Selected prfA* Mutations in Recombinant Attenuated Listeria monocytogenes Strains Augment Expression of Foreign Immunogens and Enhance Vaccine-Elicited Humoral and Cellular Immune Responses

Lin Yan,1,2† Jin Qiu,1,2† Jianbo Chen,1,2,3† Bridgett Ryan-Payseur,1,2 Dan Huang,1,2 Yunqi Wang,1,2 Lijun Rong,1 Jody A. Melton-Witt,3 Nancy E. Freitag,1 and Zheng W. Chen1,2,*

Department of Microbiology and Immunology1 and Center for Primate Biomedical Research,2 University of Illinois College of Medicine, Chicago, Illinois 60612; Department of Microbiology, Sun Yat-sen University, Guangzhou, China 510080; and Department of Molecular and Cell Biology, University of California, Berkeley, California 94720-3202

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While recombinant Listeria monocytogenes strains can be explored as vaccine candidates, it is important to develop attenuated but highly immunogenic L. monocytogenes vaccine vectors. Here, prfA* mutations selected on the basis of upregulated expression of L. monocytogenes PrfA-dependent genes and proteins were assessed to determine their abilities to augment expression of foreign immunogens in recombinant L. monocytogenes vectors and therefore enhance vaccine-elicited immune responses (a prfA* mutation is a mutation that results in constitutive overexpression of PrfA and PrfA-dependent virulence genes; the asterisk distinguishes the mutation from inactivation or stop mutations). A total of 63 recombinant L. monocytogenes vaccine vectors expressing seven individual viral or bacterial immunogens each in nine different L. monocytogenes strains carrying wild-type prfA or having prfA* mutations were constructed and investigated. Mutations selected on the basis of increased PrfA activation in recombinant L. monocytogenes prfA* vaccine vectors augmented expression of seven individual protein immunogens remarkably. Consistently, prime and boost vaccination studies with mice indicated that the prfA (G155S) mutation in recombinant L. monocytogenes prfA* vaccine strains enhanced vaccine-elicited cellular immune responses. Surprisingly, the prfA (G155S) mutation was found to enhance vaccine-elicited humoral immune responses as well. The highly immunogenic recombinant L. monocytogenes ΔactA prfA* vaccine strains were as attenuated as the recombinant parent L. monocytogenes ΔactA vaccine vector. Thus, recombinant attenuated L. monocytogenes ΔactA prfA* vaccine vectors potentially are better antimicrobial and anticancer vaccines.

Recombinant Listeria monocytogenes is a promising intracellular live vaccine vector for delivering immunogens and for inducing vaccine-elicited immune responses and immunity to infections or tumors. Compared to other bacterial vectors, such as Mycobacterium bovis BCG and Salmonella, recombinant L. monocytogenes appears to elicit CD8+ T-cell responses more readily. The gram-positive facultative intracellular organism L. monocytogenes can invade a wide range of host cells, including phagocytes and nonphagocytic cells, and can escape from the phagolysosomes to the cytoplasm by means of the pore-forming cholesterol-dependent cytolysin listeriolysin O (LLO). The ability of L. monocytogenes to escape from the endosome to the cytosol after entry into host cells allows vaccine antigens to enter both major histocompatibility complex class I and II pathways of antigen processing and presentation and therefore to elicit both CD4 and CD8+ T-cell responses (13, 20, 39, 56). In addition, both wild-type and attenuated recombinant L. monocytogenes strains appear to be gastrointestinal tract-tropic and oropharyngeal mucosa-tropic, enabling mucosa-targeted delivery of vaccine immunogens. Finally, underlying immunity to the L. monocytogenes vector itself does not prevent priming or boosting of immune responses to foreign immunogens (6, 52, 53).

Recent progress in bacterial genetics has facilitated the development of genetically manipulated L. monocytogenes strains as attenuated vaccine vectors (2, 8, 10, 16, 34, 41, 56, 57). Some of these strains have been shown to induce protective immune responses to infections and cancers (7, 34, 38, 40, 57), whereas others are currently being tested in clinical trials (2). In particular, deletion of the actA gene in L. monocytogenes results in remarkable attenuation since the actin-assembling protein encoded by actA is necessary for L. monocytogenes to utilize actin-based motility for cell-to-cell spread or transmission (14, 19, 26, 55). In fact, an attenuated L. monocytogenes ΔactA vaccine strain has been shown to exhibit diminished pathogenicity and toxicity in vivo but to maintain immune potency (8). The current efforts to develop recombinant L. monocytogenes vaccines appear to focus on at least three aspects: (i) increasing the success rate for making recombinant L. monocytogenes constructs and augmenting expression of various foreign immunogens in attenuated L. monocytogenes strains; (ii) enhancing the ability of L. monocytogenes vaccine vectors to elicit immunogen-specific cellular immune responses; and (iii) exploiting the ability of L. monocytogenes vaccine vectors to elicit humoral immune responses.

Molecular approaches targeting increased activity of L. monocytogenes transcriptional regulatory factors may make it
possible to augment expression of foreign immunogens in recombinant L. monocytogenes vectors and thereby enhance vaccine-elicited immune responses. Positive regulatory factor A (PrfA) functions as an L. monocytogenes transcriptional activator and plays a central role in the transcription of a number of virulence genes, including hly and actA (22, 24, 25, 30). PrfA is a member of the CRP/PmrA family of regulatory proteins and binds to a 14-bp palindromic DNA sequence located in the −40 region of target promoters (23, 46). Interestingly, some prfA* mutations obtained following chemical mutagenesis result in a constitutively activated form of the protein that can induce high-level expression of PrfA-dependent virulence genes and proteins (37, 45, 49) (a prfA* mutation is a mutation that results in constitutive overexpression of PrfA and PrfA-dependent virulence genes; the asterisk distinguishes the mutation from inactivation or stop mutations). A prfA(G145SS) mutant, the first prfA* mutant identified, exhibited an approximately 18-fold increase in binding affinity for the hly promoter and constitutively expressed high levels of other PrfA-dependent gene products (21, 44). prfA(A77K) and prfA(G155S) increase the production of LLO approximately 20-fold and expression of actA approximately 90- to 270-fold in broth cultures compared to the wild-type gene (49). Given that prfA* mutations increase hly expression, recombinant L. monocytogenes ΔactA prfA* vaccine vectors expressing immunogen-encoding genes via the hly promoter would be predicted to induce much higher levels of expression of immunogens and possibly enhance vaccine-elicited immune responses. On the other hand, such recombinant L. monocytogenes ΔactA prfA* vectors should remain attenuated as deletion of actA results in attenuation of L. monocytogenes virulence. The current proof-of-concept studies were designed to address these possibilities. Our data demonstrate that recombinant L. monocytogenes ΔactA prfA* vaccine vectors exhibit high levels of expression of immunogens and enhance vaccine-elicited humoral and cellular responses while they remain profoundly attenuated.

MATERIALS AND METHODS

Plasmids, bacterial strains, and media. The shuttle integration vector pPL2, which can replicate autonomously in Escherichia coli and integrate at a single location in the chromosome of L. monocytogenes (31), was used as the parent plasmid in this study. The pPL2 plasmid and all derivative recombinant plasmids were maintained in E. coli strain DH5α in Luria-Bertani (LB) broth (Fisher Biotech) with chloramphenicol (25 μg/ml) selection. E. coli SM10 was used as the donor strain for transforming recombinant plasmids from E. coli to L. monocytogenes. L. monocytogenes strains with and without prfA* mutations that were defined as mutations that resulted in low, mid, and high levels of activation were used in this study to develop vaccine constructs (Table 1). For expression of foreign proteins all recombinant L. monocytogenes strains were grown at 30°C with shaking at 280 rpm either in brain heart infusion (BHI) medium (BD) or in LB medium with chloramphenicol (7.5 μg/ml) and streptomycin (200 μg/ml) selection.

Construction of pPL6-myc shuttle vector plasmid. The vector, ribosome binding site, and downstream 75-bp signal sequence of the hly gene were amplified from the purified L. monocytogenes genome by PCR using primers HLYP-L (5'-CGGGTACCGATAATCAAAACTATCGTTGC-3') and HLYP-R (5'-CCCTTGGCCTTGGCTGGGGCCG3') and the resulting PCR product was cloned into the KpnI and BamHI sites of the integration vector pPL2. The C-Myc tag fragment plus three restriction sites (BamHI, Spel, and XbaI) were added in frame following the hly signal sequence by PCR from plasmid pPL6 using primers HLYP-L and Myc-tag (5'-CGCGGCGCGCGGCTAAAGCATCCTCCTCGAGAATAAGCCTTTGTTCTCTCAAGACTGGAGCTC3'). The purified PCR products were digested with KpnI and NotI and subcloned into pPL2. These modifications resulted in development of the shuttle vector plasmid pPL6-myc.

Construction of recombinant L. monocytogenes vaccine vectors. All pPL6 integration plasmids containing foreign genes were individually transformed into CaCl2-complement E. coli SM10. For conjugation, SM10 cultures transformed with pPL6 integration plasmids were grown in LB medium containing 25 μg/ml of chloramphenicol; L. monocytogenes strains were grown in BHI medium containing 200 μg/ml of streptomycin to an optical density at 600 nm (OD600) of 0.6. Then 1.5 ml of an L. monocytogenes strain was mixed with 3 ml of a transformed SM10 culture, centrifuged, and washed twice with LB medium, and the pellet (~30 μl) was spotted onto a BHI medium plate in the absence of antibiotic. After 4 h of incubation at 37°C, the dried spots were gently suspended in 1 ml of BHI medium, and 50 μl was plated on BHI agar supplemented with 7.5 μg/ml of chloramphenicol and 200 μg/ml of streptomycin, which was followed by incubation at 30°C for 2 to 3 days. An L. monocytogenes recombinant carrying the chromosomally integrated plasmid was selected on BHI agar plates containing streptomycin and chloramphenicol and verified by PCR using previously described primers (31).

Western blot analyses. (i) Time course of foreign gene expression. The recombinant L. monocytogenes prfA(G155S) vector expressing vaccinia virus protein H3L was used to optimize the time course of foreign immunogen expression. Typically, 500 μl of an overnight culture was added to 10 ml of fresh BHI medium and grown at 30°C with shaking. Portions (500 μl) of the culture were removed at 3, 4, 5, 6, 8, 10, 12, and 24 h and stored at −20°C until they were used for analyses. Equal volumes of samples were electrophoresed on denaturing polyacrylamide gels, electrotransferred onto nitrocellulose membranes, and probed with anti-c-myc monoclonal antibody (MAb) 9E10 (Sigma) at a 1:1,000 dilution or with anti-vaccinia virus H3L polyclonal antibody (GeneBio Tech Inc.) at a 1:500 dilution. Following incubation with peroxidase-labeled goat anti-mouse immunoglobulin G (H + L), or goat anti-(mouse IgG) H + L, and 1:10,000 dilution of horseradish peroxidase-conjugated goat anti-mouse IgG H + L, the membrane was visualized by staining with the West Pico chemiluminescent substrate (Pierce).

(ii) Immunogen expression in different L. monocytogenes strains. All recombinant L. monocytogenes strains were grown for 16 to 18 h in 20 ml of BHI medium or LB medium at 30°C with shaking. Bacterial pellets and supernatant samples of the recombinant L. monocytogenes strains were collected by centrifugation at 5,000 rpm for 20 min at 4°C. Supernatants were then concentrated approximately 150-fold by centrifugation through Amicon Ultra filter columns (Millipore). Bacterial pellets and supernatants were obtained from equal amounts of bacteria as determined by measurement of the OD600 and were analyzed by Western blotting using anti-c-myc MAb as described above.

Immunization of mice. Eight- to 10-week-old female BALB/c mice were used in immunogenicity studies. For comparisons of immunogenicity, the experiments were divided into two subsets. In subset A experiments, each group of mice received intravenously homologous recombinant L. monocytogenes prime (week 0) and boost (week 4) immunizations. In these experiments, each mouse was primed and boosted intravenously with 1 × 107 CFU of an L. monocytogenes strain in 100 μl of phosphate-buffered saline (PBS) using a 28-gauge needle unless otherwise indicated. Thirty-six mice were randomly divided into six groups. Two groups that were not immunized (control) were vaccinated with the attenuated L. monocytogenes wild-type prfA ΔactA strain (DP-L3078) or prfA(G155S) ΔactA strain (NF-L974) (no immunogen). The other four groups were incoemunized with recombinant L. monocytogenes vectors derived from DP-L3078 or NF-L974 and expressing one of two foreign immunogens: vaccinia virus protein H3L or Anthrax protective antigen (PA). All groups were boosted at week 4 after primary immunization with the same amount of L. monocytogenes. Heparinized blood (300 to 400 μl per mouse) was collected by retroorbital bleeding, and the blood from a group was pooled to isolate peripheral blood mononuclear cells (PBMCs) and plasma. Plasma and PBMCs were collected for use in enzyme-linked immunosorbent assay (ELISA) and enzyme-linked immunospot assay (ELISPOT) assays at weeks 0, 2, 4, and 6. Plasma was isolated before and after the first immunization at 2-week intervals and stored at −80°C for future ELISA analysis. At week 6, all mice were euthanized, and lymphocytes were isolated from the spleens for ELISPOT assays.

In subset B experiments, groups of mice received intravenously heterologous recombinant L. monocytogenes prime (week 0) and gram-positive enhancer matrix (GEM) particle boost (week 4) immunizations. Four groups of mice (eight each) were vaccinated with strains expressing the Mycobacterium tuberculosis-derived early 6-kDa secretory antigen (ESAT6) as follows: 1 × 107 CFU of DP-L3078 + ESAT6 (derived from wild-type L. monocytogenes), 1 × 107 CFU of DP-L3078 + ESAT6 (derived from the attenuated L. monocytogenes ΔactA strain), NF-L974 + ESAT6 (derived from the prfA(G155S) ΔactA strain), or the control strain L. monocytogenes NF-L974. Mice vaccinated with the recombinant virulent wild-type L. monocytogenes strain expressing ESAT6 were dead within 7 days, but the other groups of mice received a second immunization at week 4 with 2.5 × 107
of Lactococcus lactis GEM particles loaded with vaccine protein ESAT6 (groups 2 and 3) or the same amount of mock GEM particles. The development of a GEM-ESAT6 subunit vaccine is described elsewhere (J. Qiu, L. Yan, and J. Chen, unpublished data). Heparinized blood (300 to 400 μl per mouse) was collected by retroorbital bleeding and pooled for each group to isolate PBMCs and plasma. Plasma and PBMCs were collected for ELISA and ELISPOT assay analyses at weeks 0, 2, 4, 5 and 6. At the termination of the experiments, splenocytes were isolated for ELISPOT assay analysis.

IFN-γ ELISPOT assays. The protocols used for ELISPOT assays for measuring antigen-specific gamma interferon (IFN-γ)-secreting T cells were modified from our protocols as described previously (1, 47, 48). Briefly, a murine IFN-γ ELISPOT assay kit consisting of capture and detection antibodies (Diaclone, France) was used according to the manufacturer’s instructions, with several modifications. Ninety-six-well plates with polyvinylidene difluoride bottoms (Millipore, Billerica, MA) were coated overnight under sterile conditions with 100 μl of capture antibody at 4°C. After the plates were washed with PBS, they were blocked with PBS containing 2% dry skim milk for 6 h at 4°C. Each plate was then seeded with 5 × 10^5 PBMCs/well or 1 × 10^6 splenocytes/well, using triplicate samples. Cells were incubated with a pool of 15-mer peptides that overlapped by a 12-mer spanning the entire ESAT6, H3L, or PA protein (10 μg/ml for each peptide; synthesized by Genscript) at 37°C in the presence of 5% CO₂ for 16 to 20 h. The positive and negative controls consisted of 10 μg/ml of concanavalin A (Sigma) and RPMI 1640 containing 10% fetal bovine serum, respectively. The plates were then incubated with biotinylated detection antibody for 1.5 h at 37°C and washed, and then they were developed with 5-bromo-4-chloro-3-indolylphosphate (BCIP)-nitroblue tetrazolium buffer for 10 min at room temperature. The areas in which a single cell was stimulated to secrete IFN-γ were detected as spots on the polyvinylidene difluoride membrane. Measurements were obtained in triplicate for each stimulant. The spots on the plates were counted by using an automated ELISPOT reader system (CTL Analyzers) with the ImmunoSpot software. The number of spots for triplicate wells was expressed as the mean ± standard deviation for 1 × 10⁵ PBMCs or splenocytes.

ELISA. Antigen-specific IgG antibody titers were determined by an ELISA using the plasma pooled from each group of vaccinated mice. Briefly, high-binding-capacity 96-well ELISA plates (Costar) were coated with PA (0.25 μg/well; Alpha Diagnostic International), H3L (1 μg/well), or ESAT6 (2 μg/well) in coating buffer overnight at 4°C. Recombinant six-His-tagged H3L and ESAT6 were produced from E. coli in our lab and were purified using an Ni-nitrilotriacetic acid column (Qiagen Inc., Valencia, CA). After blocking, the plates were incubated with serial dilutions of plasma for 1 h at 37°C and washed. Horseradish peroxidase-conjugated anti-mouse IgG (KPL) was incubated for 1 h at a dilution

### TABLE 1. L. monocytogenes strains developed and used in this study

| Strain(s) | Description | Reference |
|-----------|-------------|-----------|
| 10403S | Wild-type L. monocytogenes | 31 |
| 10403S + ESAT6 | Wild-type L. monocytogenes expressing ESAT6 | This study |
| 10403S + Ag85B | Wild-type L. monocytogenes expressing antigen 85B | This study |
| Other 10403S derivatives | Wild-type L. monocytogenes expressing other immunogen(s) | This study |
| NF-L476 | 10403S actA gus plcB | 49 |
| NF-L1213 | NF-L476 prfA(Y154C) (low-level PrfA activation) | Miner et al. |
| NF-L1213 + ESAT6 | prfA(Y154C) expressing ESAT6 | This study |
| Other NF-L1213 derivatives | prfA(Y154C) expressing other immunogen(s) | This study |
| NF-L924 | NF-L476 prfA(E77K) (mid-level PrfA activation) | 49 |
| NF-L924 + ESAT6 | prfA(E77K) expressing ESAT6 | This study |
| Other NF-L924 derivatives | prfA(E77K) expressing other immunogen(s) | This study |
| NF-L943 | NF-L476 prfA(G155S) (mid-level PrfA activation) | 49 |
| NF-L943 + ESAT6 | prfA(G155S) expressing ESAT6 | This study |
| Other NF-L943 derivatives | prfA(G155S) expressing other immunogen(s) | This study |
| NF-L1124 | 10403S actA gus plcB neo | Miner et al. |
| NF-L1124 + ESAT6 | prfA(G145S) expressing ESAT6 | This study |
| Other NF-L1124 derivatives | prfA(G145S) expressing other immunogen(s) | This study |
| NF-L1167 | NF-L1124 prfA(L140F) (high-level PrfA activation) | Miner et al. |
| NF-L1167 + ESAT6 | prfA(L140F) expressing ESAT6 | This study |
| Other NF-L1167 derivatives | prfA(L140F) expressing other immunogen(s) | This study |
| NF-L1214 | NF-L1124 prfA(Y63C) (high-level PrfA activation) | Miner et al. |
| NF-L1214 + ESAT6 | prfA(Y63C) expressing ESAT6 | This study |
| Other NF-L1214 derivatives | prfA(Y63C) expressing other immunogen(s) | This study |
| DP-L3078 | 10403S with actA in-frame deletion | 50 |
| DP-L3078 + ESAT6 | ΔactA expressing ESAT6 | This study |
| Other DP-L3078 derivatives | ΔactA expressing other immunogen(s) | This study |
| NF-L974 | 10403S prfA(G155S) ΔactA | 37 |
| NF-L974 + ESAT6 | prfA(G155S) ΔactA expressing ESAT6 | This study |
| Other NF-L974 derivatives | prfA(G155S) ΔactA expressing other immunogen(s) | This study |
| NF-L972 | 10403S prfA(E77K) ΔactA | 37 |
| NF-L972 + H3L | prfA(E77K) ΔactA expressing H3L | This study |

* Parental L. monocytogenes strain with or without prfA* mutation used in this study.

* Seven foreign immunogens (ESAT6, antigen 85B, IBHA, H3L, BSR, A27L, and enhanced green fluorescent protein) were expressed in recombinant L. monocytogenes strains with and without prfA* mutations.

* M. D. G. C. P. Miner, H. G. Bouwer, J. Chang, and N. E. Freitag, submitted for publication.

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of 1:3,000. A colorimetric reaction was obtained by addition of the 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) one-component microwell peroxidase substrate (KPL) for 8 to 10 min. OD405 values were determined with an ELISA plate reader (Bio-Rad model 550). The plasma IgG endpoint titer was defined as the reciprocal plasma dilution that gave an optical density that was three times the average value obtained with bovine serum albumin. The cutoff titer was defined as the mean titer obtained for prevaccinated mouse plasma plus 3 standard deviations.

Necropsy and pathological studies. At week 6, all mice were subjected to a complete necropsy and histopathological examination. Portions of livers and spleens were fixed in 10% formalin for 24 to 48 h and then embedded in paraffin. Five-micrometer sections were cut and stained with hematoxylin and eosin. Routine histology studies were done as previously described (28).

Statistical analysis. The standard deviations of the means were determined. All data were analyzed by using a one-way analysis of variance (ANOVA) for statistical significance as previously described (28). The differences between groups were evaluated for statistical significance by calculating the P value.

RESULTS

Construction of recombinant L. monocytogenes strains. To conveniently construct all recombinant L. monocytogenes strains expressing various antigen proteins, we modified the site-specific phage-based vector pPL2 by adding the promoter, ribosome binding site, and signal sequence of the L. monocytogenes virulence gene hly encoding the highly secretory signal peptide (12, 36), followed by a c-myc tag. The resultant plasmid, designated pPL6-myc (data not shown), was used for subcloning foreign immunogen-encoding genes. By using the BamHI, SpeI, and XbaI restriction sites, each antigen gene without its stop codon was inserted downstream of the hly promoter and in frame with the signal sequence of hly and upstream of the c-myc tag (EQKLISEEDL), which served as a marker to detect protein expression and secretion.

All resulting pPL6 integration plasmids containing foreign genes were individually transformed into SM10 and integrated into the chromosome of L. monocytogenes after SM10 transformants with pPL6 integration plasmids were conjugated with various L. monocytogenes strains. A total of 63 recombinant L. monocytogenes vaccine vectors expressing seven individual immunogens in nine different wild-type and attenuated (ΔactA) L. monocytogenes backgrounds containing wild-type prfA or various prfA* mutations were developed and used in this study (Table 1).

Mutationally activated prfA alleles in recombinant L. monocytogenes prfA* strains resulted in increased expression of foreign immunogens. Although prfA* mutations resulting in PrfA activation in L. monocytogenes have been shown to enhance expression of many PrfA-dependent virulence genes, including hly and actA (37, 44, 49), it is not known whether prfA* mutations can augment the hly promoter-driven expression of foreign microbial immunogens. As an initial effort to address this question, we first examined the time course of immunogen expression for genes placed under the control of the hly promoter in an L. monocytogenes prfA* mutant strain. The recombinant L. monocytogenes constructs derived from our modified integration plasmid pPL6-myc were designed to allow immunogens to be produced and secreted into the medium for detection. Thus, the recombinant L. monocytogenes prfA (G155S) strain expressing vaccinia virus antigen H3L (NF-L974+H3L) was cultured, and the levels of secreted H3L in the culture supernatant were assessed over time by measuring the integrated densities of Western blot signals and the optical densities of culture samples collected at different time points. H3L protein was detectable with anti-myc MAb and anti-H3L antibody during the first 3 h of bacterial growth in broth, and the expression peaked at the late log stage (6 h) and plateaued in the stationary phase (24 h) without degradation (Fig. 1A).

The kinetics of H3L immunogen expression were comparable to those observed for β-glucuronidase (5) or LuxAB luciferase (9) driven by the hly promoter based on a comparison with transcriptional fusions previously reported for L. monocytogenes.

We then sought to determine whether the presence of mutationaly activated prfA alleles in recombinant L. monocytogenes prfA* mutant strains resulted in increased foreign immunogen expression compared to wild-type strains. Since a recombinant L. monocytogenes prfA* mutant stably expressed the H3L immunogen through the stationary phase without degradation, four recombinant strains, including wild-type, ΔactA, prfA(G155S) ΔactA, and prfA(E77K) ΔactA H3L-expressing strains, were grown for 16 to 18 h in either LB medium or BHI medium at 37°C, and the levels of H3L secretion were compared. The two recombinant L. monocytogenes vectors carrying wild-type prfA, 10403S+H3L and DP-L3078+H3L, secreted undetectable or very low levels of H3L into the culture supernatant, as shown by Western blotting, although supernatant that was concentrated 150-fold produced faint bands (Fig. 1B). In contrast, the two recombinant L. monocytogenes vectors carrying the prfA(E77K) or prfA(G155S) mutation, NF-L972-H3L and NF-L974+H3L, secreted significantly higher levels of the foreign immunogen H3L than the recombinant L. monocytogenes vectors carrying wild-type prfA (Fig. 1B). The prfA*-mediated increase in expression of foreign immunogen was more striking in nutrient-rich BHI broth (Fig. 1B), suggesting that the L. monocytogenes prfA(E77K) ΔactA and L. monocytogenes prfA(G155S) ΔactA vectors were less sensitive to the effect of catabolite repression than the L. monocytogenes vectors carrying wild-type prfA (49).

To confirm that there was prfA*-induced upregulation of foreign protein expression, nine parental L. monocytogenes strains carrying wild-type prfA or prfA* mutations that resulted in low-, mid-, or high-level PrfA activation (Table 1) were utilized to construct recombinant L. monocytogenes vaccine vectors expressing seven individual immunogens. A total of 63 recombinant L. monocytogenes vaccine vectors were constructed, characterized, and titrated for comparison. The recombinant L. monocytogenes vectors derived from two strains carrying wild-type prfA (10403S) and ΔactA (DP-L3078) secreted very low or undetectable levels of seven individual foreign immunogens when ~3 × 10^7 cells of each recombinant culture were analyzed by Western blotting (Fig. 1C). These immunogens were detectable only in highly concentrated culture supernatants (data not shown). In contrast, recombinant L. monocytogenes vectors derived from seven other strains carrying selected mutant prfA* genes secreted significantly higher levels of seven individual foreign immunogens than strains containing wild-type prfA (Fig. 1C). The levels of mutational PrfA activation conferred by the selected prfA* mutations did not appear to directly correlate with the levels of foreign immunogen secreted. However, the high-level PrfA activation G145S, L140F, and Y66C mutants exhibited higher levels of expression of all immunogens tested, whereas the low-level PrfA activation Y145C mutant or the mid-level E77K mu-
tantly expressed antigen 85B, heparin-binding hemagglutinin (HBHA), and H3L equally but not BR5 and A27L. High-level PrfA activation mutants might exhibit upregulation of transcription/translation of all the immunogen genes regardless of their sequence codon usage or fitness. However, a low-level PrfA activation mutant (Y145C) or a mid-level E77K mutant may not be able to overcome such a lack of sequence fitness or the toxicity of some immunogen genes, such as the BR5 and A27L genes. These results demonstrated that selected prfA* mutations mediating PrfA activation in recombinant L. monocytogenes mutant strains were capable of remarkably augmenting protein expression and secretion of foreign immunogens.

The prfA(G155S) mutation in recombinant L. monocytogenes ΔactA prfA* vaccine vectors enhanced vaccine-elicited cellular immune responses. Given that mutational PrfA activation in recombinant L. monocytogenes prfA* vaccine vectors augmented foreign immunogen expression, we sought to examine whether the PrfA*-mediated augmentation of immunogen expression enhanced vaccine-elicited immune responses. Since L. monocytogenes ΔactA strains have been shown to be ~1,000
times less virulent than wild-type *L. monocytogenes* strains, recombinant *L. monocytogenes prfA(G155S) ΔactA* vaccine vectors were directly compared with recombinant wild-type *prfA ΔactA* vaccine vectors to evaluate PrfA*-enhanced immunogenicity in paired groups of mice. Strains containing the *prfA(G155S)* mutant gene were selected for further study since these strains consistently secreted high levels of all immunogens tested and since the expression levels in recombinant *prfA(G155S) ΔactA* vaccine vectors appeared to be higher than (HBHA and ESAT6) or comparable to (other immunogens) those in the high-level activated *prfA* mutants (Fig. 1C). As controls, two groups of mice were vaccinated with *L. monocytogenes prfA(G155S) ΔactA* and wild-type *prfA ΔactA* strains lacking immunogens, respectively. The foreign immunogens used for comparison of vaccine vectors were three representative microbial antigens, H3L (vaccinia virus), PA (*Bacillus anthracis*), and ESAT6 (*M. tuberculosis*) (15, 17, 43).

The mice that received prime and boost immunizations with
the prfA(G155S) ΔactA strain expressing H3L (NF-L974+H3L) contained greater mean numbers of H3L-specific IFN-γ-producing cells than the animals immunized similarly with the wild-type prfA ΔactA strain expressing H3L (DP-L3078+H3L) (Fig. 2A and 2B). The increased numbers of cells were detected both for the PBMCs in the blood pooled at weeks 2, 4, and 6 and for the splenocytes collected at week 6, although there was a statistical difference between the two groups only at week 2 (Fig. 2A and 2B). Interestingly, the mice that received prime and boost immunizations with the L. monocytogenes prfAΔactA ΔactA strain expressing PA (NF-L974+PA) contained much higher mean numbers of PA-specific IFN-γ-producing PBMCs and splenocytes than the animals vaccinated similarly with the wild-type prfA ΔactA strain expressing PA (DP-L3078+PA) (Fig. 2C and 2D). The statistical analysis indicated that there were significant differences between these two vaccine vectors, with P values of <0.05 and <0.001 at all time points after immunization.

Similarly, the mice primed with the prfA(G155S) ΔactA strain expressing ESAT6 (NF-L974+ESAT6) and boosted with the subunit vaccine consisting of GEM-ESAT6 particles contained significantly greater numbers of ESAT6-specific IFN-γ-producing PBMCs than the mice primed with the wild-type prfA ΔactA strain expressing ESAT6 (DP-L3078+ESAT6) and boosted with the subunit vaccine consisting of GEM-ESAT6 particles at weeks 2, 4, 5, and 6 (Fig. 2E). Consistently, higher numbers of ESAT6-specific IFN-γ-producing splenocytes were detected at week 6 after the boosting vaccination in the mice primed with the prfA(G155S) ΔactA strain expressing ESAT6 (Fig. 2F). Taken together, these results demonstrate that the prfA(G155S) mutation mediating PrfA activation in recombinant L. monocytogenes ΔactA prfAΔ vaccine vectors could enhance vaccine-elicted cellular immune responses.

The prfA G155S mutation in recombinant L. monocytogenes ΔactA prfAΔ vaccine vectors resulted in enhanced vaccine-elicted humoral immune responses. The next interesting question was whether the prfA(G155S) mutation mediating PrfA activation in vaccine vectors could enhance vaccine-elicted humoral immune responses. To address this question, we measured plasma anti-H3L, anti-PA, or anti-ESAT6 antibody titers in three groups of mice vaccinated with the corresponding prfA(G155S) ΔactA vaccine vectors and compared these groups with three groups of mice immunized with the wild-type prfA ΔactA vaccine vectors. The mice that received homologous prime and boost vaccinations with the recombinant L. monocytogenes prfA(G155S) ΔactA strain expressing H3L (NF-L974+H3L) developed statistically higher anti-H3L IgG antibody titers than the mice that received prime and boost vaccinations with the recombinant L. monocytogenes wild-type prfA ΔactA strain expressing H3L (NF-L974+H3L) (Fig. 3A). Similarly, the mice that received prime and boost vaccinations with the prfA(G155S) ΔactA strain expressing PA (NF-L974+PA) had much higher anti-PA IgG antibody titers than...
FIG. 4. The *L. monocytogenes* Δ*actA* prfA*"* vaccine vector remains as attenuated as the wild-type prfA Δ*actA* vaccine vector. (A) Survival curves for three groups of BALB/c mice after intravenous vaccination with three different recombinant *L. monocytogenes* strains. All mice were vaccinated with recombinant *L. monocytogenes* starting at day 0 and were observed for 42 days. Groups of eight mice were studied. ■, 1 x 10⁴ CFU of wild-type prfA strain with ESAT6 (10403S+ESAT6); ○, 1 x 10⁷ CFU of wild-type prfA Δ*actA* strain with ESAT6 (DP-L3078+ESAT6); ▲, 1 x 10⁷ CFU
the mice vaccinated with the ΔactA strain containing wild-type prfA (DP-L3078 + PA) (Fig. 3B). Notably, the mean endpoint titers of anti-H3L antibodies rose to ~3,500 after the boost with the prfA(G155S) ΔactA strain expressing H3L, and the boost immunization with the prfA(G155S) ΔactA strain expressing PA elicited an extremely high level of anti-PA antibody (P < 0.001) (Fig. 3A and 3B). Furthermore, heterogeneous prime and boost vaccinations with L. monocytogenes prfA (G155S) ΔactA strains expressing ESAT6 and GEM-ESAT6 particles elicited much higher mean endpoint titers of anti-ESAT6 antibodies than prime and boost vaccinations with wild-type prfA ΔactA strains expressing ESAT6 and GEM-ESAT6 (P < 0.001 for all the time points tested) (Fig. 3C). These data demonstrate that the prfA(G155S) mutation mediating PrfA activation in recombinant vaccine vectors enhanced vaccine-elicited humoral immune responses, as well as cellular immune responses.

The recombinant L. monocytogenes ΔactA prfA* vaccine vectors remained as attenuated as wild-type L. monocytogenes ΔactA vaccine vectors. The finding that the prfA(G155S) mutation in recombinant L. monocytogenes ΔactA prfA* vaccine vectors could enhance vaccine-elicited cellular and humoral immune responses raised an important question concerning whether the recombinant prfA(G155S) ΔactA vaccine vectors were safe. It is important to note that L. monocytogenes ΔactA vaccine vectors are at least 1,000-fold less virulent than wild-type L. monocytogenes in mice (11, 32, 51). On the other hand, introduction of the prfA(G155S) mutation into wild-type L. monocytogenes has been shown to increase bacterial virulence in mice approximately 5- to 10-fold (22, 49). It was therefore crucial to determine whether recombinant L. monocytogenes prfA(G155S) ΔactA vaccine vectors remained as attenuated as recombinant ΔactA strains while they were enhancing immunogenicity.

Three groups of BALB/c mice were included in the safety studies. One group of mice was vaccinated with 1 × 10⁷ CFU of the L. monocytogenes prfA(G155S) ΔactA strain expressing ESAT6 (NF-L974 + ESAT6), as described above. Two groups served as controls; one of these groups received 1 × 10⁷ CFU of the wild-type prfA ΔactA strain expressing ESAT6 (DP-L3078 + ESAT6), whereas the other received 1 × 10⁷ CFU of the wild-type L. monocytogenes strain expressing ESAT6 (10403S + ESAT6). All the mice vaccinated with the recombinant wild-type L. monocytogenes strain expressing ESAT6 became moribund and died on days 3, 5, and 7 after vaccination (Fig. 4A). In the mice wild-type L. monocytogenes strain expressing ESAT6 appeared to be more virulent than the wild-type L. monocytogenes strain. In contrast, the two groups of mice that received 1 × 10⁷ CFU of the L. monocytogenes prfA(G155S) ΔactA strain expressing ESAT6 or 1 × 10⁷ CFU of the wild-type prfA ΔactA strain expressing ESAT6 remained healthy and gained body weight without any noticeable clinical signs of illness during a 42-day prime-boost vaccination period.

Complete necropsy and histology studies were performed at the time that the mice vaccinated with wild-type L. monocytogenes expressing ESAT6 died and at the endpoint (42 days) for the mice vaccinated with the prfA(G155S) ΔactA strain expressing ESAT6 and for the mice vaccinated with the wild-type prfA ΔactA strain expressing ESAT6. A group of healthy unvaccinated mice were also euthanized as controls. While the mice that received 1 × 10⁴ CFU of the wild-type L. monocytogenes strain expressing ESAT6 had multiple acute abscesses and severe inflammatory lesions in the spleen and liver and had spleens that were two times larger than normal spleens, the mice vaccinated with the prfA(G155S) ΔactA strain expressing ESAT6 and the mice vaccinated the wild-type prfA ΔactA strain expressing ESAT6 did not show any abnormal morphologies in the liver, spleen, intestine, lung, or other organs compared to the healthy unvaccinated mice (Fig. 4B). Histopathologic studies showed that the livers and spleens from the mice vaccinated with the wild-type L. monocytogenes strain expressing ESAT6 had intensive inflammatory lesions, multiple acute abscesses, or focal microabscesses. Abscess cavities were surrounded by granulation tissues and a large number of leukocytes, and the central area consisted of purulent, necrotic tissues. In contrast, no apparent lesions were seen in the tissue sections of the livers and spleens from the mice vaccinated with the wild-type prfA ΔactA or prfA(G155S) ΔactA strain expressing ESAT6 (Fig. 4C and 4D). Thus, these results demonstrated that recombinant L. monocytogenes prfA(G155S) ΔactA vaccine vectors were attenuated, while the vaccine-elicited immune responses were enhanced, and that the prfA(G155S) ΔactA strain expressing ESAT6 was as safe as the ΔactA strain containing wild-type prfA expressing ESAT6 in the preliminary safety study.

**DISCUSSION**

The current study took advantage of the combination of prfA*-induced upregulation of PrfA-dependent hly- and ΔactA-mediated attenuation of L. monocytogenes for use in development of vaccines for tuberculosis (ESAT6), vaccinia/smallpox (H3L), and anthrax (PA). While PrfA functions as a positive regulatory factor that regulates the transcription of the hly gene encoding LLO (33), L. monocytogenes prfA* mutants carrying prfA(E77K) or prfA(G155S) mutations have been reported to exhibit substantially increased in vitro expression of PrfA-dependent genes, including actA, hly, inlA, and plcB (37, 49). L. monocytogenes strains with various degrees of PrfA activation resulting from prfA* mutations (Table 1) allowed us to demonstrate PrfA*-induced augmentation of the expression of prfA(G155S) ΔactA strain with ESAT6 (NF-L974 + ESAT6). (B, C, and D) Representative histopathologic evaluation of tissue sections of livers and spleens infected with wild-type prfA or prfA(G155S) strains. Livers and spleens were isolated from a naive mouse or mice vaccinated intravenously with 1 × 10⁴ CFU of a wild-type prfA strain expressing ESAT6 (10403S + ESAT6), with 1 × 10⁷ CFU of a wild-type prfA ΔactA strain expressing ESAT6 (DP-L3078 + ESAT6), or with 1 × 10⁷ CFU of a prfA(G155S) ΔactA strain expressing ESAT6 (NF-L974 + ESAT6). (B) Digital images. (C and D) Hematoxylin and eosin (HE) staining. (C) Liver sections. Magnification, ×200. (D) Spleens. Magnification, ×100. WT, wild type.
and secretion of foreign immunogens in recombinant *L. monocytogenes* strains. The *prfA* alleles facilitated development of recombinant *L. monocytogenes ΔactA* vaccine constructs, since wild-type and attenuated ΔactA strains often express very low levels of foreign immunogen genes (Fig. 1C). The ability of the *L. monocytogenes* ΔactA *prfA*Δ vaccine vector strain to express and secrete increased levels of foreign antigens allowed us to readily develop recombinant *L. monocytogenes ΔactA prfA*Δ vaccine constructs expressing a total of 14 different foreign immunogens derived from both viruses (vaccinia virus, human immunodeficiency virus type 1) and bacteria (*M. tuberculosis*, *B. anthracis*, and *Yersinia pestis*) (this study and data not shown).

An interesting and important observation from the immunologic studies with mice is that recombinant *L. monocytogenes* ΔactA vaccine vectors enhance vaccine-elicted cellular immune responses compared to wild-type *prfA ΔactA* vaccine vectors. The *prfA*Δ (G155S)-enhanced immunogenic responses were observed in mice immunized with three different vaccine candidates that featured an *L. monocytogenes* *prfA* (G155S) ΔactA strain expressing ESAT6, PA, or H3L. It is likely that the *prfA*Δ (G155S)-enhanced immune responses occur as a result of *PrfA*-augmented expression of the microbial immunogens in host cells. In fact, it has been shown that the magnitude of *L. monocytogenes*–specific immune responses depends on the amount of bacterial antigens produced during the first 24 h of *L. monocytogenes* infection (4, 8, 35). Since the use of selected *prfA* mutations mediating *PrfA* activation in recombinant *L. monocytogenes ΔactA* vectors dramatically increased the expression of immunogens compared to that in the wild-type *prfA* Δ*actA* *L. monocytogenes* strains, it is anticipated that increased production and secretion of immunogens in vivo in mice vaccinated with recombinant *L. monocytogenes ΔactA prfA*Δ vaccine vectors would lead to enhancement of major histocompatibility complex–peptide presentation to CD4+ and CD8+ T cells. It is also important to note that while *L. monocytogenes* *prfA*Δ (G155S) strains have been shown to dramatically upregulate many PrfA-dependent genes, including *actA* and *hly*, these strains are also 10 to 50 times more invasive for certain types of host cells without apparent damage to the plasma membrane (37, 49). The potential for enhanced entry into antigen-presenting cells by recombinant *L. monocytogenes ΔactA prfA*Δ vaccine vectors may also facilitate immunogen presentation and elicit stronger immune responses.

Other interesting findings were that both the *L. monocytogenes ΔactA* and Δ*actA prfA*Δ vaccine vectors elicited appreciable antibody responses and that the *prfA*Δ strain induced even higher titers of immunogen-specific antibodies. It is generally believed that humoral responses to *L. monocytogenes* antigens are very weak (56). Mice infected either parenterally or orally with *L. monocytogenes* strains often express very low antibody titers either in the peripheral blood or in the gut-associated lymphoid tissue (40). This may be attributed to the intracellular location of *L. monocytogenes* and its ability to spread from cell to cell without leaving its intracellular niche, thus sequestering the organism from the humoral arm of the immune response (56). However, an *L. monocytogenes ΔactA* mutant has been observed to grow as microcolonies within the cytosol due to loss of the ability to move intracellularly and to spread to adjacent cells (18, 29). Intracellular growth of Δ*actA* strains may result in eventual host cell lysis and the subsequent release of the bacteria and bacterial antigens into the extracellular milieu, which would promote antigen exposure to the humoral immune system (11).

Hence, it is possible that recombinant *L. monocytogenes ΔactA* vaccine immunogens may be released into the extracellular milieu after host cell lysis and directly ingested by B cells for initiation of humoral immune responses. Since recombinant *L. monocytogenes ΔactA prfA*Δ strains have an increased capability for immunogen production and/or secretion and may have an enhanced ability to invade antigen-presenting cells (as discussed above), the Δ*actA prfA*Δ strains may thus elicit stronger antibody responses than wild-type *prfA ΔactA* strains. Further studies are needed to elucidate the precise mechanisms leading to enhanced immune responses.

The data obtained in the current study suggest that *L. monocytogenes ΔactA prfA*Δ vaccine vectors are highly immunogenic and as attenuated as Δ*actA* vaccine vectors. The increased PrfA activation in the *L. monocytogenes ΔactA prfA* (G155S) Δ*actA* strain did not induce any detectable toxicity or visible lesions in BALB/c mice even though *prfA* (G155S) has been shown to enhance *L. monocytogenes* virulence (49). An *L. monocytogenes ΔactA* strain is highly attenuated, with toxicity that is at least 1,000-fold less than that of wild-type *L. monocytogenes* strains (11, 42), and has been found to be safe even for IFN-γ-deficient mice (3, 27, 54). This may help to explain why increased PrfA activation in the *prfA*Δ (G155S) Δ*actA* vaccine vectors enables efficient antigen delivery but does not induce detectable virulence in mice despite the enhanced invasive capacity of *prfA*Δ (G155S) Δ*actA* strains. This finding from the proof-of-concept studies raises the possibility that in-depth toxicological evaluations of recombinant *L. monocytogenes ΔactA prfA*Δ strains can be performed.

In conclusion, the increased PrfA activation in recombinant attenuated *L. monocytogenes ΔactA prfA*Δ vaccine vectors confers the capacity to augment the expression and secretion of foreign immunogens, as well as the capacity to enhance vaccine-elicted cellular and humoral immune responses in mice. Deletion of *actA* renders recombinant *prfA*Δ (G155S) Δ*actA* strains markedly attenuated, and the strains appear to be as safe as wild-type *prfA ΔactA* vaccine vectors in mice. The recombinant attenuated *L. monocytogenes prfA*Δ (G155S) Δ*actA* vaccine vector may therefore be a superior candidate for further clinical development of both therapeutic and prophylactic vaccines against infections or cancers.

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**REFERENCES**

1. **Ali, Z., L. Shao, L. Halliday, A. Reichenberg, M. Hintz, H. Jomaa, and Z. W. Chen. 2007. Prolonged (E)-4-hydroxy-3-methyl-but-2-enyl pyrophosphate–driven antiviral and cytotoxic responses of pulmonary and systemic Vγ2Vδ2 T cells in macaques. J. Immunol. 179:8287–8296.**

2. **Angelakopoulos, H., K. Looch, D. M. Sisul, E. R. Jensen, J. F. Miller, and E. L. Hofmann. 2002. Safety and shedding of an attenuated strain of *Listeria monocytogenes* with a deletion of *actA*Δ*prfA* in adult volunteers: a dose escalation study of oral inoculation. Infect. Immun. 70:3592–3601.**

3. **Badovinac, V. P., and J. T. Harty. 2000. Adaptive immunity and enhanced...**
CD8+ T cell response to Listeria monocytogenes in the absence of perforin and IFN-gamma. J. Immunol. 164:4444–4452.

4. Badovinac, V. P., B. B. Porter, and J. T. Harty. 2002. Programmed contraction of CD8+ T cells after infection. Nat. Immunol. 3:196–202.

5. Behar, J., and M. Noegel. 1996. Regulation of transcription by the cell cycle: mechanisms mediated by PrfA. Infect. Immun. 66:3635–3642.

6. Bouwer, H. G., H. Shen, X. Fan, J. F. Miller, R. A. Barry, and D. J. Hinrichs. 1998. Existing antimicrobial immunity does not inhibit the development of a Listeria monocytogenes-specific primary cytotoxic T-lymphocyte response. Infect. Immun. 66:253–258.

7. Boyer, J. D., T. M. Robinson, P. C. Maciag, X. Peng, R. S. Johnson, G. Pavlovic, D. G. Brockstedt, H. Shen, S. R. Siliciano, S. L. K. Brown, D. V. Schmutz, J. L. LeFort, M. Huerre, P. Gounon, C. Dugy, C. Babinet, and P. Cossart. 2001. A transgenic model for listeriosis: role of internalin in crossing the intestinal barrier. Science 292:1722–1725.

8. Breiteneder, M. C., C. Hoffner, E. Domann, W. Goebel, and T. Chakraborty. 1990. Identification of a gene that negatively regulates expression of listeriolysin O, the major virulence factor of Listeria monocytogenes. Proc. Natl. Acad. Sci. USA 87:8336–8340.

9. Li, Z., X. Zhao, D. E. Higgins, and F. R. Frankel. 2005. Conditional lethality yields a new vaccine strain of Listeria monocytogenes for the induction of cell-mediated immunity. Infect. Immun. 73:5065–5073.

10. Mercado, R. S., V. J. S. Allen, K. Kerkisik, I. M. Pilip, and E. G. Pamer. 2000. Early programming of T cell populations responding to bacterial infection. J. Immunol. 164:6583–6593.

11. Moore, M. A., B. Levri, P. Youngman, and D. A. Portnoy. 1999. Expression of lysteriolysin O and ActA by intracellular and extracellular Listeria monocytogenes. Infect. Immun. 67:131–139.

12. Mueller, K. J., and E. N. Freitag. 2005. Pleiotropic enhancement of bacterial pathogenesis resulting from the constitutive activation of the Listeria monocytogenes regulatory factor PrfA. Infect. Immun. 73:1917–1926.

13. Orr, M. T., N. N. Orgun, C. B. Wilson, and S. S. Way. 2007. Cutting edge: recombinant Listeria monocytogenes expressing a single immune-dominant peptide confers protective immunity to herps simplex virus-1 infection. J. Immunol. 178:4731–4735.

14. Pamer, E. G. 2004. Immune responses to Listeria monocytogenes. Nat. Rev. 8:823–823.

15. Pan, K., G. Ikonomidou, A. Lazenby, D. Pollard, and Y. Paterson. 1995. A recombinant Listeria monocytogenes vaccine expressing a model tumour antigen protects mice against lethal tumour challenge and causes regression of established tumours. Nat. Med. 1:471–477.

16. Papen, V., and R. S. Johnson. 2004. Progress towards the use of Listeria monocytogenes as a live bacterial vaccine vector for the delivery of HIV antigens. Expert Rev. Vaccines 3:519–513.

17. Peters, C., E. Domann, A. Darbouche, T. Chakraborty, and M. E. Mielke. 2003. Tailoring host immune responses to Listeria by manipulation of virulence genes—the interface between innate and acquired immunity. FEBS Immunol. Med. Microbiol. 35:243–253.

18. Pym, A. S., P. Brodin, L. Majlessi, R. Brosch, C. Demangel, A. Williams, K. E. Griffiths, G. Marchal, C. Leclerc, and S. T. Cole. 2003. Reombinent BCG expressing ESA1-6 confers enhanced protection against tuberculosis. Nat. Med. 9:533–539.

19. Rigol, M. T., G. Dominguez-Bernal, M. Lara, M. Suarez, and J. A. Vazquez-Boland. 1997. A Gly145Ser substitution in the transcriptional activator PrfA regulates virulence genes—the interface between innate and acquired immunity. FEMS Immunol. Med. Microbiol. 19:21–21.

20. Rigol, M. T., G. Dominguez-Bernal, M. Suarez, B. Kehm, P. Berche, and J. A. Vazquez-Boland. 1999. The bocAs of L. monocytogenes mutants with high virulence induce a cellular immune response in human monocytes, suggesting that they contribute to intracellular growth and cell-to-cell spread. Infect. Immun. 67:11724–11733.

21. Rigol, M. T., G. Dominguez-Bernal, M. Suarez, B. Kehm, P. Berche, and J. A. Vazquez-Boland. 1999. Transcriptional activation of virulence genes in wild-type strains of Listeria monocytogenes in response to a change in the extracellular medium composition. Res. Microbiol. 150:1423–1432.

22. Skoble, J., D. A. Portnoy, and M. D. Welch. 2000. Three regions within ActA contribute to intracellular growth and cell-to-cell spread. Infect. Immun. 68:2537–2544.

23. Freitag, N. E., L. Rong, and D. A. Portnoy. 1993. Regulation of the PrfA transcriptional activator of Listeria monocytogenes: multiple promoter elements contribute to intracellular growth and cell-to-cell spread. Infect. Immun. 61:2537–2544.

24. Freitag, N. E., P. Youngman, and D. A. Portnoy. 1992. Transcriptional activation of the Listeria monocytogenes hemolysin gene in Bacillus subtilis. J. Bacteriol. 174:1293–1298.

25. Gray, M. J., N. E. Freitag, and K. J. Boor. 2006. How the bacterial pathogen Listeria monocytogenes mediates the switch from environmental Dr. Jekyll to pathogenic Mr. Hyde. Infect. Immun. 74:2505–2512.

26. Hannon, M., B. Hieren, and P. Cossart. 2006. Listeria monocytogenes: a multifaceted model. Nat. Rev. Microbiol. 4:423–434.

27. Hartj, J. T., and M. J. Bevan. 1995. Specific immunity to Listeria monocytogenes in Balb/c mice. Immunol. Lett. 48:109–117.

28. Huang, D., Y. Shen, L. Qiu, C. Y. Chen, L. Shen, J. Estep, R. Hunt, D. Vasconcelos, G. Du, P. Aye, A. A. Lackner, M. H. Larsen, W. R. Jacobs, Jr., B. F. Haynes, N. L. Letvin, and Z. W. Chen. 2008. Immune distribution and localization of phosphoantigen-specific VγVδ2 T cells in lymphoid and nonlymphoid tissues in Mycobacterium tuberculosis infection. Infect. Immun. 76:452–456.

29. Kocks, C., E. Gouin, M. Tabouret, P. Berche, H. Ohayon, and P. Cossart. 1992. L. monocytogenes-induced actin assembly requires the actA gene product, a surface protein. Cell 68:521–531.

30. Kreft, J., and J. A. Vazquez-Boland. 2001. Regulation of virulence genes in Listeria monocytogenes. Int. J. Med. Microbiol. 291:145–157.

31. Lauer, P., M. Y. Chow, M. J. Loensser, D. A. Portnoy, and R. Calendar. 2002. Construction, characterization, and use of two Listeria monocytogenes site-specific phage integration vectors. J. Bacteriol. 184:4177–4186.

32. Leimeister-Wachter, M., W. Goebel, and T. Chakraborty. 2001. A transgenic model for listeriosis: role of internalin in crossing the intestinal barrier. Science 292:1722–1725.

33. Leimeister-Wachter, M., C. Hoffner, E. Domann, W. Goebel, and T. Chakraborty. 1990. Identification of a gene that negatively regulates expression of listeriolysin O, the major virulence factor of Listeria monocytogenes. Proc. Natl. Acad. Sci. USA 87:8336–8340.
Bouwer. 2004. Listeria monocytogenes as a vaccine vector: virulence attenuation or existing antivector immunity does not diminish therapeutic efficacy. J. Immunol. 173:420–427.

53. Stevens, R., A. Lavoy, S. Nordone, M. Burkhard, and G. A. Dean. 2005. Pre-existing immunity to pathogenic Listeria monocytogenes does not prevent induction of immune responses to feline immunodeficiency virus by a novel recombinant Listeria monocytogenes vaccine. Vaccine 23:1479–1490.

54. Tvinnereim, A. R., and J. T. Harty. 2000. CD8+ T-cell priming against a nonsecreted Listeria monocytogenes antigen is independent of the antimicrobial activities of gamma interferon. Infect. Immun. 68:2196–2204.

55. Vazquez-Boland, J. A., M. Kuhn, P. Berche, T. Chakraborty, G. Dominguez-Bernal, W. Goebel, B. Gonzalez-Zorn, J. Wehland, and J. Kreft. 2001. Listeria pathogenesis and molecular virulence determinants. Clin. Microbiol. Rev. 14:584–640.

56. Weiskirch, L. M., and Y. Paterson. 1997. Listeria monocytogenes: a potent vaccine vector for neoplastic and infectious disease. Immunol. Rev. 158:159–169.

57. Zhao, X., Z. Li, B. Gu, and F. R. Frankel. 2005. Pathogenicity and immunogenicity of a vaccine strain of Listeria monocytogenes that relies on a suicide plasmid to supply an essential gene product. Infect. Immun. 73:5789–5798.

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