cytoLLO <i>Lm</i>	+	4 of 4	ND

ND, not determined.

*BALB/c or C57BL/6 mice were immunized with WT, Δ LLO, or cytoLLO *Lm* as indicated.

†Ten days after primary immunization, animals were given a secondary injection with the same numbers of CFU as given for the primary immunization.

‡Twenty-eight days after the boost injection, animals were challenged with either a 10- or 40-LD₅₀ dose of *Lm* strain 10403. The animals were monitored daily and killed when moribund. Data are presented as the number of surviving animals per number of animals in the group.

animals survived a lethal challenge. BALB/c mice were immunized with WT, Δ LLO, or cytoLLO *Lm*. Twenty-eight days after the boost, animals were challenged with either a 10- or 40-LD₅₀ dose of *Lm* strain 10403. All animals immunized with WT *Lm* survived lethal challenge, whereas none of the animals immunized with Δ LLO *Lm* (prime-boost) or a single immunization with cytoLLO *Lm* survived lethal challenge (Table 1). However, 9 of 10 of the animals given the prime-boost with cytoLLO *Lm* survived a 10-LD₅₀ challenge, and 4 of 4 animals survived a 40-LD₅₀ challenge dose. These results demonstrate that highly effective protective immunity is stimulated after immunization with cytoLLO *Lm*.

An advantage of our directed antigen-delivery strategy is that the cytoLLO *Lm* vaccine strain should effectively deliver native bacterial antigens to the cytosolic MHC Class I pathway regardless of MHC haplotype; thus, identification of pathogen-specific antigenic determinants for each host/haplotype is not required. As a test of this premise, C57BL/6 mice (H-2^b), where target antigens for CD8⁺ effector T cells are not well characterized, were immunized with WT, Δ LLO, or cytoLLO *Lm* and challenged 28 days after the boost with a 10-LD₅₀ dose of *Lm* strain 10403 (Table 1). WT *Lm*-immunized animals survived lethal challenge, whereas none of the animals immunized with Δ LLO (prime-boost) or single immunization with cytoLLO *Lm* survived challenge. However, all C57BL/6 mice primed and boosted with the cytoLLO *Lm* strain survived a 10-LD₅₀ lethal challenge, demonstrating stimulation of antilisterial immunity after immunization with cytoLLO *Lm* in a genetic background where the identity of protective *Lm* antigens are unknown.

Discussion

In this report, we demonstrate stimulation of protective immunity against an intracellular bacterial pathogen by using a highly atten-

uated strain that is deficient for replication within host cells, yet delivers native bacterial antigens directly into the endogenous MHC class I processing pathway for presentation to CD8⁺ T cells. By using this directed antigen-delivery strategy, key hallmarks of vaccine efficacy have been achieved, including (i) stimulation of memory CD8⁺ T cells, (ii) stimulation of protective antilisterial immunity, and (iii) no identifiable risk to the host because of the intracellular replication deficiency of the vaccine strain.

The intracytosolic lifestyle of *Lm* has prompted the development of several attenuated strains as vaccine vectors (22–26). However, it remains questionable whether such strains are suitably attenuated for general use as a vaccine, especially in immunocompromised individuals. One attenuated *Lm* strain was found to be 1,000-fold less virulent than the WT parent *Lm* in mice, yet replication within primary infected host cells was identical to the parental strain, raising questions concerning the potential infectious risk to patients after administration (25).

The cytoLLO *Lm* vaccine strain we have developed does not detectably replicate within host cells (Figs. 2 *A* and *D* and 5). Further, cytoLLO *Lm* is attenuated 100,000-fold in BALB/c mice compared with WT *Lm* and is cleared rapidly after immunization. Adaptive immune responses are not required for cytoLLO *Lm* clearance, because SCID mice eliminated cytoLLO *Lm* with the same kinetics as did normal animals (Fig. 2*E*). These findings address an important safety concern for general clinical use of cytoLLO *Lm*. Because viable cytoLLO *Lm* do not actively escape from the phagosome into the cytosol, it is presumed that host APCs are recognized as immunologic targets only after degradation of cytoLLO *Lm* within the phagosome. In support, we found the clearance rate of cytoLLO *Lm* to be unaltered in animals with preexisting antilisterial immunity (data not shown). Thus, bacterial uptake alone does not trigger immune recognition.

Immunization with *Lm* strains that are unable to escape the phagosome into the cytosol, such as LLO-negative or nonviable (heat killed) strains, stimulates *Lm*-specific T cells that lack effector functions necessary for protection against lethal infection with WT *Lm* (12). Studies of Toll-like receptors, adaptor molecules, and cDNA microarray analyses of gene expression profiles demonstrate distinct differences in both signaling patterns and intensities after infection with cytosolic versus vacuole-localized *Lm* (27, 28). Furthermore, invasion of *Lm* into the cytosol stimulates NF- κ B activation, an event also observed after exposure to purified LLO (29, 30). Although our cytoLLO *Lm* vaccine strain is unable to actively escape the phagosome to the cytosol, the process of vacuole disruption and/or delivery of antigen to the cytosol of APCs appears sufficient to initiate the appropriate gene expression program to drive the stimulation of antilisterial T cells possessing the full range of effector functions required to mediate protection against lethal challenge. Additional manipulations to the host, such as α -CD4 (31) or α -CD40 (32) treatment, or supplemental IL-12 injections (33) that are required for the stimulation of protective immunity after immunization with nonviable preparations of *Lm*, are unnecessary with our cytoLLO *Lm* vaccine strain. Recently, vaccination with a metabolically active, yet replication-defective *Lm* strain (because of DNA crosslinking), has been shown to be similarly capable of priming CD8⁺ T cells (34). Yet, cytoLLO *Lm* is the most attenuated *Lm* vaccine strain generated to date that can be administered as a viable organism, does not require any additional modifications to the bacteria or host, effectively stimulates protective antilisterial immunity, and is suitable for clinical safety testing in humans (25).

The efficiency of vacuole lysis after degradation of cytoLLO *Lm* within host cells is currently unknown, although our data suggest that the efficiency may be lower than typically seen after WT infection. Although APCs that have internalized cytoLLO *Lm* are immunologic targets for peptide-specific effector T cells, responses were diminished compared with those observed by using APCs infected with WT *Lm* (Fig. 3 *B* and *D*). It is possible that not all

macrophages harboring cytoLLO *Lm* become targets for peptide-specific effector T cells, whereas the majority of BM-MAC infected with WT *Lm* are immunologic targets. Alternatively, at least for LLO_{91–99}-specific responses, the level of LLO antigen derived from cytoLLO *Lm* may be significantly reduced, because cytoLLO protein used for phagosomal lysis may not be available for MHC Class I processing and presentation. In contrast, WT *Lm* continues to secrete LLO in the cytosol during intracellular replication (35). We are currently determining whether the efficiency of directed antigen delivery and overall vaccine efficacy might be enhanced with increased levels of cytoLLO production or by using an alternative pore-forming cytolysin.

Stimulation of protective antilisterial responses after immunization with cytoLLO *Lm* depended on a prime-boost regimen (10-day interval). We found that enhancement of protective antilisterial responses did not occur when animals were similarly primed and boosted with WT *Lm* over this time period (Fig. 4*E*). A recent report showed that DC-primed responses were enhanced with a secondary *Lm* infection within 1–2 weeks of priming (36). To account for these observations, the authors concluded that some immunization strategies may accelerate an IFN- γ sensitive pathway for memory-cell development. Immunization conditions that do not stimulate optimal inflammatory signals may accelerate transition of activated cells to the memory phenotype. These findings suggest that the kinetics of T cell expansion and contraction that occur after immunization by various methods must be taken into consideration when evaluating efficacy and the timing of repeat administration.

We have described a vaccine strategy for directed antigen delivery using an intracellular replication-deficient bacterial strain. This strategy effectively overcomes many obstacles to the rational design of vaccine formulations: The vaccine is safe because of defective intracellular replication, stimulates protective cell-mediated immunity to native target antigens, alleviating the need to identify specific antigenic determinants relevant for each host, and does not require additional modification to the vaccine or host. It will be of interest to determine whether the directed antigen-delivery strategy can be applied to other genetically tractable intracellular bacterial pathogens, where CD8⁺ T cell responses are a component of the protective immune response. Furthermore, development of genetic systems for manipulation of other intracellular pathogens, coupled with an understanding of the virulence factors required for their intracellular survival, may allow cytoLLO-expressing intracellular replication-deficient constructs to be used as safe, effective vaccine formulations.

Materials and Methods

Mice and Immunization. Six-week-old female BALB/c, C57BL/6, B6.129S2-*Tap1*^{tm1Atp} (*TAP1*^{-/-}) mice (The Jackson Laboratory) or SCID mice (provided by Richard Jones, Portland Veterans Affairs Medical Center) were provided unrestricted access to food and water. Eight-week-old mice were immunized by injection via tail vein with viable *Lm* in 0.2 ml of PBS. All animal experiments were conducted with approval from the Institutional Animal Care and Use Committee.

Cell Lines and Reagents. The RMA-S-K^d cell line (provided by Michael Bevan, University of Washington, Seattle) was maintained in RPMI medium 1640 (Invitrogen) supplemented with 10% FCS (Tissue Culture Biologicals, Tulare, CA) and 200 μ g/ml G-418 (Sigma). The LLO_{91–99} (GYKDGNEYI) and p60_{217–225} (KYGVS-VQDI) peptides were synthesized at the Portland Veterans Affairs Medical Center. The OVA_{257–264} (SIINFEKL) peptide was purchased commercially (Invitrogen).

Construction of cytoLLO *Lm* Strains. Splice-overlap extension (SOE) PCR (37) was used to generate the cytoLLO construct lacking the LLO secretion signal sequence. Chromosomal DNA isolated from WT *Lm* (strain 10403S) was used as the starting template for

amplification with primer pair 260, 5'-CGGAGCTCTCCTATCT-TAAAGTTACTTTTATGTGGAGG-3' and 417, 5'-ATGCATC-CTTCATGGGTTTCACTCTCCTTCTACATTTTTT-3'. Plasmid pJP-02 harboring the *hly* gene without the native transcriptional/translational control elements was used as the starting template for amplification with primer pair 418, 5'-GAAACCCATGAAGGATGCATCTGCATTCAATAAAG-AAAAT-3' and 038, 5'-ATTCGGATCCTTATTATTCGATTG-3'. The PCR products were gel purified and used as templates in a second SOE PCR using primers 260 and 038. The PCR product was digested with SacI and BamHI and ligated with the site-specific integration vector pPL2 (14) digested with the same enzymes. The resulting cytoLLO expression vector, pN8-02, was introduced into the chromosome of LLO-negative *Lm* strain DH-L377 as described in ref. 14 to generate strain DH-L1233. The cytoLLO allele was confirmed by DNA sequencing. A cytoLLO expression vector carrying the erythromycin (*ermC*)-resistance gene was generated by marker exchange. The Gram-positive chloramphenicol-resistance gene was excised from pN8-02 by using ApaLI and PvuI and was replaced by the *ermC* gene that had been excised from plasmid pPL3e (38) by using the same restriction enzymes. The resulting plasmid (pN8-02*erm*) was introduced into the chromosome of DH-L377 as described in ref. 14 to generate strain DH-L1285.

In Vivo Cytotoxicity Assay. To evaluate the *in vivo* cytotoxic function of CD8⁺ T cell populations, clearance of peptide-loaded target cells was determined (39, 40). Naïve BALB/c splenocyte target cells were suspended at 10⁷ cells per ml in RPMI medium 1640 and then labeled for 10 min with Vybrant DiI solution (Molecular Probes).

Target cells were washed and divided equally into three tubes. Target cells were then labeled with 10 μM, 1 μM, or 1 nM 5,6-carboxyfluorescein diacetate-succinimyl ester (CFSE; Molecular Probes). Finally, the dual-labeled cells were pulsed with 1 μM LLO₉₁₋₉₉ peptide, 1 μM p60₂₁₇₋₂₂₅ peptide, or no peptide for 1 h at room temperature. Each target cell population (5 × 10⁶ cells) was injected intravenously into recipient mice. Animals were rested 18 h before recipient spleens were analyzed by flow cytometry for target cell clearance. Gating on Vybrant DiI⁺ cells, the percent killing was calculated as: 100% - [(% peptide pulsed in immune/% unpulsed in immune)/(% peptide pulsed in naïve/% unpulsed in naïve)] × 100).

Statistical Analysis. Statistical probabilities were evaluated by a two-tailed Student's *t* test, with probability values of *P* < 0.05 considered to be significant.

Additional Methods. See *Supporting Methods*, which is published as supporting information on the PNAS web site for determination of LLO protein levels, infection of host cells, construction of OVA-expressing *Lm* strains, B3Z T cell activation, ELISPOT assays, and determination of protection and survival after *Lm* challenge.

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